ABSTRACT

A physiological red blood cell (RBC) kinetic model is proposed for the adenosine (ADO) transport into erythrocytes and its subsequent intracellular deamination into inactive inosine (INO) and further breakdown into hypoxanthine (HYPO). The model and its parameters were based on previous studies investigating the kinetics of the biochemical mechanism of uptake and metabolism of ADO in human erythrocytes. Application of the model for simulations of the breakdown of ADO in a RBC suspension revealed that the predicted adenosine breakdown inhibition (ABI) of draflazine corresponded well with the ABI measured ex vivo. The model definitely explained the apparent discrepancy between the ex vivo measured ABI and the nucleoside transporter occupancy of draflazine. Intracellular deamination of ADO rather than its transport by the nucleoside transporter is the rate-limiting step in the overall catabolism of ADO. Consequently, at least 90% occupancy of the transporter by draflazine is required to inhibit adenosine breakdown ex vivo substantially. Simulations on basis of the validated model were performed to evaluate the ABI for different experimental conditions and to mimic the clinical situation. The latter may be very helpful for the design of optimal dosing schemes of draflazine. It was demonstrated that the short half-life of released ADO was prolonged substantially in a dose-related manner after a continuous infusion of draflazine. Finally, the previously found different sigmoidal $E_{\text{max}}$ relationships between the measured ABI and the concentrations of draflazine in plasma and whole blood could be explained by the ADO transport and breakdown RBC kinetic model and the capacity-limited specific RBC binding characteristics of draflazine.

The purine nucleoside ADO has a multitude of pharmacological activities (Sollevi, 1986; Belardinelli et al., 1989; Schrader, 1990). Many of these actions are homeostatic and protective in nature (Ely and Berne, 1992). Because of these properties, ADO has been termed a “retaliatory metabolite” (Newby, 1984), a “homeostatic metabolite in cardiac energy metabolism” (Schrader, 1990) and a “signal of life” (Engler, 1991). In the heart and in other vital organs such as brain and kidney, ADO is released after a higher energy demand compared with the energy supply. Because of its pharmacological activities, the released ADO forms a natural defense mechanism against myocardial damage from ischemia and reperfusion (Belardinelli et al., 1989; Van Belle, 1994). Because of its interesting pharmacological profile, exogenous application of ADO could have many therapeutic implications (Sollevi, 1986). However, the only approved application thus far is termination of paroxysmal supraventricular tachycardia. One reason for failure is the very short plasma half-life of exogenously administered ADO of about 10 sec (Klabunde, 1983), which would require continuous infusions of relatively high doses of ADO to achieve therapeutic efficacy for other potential indications (Van Belle, 1993a). In addition, when present in the systemic circulation, ADO may stimulate the receptors all over the body, which possibly could lead to many unwanted side effects.

The short plasma half-life of ADO is a consequence of its rapid catabolism into pharmacologically inactive INO and HYPO in endothelial cells and erythrocytes, which possess high activities of ADO deaminase and purine nucleoside phosphorylase (Van Belle, 1969; Nees, 1989a; Schrader and West, 1990). In the myocardium, deamination of ADO occurs almost exclusively in endothelial cells (Nees, 1989b). A key role in the breakdown of ADO is played by the nucleoside transporters.
transporter which facilitates the uptake of ADO (Van Belle, 1993b, c). Nucleoside transporters are located on the endothelial cells lining the microvessels and on the RBCs.

The inhibition of the nucleoside transporter will prolong considerably the presence of adenosine at its site of release by preventing the first step (uptake) in the catabolism in the endothelial cells. Experimental evidence has been obtained in isolated rabbit and cat hearts (Van Belle et al., 1987, 1989) and in dog hearts in situ (Van Belle et al., 1986). In addition, the action will be expressed only when (ischemia) and where (e.g., myocardium) ADO is produced. This makes the concept of nucleoside transport inhibition almost an ideal example of site- and event-specific drug intervention (Ver Donck, 1994).

Potent nucleoside transport inhibition is an extremely rare property of organic molecules (Van Belle and Janssen, 1991). Draflazine is such a nucleoside transport inhibitor, and cardioprotective effects have been documented in various models for cardioprotection (Van Belle, 1993c). In vitro studies revealed that draflazine exhibits a specific capacity-limited high-affinity binding to the nucleoside transporters located on the erythrocytes (Van Belle et al., 1991; Beukers et al., 1994; Böhm et al., 1994). Draflazine specifically bound to the transporter will inhibit the transport into erythrocytes and thus the breakdown of ex vivo added adenosine.

In previous studies of draflazine in healthy subjects, the ABI was determined ex vivo and was used as a pharmacodynamic endpoint (Snoeck et al., 1996, 1997a). A sigmoidal E\text{max} relationship was observed between the concentration of draflazine and the measured ABI. In human plasma, population typical values (%CV) of the pharmacodynamic parameters were: IC_{50}, 3.76 ng/ml and Hill factor $\gamma$, 1.06. In blood, the relationship was much steeper with typical values IC_{50}, 65.7 ng/ml and Hill factor $\gamma$, 4.47. The interindividual variability (%CV) for the IC_{50} in plasma and blood was 45.1% and 15.4%, respectively. The concentration-dependent RBC/plasma distribution of draflazine in healthy subjects was characterized as a capacity-limited specific binding to the transporters on the RBCs with a K_d of 0.385 ng/ml plasma and a B_{max} of 158 ng/ml RBC corresponding to about 14,000 nucleoside transporters per RBC (Snoeck et al., 1997a). The interindividual variability (%CV) in K_d and B_{max} was 13.1% and 9.8%, respectively. Based on the specific binding parameters, the RBC nucleoside transporter occupancy was calculated serving as a second pharmacodynamic endpoint. RBC occupancy did not coincide with ABI, which was reflected in an almost 10-fold difference between K_d and IC_{50}. Only low inhibition of adenosine breakdown ex vivo was observed below a RBC occupancy of about 60%, and a substantial ABI was found only from a RBC occupancy of 90% onward (Snoeck et al., 1997a).

The purpose of the present report was to find a cell physiological explanation for the apparent discrepancy between the ex vivo measured ABI and the RBC occupancy of draflazine. For this aim, a physiological ADO transport and breakdown RBC kinetic model was constructed. After validation, simulations on basis of this RBC model were performed to predict and further evaluate the ABI for different experimental conditions and to mimic the clinical situation. In addition, the model was used together with the specific RBC binding parameters of draflazine to explain the difference in relationship between plasma and blood concentrations of draflazine and the ex vivo measured ABI. Finally, the ADO transport and breakdown RBC kinetic model was used for further explorative simulations.

**ADOS Transport and Breakdown RBC Kinetic Model**

The proposed ADO transport and breakdown RBC kinetic model (fig. 1) was based on the results of previously reported kinetic and biochemical studies with human erythrocytes (Agarwal et al., 1977; Plagemann et al., 1985; Plagemann and Wohlhueter, 1985). The uptake and metabolism of ADO in human erythrocytes was investigated by Plagemann et al. (1985). The uptake and in situ phosphorylation of ADO was investigated in studies with RBCs treated with 2’-deoxycoformycin to inhibit the deamination of ADO (Agarwal et al., 1977; Plagemann and Wohlhueter, 1985).

Extracellular ADO either can be phosphorylated or transported into the erythrocytes where a subsequent intracellular deamination will take place. The values of K_{m} and V_{max} for ADO transport were about 300 times higher than those for the in situ phosphorylation of ADO by ADA kinase. The first-order rate constant for ADA was only 10 to 20% of that for ADA kinase, whereas the K_{m} was about 100 times higher for deamination than for phosphorylation. These kinetic parameters show that ADO will be phosphorylated preferentially at physiological concentrations. However, at higher extracellular concentrations of ADO, ADA kinase will be inhibited and practically all ADO will enter the RBCs and will be deaminated. For the ex vivo determination of the ABI, a concentration of 40 μM ADO was used so that the phosphorylation of ADO was negligible.

The deamination of ADO by ADA will produce INO. The nucleoside INO in turn either will be transported out of the RBCs or will be catabolized further to HYPO by PNP (fig. 1). The PNP activity of RBCs is about four times higher than that of ADA, so that HYPO will be a more prominent product than INO (Agarwal and Parks, 1978; Stoeckler et al., 1978).

For the ADO transport and breakdown RBC kinetic model (fig. 1), the change of the extracellular amount of ADO (A_{ADO_{ox}}) as a function of time can be described by the following equation:

$$\frac{dA_{ADO_{ox}}}{dt} = \frac{V_{max}}{K_m} \cdot \frac{A_{ADO_{ox}}}{1 + \frac{C_{ADO_{ox}}}{K_{m}}} + C_{ADO_{ox}} + \frac{V_{max}}{K_m} \cdot \frac{A_{ADO_{ox}}}{1 + \frac{C_{ADO_{ox}}}{K_{m}}} + C_{ADO_{ox}}$$

![Fig. 1. Physiological ADO transport and breakdown RBC kinetic model.](image-url)
The $V_{\text{max}}$ (25°C) of the RBC transporter ($V_{\text{max}}$), transporting ADO and INO was assumed to be 28 pmol/µl cell water · sec and the $K_m$ of the transporter ($K_m$) was assumed to be 60 µM (Plagemann et al., 1985). $V_{\text{max}}$ and $K_m$ were similar for both ADO and INO. A transport competition between ADO and INO clearly is assumed from equation 1. $C_{\text{ADO}_{\text{out}}}$ and $C_{\text{ADO}_{\text{RBC}}}$ represent the ADO concentration outside and inside the RBCs, respectively. Similarly, $C_{\text{INO}_{\text{out}}}$ and $C_{\text{INO}_{\text{RBC}}}$ represent the INO concentration outside and inside the erythrocytes, respectively. Finally, the RBC nucleoside transporter occupancy of draflazine ($O_{\text{ect}}$) in equation 1 was expressed as a fraction.

The change of the amount of ADO in the erythrocytes ($A_{\text{ADO}_{\text{RBC}}}$) as a function of time was expressed as:

$$\frac{dA_{\text{ADO}_{\text{RBC}}}}{dt} = \frac{-dA_{\text{ADO}_{\text{out}}}}{dt} - \frac{V_{\text{max}} \cdot C_{\text{ADO}_{\text{out}}}}{K_m + C_{\text{ADO}_{\text{out}}}}$$

where $V_{\text{max}}$ and $K_m$ represent the kinetic parameters of ADO. The $V_{\text{max}}$ (25°C) was assumed to be 1.5 pmol/µl cell water · sec and the $K_m$ was assumed to be 32 µM (Plagemann et al., 1985).

For the change of the extracellular amount of INO ($A_{\text{INO}_{\text{out}}}$) as a function of time, an equation was applied which resembled equation 1:

$$\frac{dA_{\text{INO}_{\text{out}}}}{dt} = - \frac{V_{\text{max}} \cdot C_{\text{INO}_{\text{out}}}}{K_m} \cdot (1 - \frac{C_{\text{INO}_{\text{out}}}}{K_m}) + C_{\text{INO}_{\text{out}}}$$

The change of the cellular concentration of INO ($A_{\text{INO}_{\text{RBC}}}$) as a function of time was calculated as:

$$\frac{dA_{\text{INO}_{\text{RBC}}}}{dt} = \frac{-dA_{\text{INO}_{\text{in}}}}{dt} - \frac{V_{\text{max}} \cdot C_{\text{INO}_{\text{in}}}}{K_m + C_{\text{INO}_{\text{in}}}} + \frac{V_{\text{max}} \cdot C_{\text{INO}_{\text{out}}}}{K_m} \cdot (1 - \frac{C_{\text{INO}_{\text{out}}}}{K_m}) + C_{\text{INO}_{\text{in}}}$$

$V_{\text{max}}$ and $K_m$ represent the kinetic parameters of PNP. The $V_{\text{max}}$ (25°C) was assumed to be 6 pmol/µl cell water · sec and the $K_m$ was assumed to be 45 µM (Stoeckler et al., 1978).

Finally, the formation of HYPO in the erythrocytes ($A_{\text{HYPO}_{\text{RBC}}}$) was expressed as:

$$\frac{dA_{\text{HYPO}_{\text{RBC}}}}{dt} = \frac{V_{\text{maxPNP}} \cdot C_{\text{INO}_{\text{RBC}}}}{K_m + C_{\text{INO}_{\text{RBC}}}}$$

HYPO reaches the extracellular space by diffusion and is not transported by the nucleoside transporter. The diffusion of HYPO was not taken into account in the ADO transport and breakdown RBC kinetic model (fig. 1), because this diffusion will not have an impact on the ADO transport and breakdown.

**Methods**

**Simulation of the ex vivo determination of ABI.** In previous studies, ABI was measured ex vivo by standard incubation of erythrocytes with 40 µM ADO (Ver Donck et al., 1991; Wainwright et al., 1993). Venous blood samples for the ex vivo determination of ABI were taken from eight healthy subjects before and at different time points after i.v. administration of draflazine. After centrifugation of the samples, an erythrocyte suspension was made by suspending 100 µl of packed RBCs in 500 µl 3-(N-morpholino)propanesulfonic acid (MOPS)-NaCl buffer. Then, a total volume of 1 ml containing 40 µM ADO and 100 µl RBC suspension was incubated for 20 min at 25°C. Finally, the pellet was discarded and the supernatant was stored at −20°C until assayed for ADO, INO and HYPO. Concentrations of ADO, INO and HYPO were determined by high-performance liquid chromatography (Wynants and Van Belle, 1985). The ADO concentration remaining after 20 min incubation was expressed as a fraction ($f_{\text{ADO}}(t)$) by dividing the concentrations of ADO by the sum of the concentrations of ADO, INO and HYPO. The ABI was calculated as:

$$\text{ABI} = \frac{f_{\text{ADO}}(t) - f_{\text{ADO}}(0)}{1 - f_{\text{ADO}}(0)} \times 100$$

where $f_{\text{ADO}}(0)$ and $f_{\text{ADO}}(t)$ represent the fraction of ADO remaining after a 20-min incubation with erythrocytes from a blood sample taken before (0) and at time (t) after the start of the administration of draflazine. The coefficient of variation (CV) of the ABI measured ex vivo at several time points after the start of the infusion ranged from approximately 5 to 40%.

This experimental procedure for the ex vivo determination of the ABI was mimicked by the ADO transport and breakdown RBC kinetic model. Concentrations of ADO, INO and HYPO were simulated first in the absence of draflazine during a 120-min incubation period. The concentrations of ADO, INO and HYPO then were simulated for different RBC occupancies of draflazine during an incubation period of 20 min. Then, the ABI was predicted as a function of the RBC occupancy and was compared with the ex vivo measured ABI. The ABI also was simulated for different ADO concentrations added (1, 10, 40 and 100 µM) and for different incubation times (15, 20, 30, 45 and 60 min). Finally, the ABI was simulated for whole blood instead of a 1.67% (v/v) RBC suspension. An incubation time of 10 sec was used for whole blood, and an incubation time of 20 min was used for the 1.67% RBC suspension.

**Simulation of the draflazine concentration-ABI relationship.** Previous studies demonstrated that draflazine exhibited a capacity-limited specific binding to the transporters located on the RBCs (Snoeck et al., 1996, 1997a). By assuming a single-affinity binding site on the erythrocytes and an equilibrium between bound and unbound drug, the total RBC draflazine concentration ($C_{\text{RBC}}$) was calculated from the plasma concentration of draflazine as:

$$C_{\text{RBC}} = B_{\text{max}} \cdot C_p \cdot \frac{1}{K_d + C_p}$$

The RBC binding parameters of draflazine used to calculate the population average $C_{\text{RBC}}$ as a function of the $C_p$ were taken from a previous study of draflazine in healthy subjects (Snoeck et al., 1997a). The population parameter typical value of the maximal concentration of draflazine that was specifically bound to the RBCs ($B_{\text{max}}$) was 158 ng/ml RBC. The estimate of the population parameter typical value of the dissociation constant $K_d$ was 0.385 ng/ml plasma which expresses the very high affinity of the specific binding, and the estimate of the nonspecific binding constant was 0.615.

The percentage of RBC nucleoside transporters occupied by draflazine was calculated as a function of $C_p$ as:

$$\text{Occ}_{\text{RBC}}(\%) = \frac{C_{\text{RBC}} \cdot C_{\text{specific}}}{C_p \cdot K_d + C_p} \cdot 100$$

The whole blood concentration of draflazine ($C_b$) was calculated from the hematocrit ($H$) and the plasma and total RBC concentrations as:

$$C_b = H \cdot C_{\text{RBC}} + (1 - H) \cdot C_p$$

To simulate the draflazine plasma concentration-ABI relationship, the $\text{Occ}_{\text{RBC}}$ was calculated as a function of $C_b$ (equation 8). The $\text{Occ}_{\text{RBC}}$ was expressed as a fraction and the ABI was calculated as a function of $\text{Occ}_{\text{RBC}}$ (equations 1–5), by use of the ADO transport and breakdown RBC kinetic model (fig. 1). Finally, the whole blood concentration was calculated as a function of the plasma concentration of draflazine (equations 7 and 9), to simulate the draflazine whole blood concentration-ABI relationship.
Additional simulations on basis of the RBC model. The model finally was used to mimic the clinical situation. One of the possible indications for draflazine is cardioprotection during a CABG. In response to myocardial ischaemia, ADO is released rapidly reaching local concentrations up to 10 to 100 μM (Smolenski et al., 1992). Unfortunately, the half-life of ADO ($t_{1/2,ADO}$) is very short because of its rapid breakdown. However, $t_{1/2,ADO}$ will increase in the presence of draflazine. On basis of the ADO transport and breakdown kinetic RBC model, the prolongation of $t_{1/2,ADO}$ was simulated as a function of the steady-state $O_{c,RBC}$ of draflazine resulting from continuous infusions of the drug.

Computation. The ADO transport and breakdown RBC kinetic model was built with ADAPT II, a mathematical software for pharmacokinetic/pharmacodynamic system analysis (D’Argenio and Schumitzky, 1979; D’Argenio et al., 1988; D’Argenio and Schumitzky, 1990). The DOS Release version of ADAPT II was used and was run under the Microsoft Fortran Powerstation Compiler.

Results

Figures 2 to 8 depict various simulations based on the ADO transport and breakdown RBC kinetic model (fig. 1). The previously published biochemical parameters describing the kinetics of ADO transport and breakdown in the erythrocytes were used for these simulations (Agarwal et al., 1977; Plagemann et al., 1985; Plagemann and Wohlhueter, 1985). The ADO transport and breakdown RBC kinetic model was run under the Microsoft Fortran Powerstation Compiler.

Ex vivo determination of ABI. Figure 2 depicts a simulation of the total concentrations of ADO and its breakdown products INO and HYPO, as well as the separate RBC and incubate concentrations of ADO and INO as a function of time after addition to 40 μM ADO in a 1.67% (v/v) RBC suspension and incubation for 120 min. The initial $C_{ADO,init}$ was about 40 μM and was very similar to the total concentration of ADO ($C_{ADO}$). The initial $C_{ADO,RBC}$ was zero but increased very rapidly and reached the maximal concentration of about 36 μM within 30 sec after addition of 40 μM ADO (fig. 2). $C_{ADO}$ declined linearly, and consequently the sum of the total concentrations of the breakdown products INO and HYPO ($C_{INO+HYPO}$) increased linearly during the first 20 to 30-min incubation period. Thereafter, $C_{ADO}$ declined exponentially, whereas $C_{INO+HYPO}$ increased exponentially. $C_{INO,RBC}$ and $C_{INO,incub}$ increased relatively slowly and reached a maximal concentration of about 5 μM at about 25 min after the addition of ADO. Thereafter, $C_{INO,RBC}$ and $C_{INO,incub}$ decreased very slowly (fig. 2). In contrast, $C_{HYPO}$ steadily increased during the 120-min incubation period and reached a final concentration of about 38.8 μM. At 120 min after the addition of 40 μM ADO, $C_{ADO}$ was about 0.7 μM and consequently $C_{INO+HYPO}$ was about 39.3 μM, which indicates that during this incubation period nearly all ADO was catabolized to INO and HYPO.

Figure 3 shows a simulation of $C_{ADO}$ and $C_{INO+HYPO}$ as a function of time after a 20-min incubation period with 40 μM ADO in the absence and presence of different RBC occupancies of draflazine. For all RBC occupancies of draflazine, $C_{ADO}$ decreased linearly as a function of time. Consequently, $C_{INO+HYPO}$ increased linearly as a function of time during the 20-min incubation period (fig. 3). Up to a RBC occupancy of about 50%, the decrease in $C_{ADO}$ was nearly similar to the decrease in $C_{ADO}$ in the absence of draflazine, which indicates that hardly any ABI was present for a RBC occupancy of less than 50%. From a RBC occupancy of 80% onward, the rate of decrease in $C_{ADO}$ gradually diminished. The most substantial differences in the inhibition of the breakdown of ADO occurred with a RBC occupancy ranging from 90 to 99% (fig. 3). After a 20-min incubation period, a RBC occupancy of 90, 95, 98 and 99% resulted in a final $C_{ADO}$ of 30.4, 33.2, 36.4 and 38.0 μM, respectively, corresponding to an ABI of 32.9, 52.3, 75.0 and 86.1%, respectively.

The predicted and ex vivo measured ABI as a function of the RBC occupancy of draflazine were compared in figure 4. As already indicated by figure 3, a plot of the ABI as a function of the RBC occupancy of draflazine revealed no 1:1 relationship of about 36 μM within 30 sec after addition of 40 μM ADO. The initial $C_{ADO,init}$ was about 40 μM and was very similar to the total concentration of ADO ($C_{ADO}$). The initial $C_{ADO,RBC}$ was zero but increased very rapidly and reached the maximal concentration of about 36 μM within 30 sec after addition of 40 μM ADO (fig. 2). $C_{ADO}$ declined linearly, and consequently the sum of the total concentrations of the breakdown products INO and HYPO ($C_{INO+HYPO}$) increased linearly during the first 20 to 30-min incubation period. Thereafter, $C_{ADO}$ declined exponentially, whereas $C_{INO+HYPO}$ increased exponentially. $C_{INO,RBC}$ and $C_{INO,incub}$ increased relatively slowly and reached a maximal concentration of about 5 μM at about 25 min after the addition of ADO. Thereafter, $C_{INO,RBC}$ and $C_{INO,incub}$ decreased very slowly (fig. 2). In contrast, $C_{HYPO}$ steadily increased during the 120-min incubation period and reached a final concentration of about 38.8 μM. At 120 min after the addition of 40 μM ADO, $C_{ADO}$ was about 0.7 μM and consequently $C_{INO+HYPO}$ was about 39.3 μM, which indicates that during this incubation period nearly all ADO was catabolized to INO and HYPO.

Figure 3 shows a simulation of $C_{ADO}$ and $C_{INO+HYPO}$ as a function of time after a 20-min incubation period with 40 μM ADO in the absence and presence of different RBC occupancies of draflazine. For all RBC occupancies of draflazine, $C_{ADO}$ decreased linearly as a function of time. Consequently, $C_{INO+HYPO}$ increased linearly as a function of time during the 20-min incubation period (fig. 3). Up to a RBC occupancy of about 50%, the decrease in $C_{ADO}$ was nearly similar to the decrease in $C_{ADO}$ in the absence of draflazine, which indicates that hardly any ABI was present for a RBC occupancy of less than 50%. From a RBC occupancy of 80% onward, the rate of decrease in $C_{ADO}$ gradually diminished. The most substantial differences in the inhibition of the breakdown of ADO occurred with a RBC occupancy ranging from 90 to 99% (fig. 3). After a 20-min incubation period, a RBC occupancy of 90, 95, 98 and 99% resulted in a final $C_{ADO}$ of 30.4, 33.2, 36.4 and 38.0 μM, respectively, corresponding to an ABI of 32.9, 52.3, 75.0 and 86.1%, respectively.

The predicted and ex vivo measured ABI as a function of the RBC occupancy of draflazine were compared in figure 4. As already indicated by figure 3, a plot of the ABI as a function of the RBC occupancy of draflazine revealed no 1:1

Fig. 2. Simulation of the total concentrations of ADO (●) and the sum of the breakdown products INO and HYPO (■) as a function of time after addition of 40 μM ADO added to a 1.67% (v/v) RBC suspension and incubated for 120 min. Intra- (□) and extracellular (○) concentrations of ADO, intra- (▲) and extracellular (●) concentrations of INO as well as the total concentration of HYPO (●) also are plotted. Time profiles of the total and extracellular concentrations of ADO and those of the intra- and extracellular concentrations of INO are very similar and are hardly distinguishable.

Fig. 3. Simulation of the total concentrations of ADO ($C_{ADO}$) and the sum of the breakdown products INO and HYPO ($C_{INO+HYPO}$) as a function of time after 40 μM ADO added to a 1.67% (v/v) RBC suspension and incubated for 20 min. Concentrations were predicted in the absence (thick lines) and presence (thin lines) of draflazine at a RBC occupancy of 50%, 80%, 85%, 90%, 95%, 98%, 99% and 100%. [Image 57x132 to 265x341]
relationship between both pharmacodynamic endpoints (fig. 4). A RBC occupancy of 70% resulted in an ABI of about 10%. Thereafter, the ABI rapidly increased up to 92.7% for a RBC occupancy of 99.5%. From this figure, it is also clear that the predicted ABI based on the ADO transport and breakdown RBC kinetic model very much resembled the ABI measured ex vivo after different infusion schemes of draflazine administered to healthy subjects (Snoeck et al., 1997a).

Figure 5A depicts the predicted ABI as a function of the RBC occupancy of draflazine after a 20-min incubation period of 1, 10, 40 and 100 μM ADO. A small and negligible shift in the ABI was observed for the incubations with different ADO concentrations. For a given RBC occupancy of draflazine, the ABI decreased slightly when increasing concentrations of ADO were added to the RBC suspension (fig. 5A). In addition, the f_{ADO(0)} increased when increasing concentrations of ADO were added to the incubate (data not shown). The predicted ABI as a function of the RBC occupancy of draflazine after a 15-, 20-, 30-, 45- and 60-min incubation period of 40 μM ADO is depicted in figure 5B. Again, a small and negligible shift in the ABI was observed for the different incubation periods. For a given RBC occupancy of draflazine, the ABI decreased when the incubation period was prolonged (fig. 5B).

The relationship between the RBC occupancy of draflazine and the predicted ABI in whole blood with a 42% (v/v) erythrocyte volume was compared with the predicted ABI in a 1.67% (v/v) RBC suspension (fig. 6). Hardly any difference in the ABI as a function of the RBC occupancy between whole blood and the RBC suspension was present. From a RBC occupancy of 70% onward, the ABI in whole blood was slightly higher than that in the RBC suspension (fig. 6).

**Draflazine concentration-ABI relationship.** Figure 7 depicts the ABI as a function of the plasma and whole blood concentration of draflazine, predicted on the basis of the ADO transport and breakdown RBC kinetic model and the specific RBC binding parameters. A sigmoidal E_{max} relationship was present between the concentrations of draflazine in plasma and whole blood and the predicted ABI (fig. 7). However, the plasma concentration-ABI relationship was very different from that in whole blood. The predicted maximal ABI (E_{max}) was 100%, and was reached at a plasma and whole blood concentration of about 1000 ng/ml. The estimated plasma concentration of draflazine that produces 50% of the maximal ABI (IC_{50}) was 6.8 ng/ml plasma. The estimated IC_{50} of draflazine in whole blood was 67.6 ng/ml. The predicted Hill factor describing the steepness of the sigmoidal E_{max} relationship was 1.0 for draflazine in plasma and 3.1 for draflazine in whole blood, which demonstrates that the blood concentration-ABI relationship was much steeper than the plasma concentration-ABI relationship (fig. 7).

**Simulations on basis of the RBC model.** Figure 8 shows the predicted increase in t_{1/2,ADO} as a function of the steady-state RBC occupancy of draflazine. Hardly any increase in t_{1/2,ADO} was predicted below a RBC occupancy of about 60% (fig. 8). A 50% increase in t_{1/2,ADO} was predicted for a RBC occupancy of about 87%. A 2- and 3-fold increase in t_{1/2,ADO} was predicted for RBC occupancies of about 94% and 96%, respectively (fig. 8). Finally, the increase in t_{1/2,ADO} was predicted to be almost 5-fold and 9-fold for a RBC occupancy of 98% and 99%, respectively (data not depicted).
Discussion

The ex vivo determination of the ABI was mimicked by the ADO transport and breakdown RBC kinetic model. In this model, ADO and INO was transported inside and outside the erythrocyte by the nucleoside transporter with a $V_{\text{maxT}}$ of 28 pmol/μl cell water · sec and a $K_{\text{mT}}$ of 60 μM. Draflazine was assumed to be a competitive inhibitor of the nucleoside transporter, which implies that the inhibitor draflazine displaces ADO as well as INO from the transporter (Segal, 1959; Wigler and Alberty, 1960). The additional inhibitory effects of nucleoside transporter inhibitors on the rate of efflux of INO was demonstrated previously by Van Belle and Janssen (1991). Intracellular ADO either will be transported outside the cell by the nucleoside transporter or will be deaminated by ADA into inactive INO. The $V_{\text{maxADA}}$ was reported to be 1.5 pmol/μl cell water · sec, which indicates that the maximal velocity of ADA was more than 18 times lower than the $V_{\text{maxT}}$. The $K_{\text{mADA}}$ was 32 μM and was in the same order of magnitude as the $K_{\text{mT}}$. The comparison of the kinetic parameters of ADO transport and ADO deamination thus demonstrated that, in the absence of draflazine, ADA is the rate-limiting step in the catabolism of ADO. The $K_{\text{mPNP}}$ was also in the same order of magnitude as the $K_{\text{mT}}$ and the $K_{\text{mADA}}$. However, the $V_{\text{maxPNP}}$ was about 5-fold higher than the $V_{\text{maxDET}}$. Consequently, INO will be catabolized further into HYPO relatively rapidly (fig. 2).

Figure 2 further depicts that the ADO, INO and HYPO concentration-time profiles could be predicted until all added ADO was transported into the erythrocytes and subsequently converted by intracellular ADA and PNP. In the absence of draflazine, all ADO was broken down after an incubation period of about 120 min. At that time point, only HYPO was present in the incubate (fig. 2). It is also clear from this figure that after an incubation period of 20 min, which is used for the ex vivo determination of the ABI, still a total concentration of more than 25 μM ADO was present in the RBC suspension. It is obvious that a relatively extensive series of ex vivo pharmacological experiments in human erythrocytes as well as several HPLC assays are needed to obtain results similar to those depicted in figure 2. Moreover, at each incubation time point, extra- as well as intracellular concentrations of ADO and INO can be predicted by the RBC model, whereas only total concentrations of these nucleosides can be measured ex vivo.

When not only ADO but also draflazine was present in the incubate, the competitive transport inhibitor draflazine prevented the transport of ADO and INO into the erythrocytes. Consequently, less ADO was converted and consequently less INO and HYPO was present in the incubate. However, figure 3 depicts that hardly any change in the conversion of ADO was present for a RBC occupancy of draflazine of less than 50%. The most substantial changes were observed between the RBC occupancy range of 90 to 99% (fig. 3). From equations 1 to 4 of the ADO transport and breakdown RBC model it becomes clear that draflazine reduced the $V_{\text{maxT}}$ by the
term “1 – OccRBC.” A 10- to 100-fold reduction of the $V_{\text{max}}$, (RBC occupancy between 90 and 99%) will provide substantially less intracellular ADO, so that ADO no longer is deaminated at the maximal rate. In this situation, the nucleoside transport into the erythrocytes becomes the rate-limiting step so that the breakdown of ADO will be inhibited substantially (fig. 3).

Because nucleoside transport becomes the rate-limiting step in the ADO breakdown only from a substantial RBC occupancy of draflazine onward, no 1:1 relationship was found when the RBC occupancy was plotted against the predicted ABI (fig. 4). The absence of this 1:1 relationship explains the apparent discrepancy between the previously reported dissociation constant $K_d$ for RBC occupation of 0.385 ng/ml plasma (Snoeck et al., 1997a) and the more than 17-fold higher predicted IC$_{50}$ for ABI of 6.8 ng/ml plasma. When the plasma concentration of draflazine is equal to the $K_d$, the RBC occupancy of draflazine will be 50%, whereas the ABI is only about 5% (fig. 4). On the other hand, when the plasma concentration of draflazine is equal to the IC$_{50}$, the ABI will be 50%, whereas the RBC occupancy in that case is almost 95% (fig. 4).

Figure 4 also demonstrates the validity of the RBC model, because the predicted ABI corresponded well with the ABI measured ex vivo after different infusion schemes of draflazine administered to healthy subjects. The good resemblance between predicted and measured ABI allowed us to estimate the ABI without taking additional blood samples for the determination ex vivo. For future studies, the RBC occupancy of draflazine can be predicted based on the measured plasma concentration of draflazine and by use of the population parameter typical value of $K_d$ (equation 8). Then, the ABI can be predicted based on the calculated RBC occupancy of draflazine by use of the RBC model. Because the estimated interindividual variability in $K_d$ is low (CV 13.1%), the determination of the plasma concentrations of draflazine during clinical studies may permit us to estimate the RBC occupancy of draflazine as well as the ABI, with both serving as pharmacodynamic endpoints.

After validation of the ADO transport and breakdown RBC kinetic model, this model may be used to further predict and evaluate the ABI for different experimental conditions. Different ADO concentrations added to the incubate as well as different incubation times had no relevant influence on the predicted ABI (fig. 5, A and B). Previously, it was demonstrated that the addition of 100 μM ADO instead of 40 μM ADO indeed resulted in minor changes in the ABI (Van Belle et al., 1991). The minimal changes in the ABI caused by alterations in the experimental conditions make the ABI a valid and robust pharmacodynamic endpoint. The ABI measured ex vivo was determined in a RBC suspension. However, in humans, whole blood is present instead of a RBC suspension. For this reason, the ABI was predicted for whole blood and was compared with the ABI for the RBC suspension (fig. 6). Because the total number of erythrocytes in whole blood was much higher than that of the RBC suspension, the total amount of ADO breakdown capacity was much higher than that of the RBC suspension. Consequently, the incubation time needed for a final ADO concentration of 25 μM was only 10 sec in whole blood and 20 min in a 1.67% RBC suspension. For this reason, the ABI was simulated with a 10-sec incubation time in whole blood and with a 20-min incubation time in the RBC suspension (fig. 6). For a RBC occupancy of 70% onward, the ABI in whole blood was somewhat higher than the ABI in the RBC suspension. However, because the differences were only small, the ABI in the RBC suspension may serve as a useful pharmacodynamic endpoint in dose-ranging studies of draflazine.

A sigmoidal $E_{\text{max}}$ relationship was present between the plasma concentration of draflazine and the predicted ABI, and between the predicted whole blood concentration of draflazine and the predicted ABI. In a previous study with healthy subjects, the population typical values of IC$_{50}$ and Hill factor $\gamma$ in plasma were estimated to be 3.76 ng/ml and 1.06, respectively (Snoeck et al., 1997a). The typical values of IC$_{50}$ and $\gamma$ in whole blood were estimated to be 65.7 ng/ml and 4.47, respectively. By use of the estimates of the population parameter typical values of the specific RBC binding parameters of draflazine ($K_d = 0.385$ ng/ml plasma; $B_{\text{max}} = 158$ ng/ml RBC) in combination with the ADO transport and breakdown RBC kinetic model, the predicted IC$_{50}$ value was 6.8 ng/ml for plasma and 67.6 ng/ml for whole blood. The predicted Hill factor $\gamma$ was 1.0 for plasma and 3.1 for whole blood. The predicted parameters of the sigmoidal $E_{\text{max}}$ relationship in plasma and whole blood corresponded well with the previously determined parameters in studies with healthy subjects, which demonstrates that these relationships could be described well by the specific capacity-limited high-affinity binding of draflazine to the erythrocytes and the kinetics of ADO transport and breakdown in the erythrocytes. In most reported combined PK-PD studies, the parameters of PK-PD relationships are evaluated only descriptively. Unfortunately, only a few examples of drugs are known for which the reasons the observed PK-PD parameters are as they are explained. Draflazine is one of these rare examples.

Figure 8 shows an example of the use of the ADO transport and breakdown RBC model to mimic the clinical situation. In the absence of a draflazine infusion (OccRBC, 0%), ADO will be broken down rapidly by the erythrocytes, which results in a $t_{1/2,\text{ADO}}$ of only about 10 sec (Klabunde, 1983). However, when draflazine is infused, the $t_{1/2,\text{ADO}}$ will increase with increasing RBC occupancies and thus in a dose-related manner. A steady-state RBC occupancy of 90 to 92%, 93 to 96% and 96% was reported in a previous study with healthy subjects after a 15-min i.v. infusion of 1 mg draflazine followed by continuous infusions of 0.25, 0.5 and 1 mg/h, respectively (Snoeck et al., 1997b). On basis of the model, a 70% increase in $t_{1/2,\text{ADO}}$ is predicted as the steady state of a draflazine infusion of 0.25 mg/h, and a 2- and 3-fold increase in $t_{1/2,\text{ADO}}$ is predicted at the steady state of an infusion of 0.5 and 1 mg/h, respectively. These simulations may be very helpful for the design of optimal dosing schemes. In CABG patients, however, the myocardial rather than the systemic ADO concentration will be increased and must be prolonged. ADO is released in the interstitial space of the heart and will be transported and metabolized by the endothelial cells. To validate the model completely, the in vitro specific binding characteristics of draflazine to the nucleoside transporters located on the endothelial cells of the myocardium also must be investigated. In a previous displacement study with [3H]nitrobenzylthioinosine, the specific binding characteristics to the human myocardium were investigated (Böhm et al., 1994). Unfortunately, this in vitro experiment was per-
formed with the racemate instead of the active (−)-enantio-
mer drafalazine.

In the present study, the apparent discrepancy between the RBC occupancy and the ex vivo measured ABI was explained. Furthermore, the relationships between the ex vivo measured ABI and the plasma and whole blood concentrations of drafalazine also were explained. However, the plasma and whole blood concentration-time profiles and consequently also the RBC occupancy-time profiles of drafalazine after different infusions of the drug still cannot be predicted. For drugs that are bound with high affinity to pharmacological target sites, a considerably large fraction of the dose is bound to target sites so that the specific binding will have an impact on the pharmacokinetic characteristics of the drug (Levy, 1994). To predict the disposition of drafalazine and its RBC occupancy, a model that integrates both pharmacokinetic and specific target site binding phenomena of drafalazine still needs to be developed.

In summary, the predicted ABI based on the physiological ADO transport and breakdown RBC kinetic model corresponded well with the ABI measured ex vivo. The apparent discrepancy between the ex vivo measured ABI and the nucleoside transporter occupancy of drafalazine could be explained on the basis of the model. ADO deamination by intracellular ADA rather than ADO transport by the nucleoside transporter was the rate-limiting step in the overall metabolism of ADO. Consequently, a RBC occupancy of at least 90% is needed for a substantial inhibition of ADO breakdown. The validated model was used to predict the ABI for different experimental conditions and to mimic the clinical situation. The latter could be very helpful for the design of optimal dosing schemes of drafalazine. It was demonstrated that the short half-life of released ADO was prolonged substantially as a function of increasing infusion rates of drafalazine. Finally, the previously reported very different sigmoidal E°max relationships between the ex vivo measured ABI and the concentrations of drafalazine in plasma and whole blood could be explained by the specific RBC binding characteristics of drafalazine and the kinetics of ADO transport and breakdown in the erythrocytes.

References


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