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REDUCTION OF DAPSONE HYDROXYLAMINE TO DAPSONE DURING METHAEMOGLOBIN FORMATION IN HUMAN ERYTHROCYTES *IN VITRO*—II*

MOVEMENT OF DAPSONE ACROSS A SEMIPERMEABLE MEMBRANE INTO ERYTHROCYTES AND PLASMA

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Abstract—We have used an *in vitro* two-compartment model, to investigate the ability of dapsone, formed by erythrocyte-mediated detoxification of its hydroxylamine metabolite, to escape the cells and cross a semi-permeable membrane into both plasma and other erythrocytes. Both diethyl dithiocarbamate (DDC) treated and untreated erythrocytes were incubated with dapsone hydroxylamine and dialysed against either fresh cells or plasma. Methaemoglobin was predominantly detectable in compartment A although the presence of low levels of methaemoglobin in compartment B indicated that the hydroxylamine itself had crossed the membrane. In contrast to methaemoglobin disposition, recovery of dapsone was higher ($P < 0.05$) in compartment B compared with A for all three treatment groups at 30 and 60 min, but not at the remaining time points. Regression analysis of the cumulative recovery of dapsone over 150 min in all three treatment groups for both compartments A and B showed correlation coefficients close to unity. In compartment A, analysis of the mean slopes of the regression lines indicated that, overall, significantly more dapsone was recovered from group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) (0.22 ± 0.05 vs 0.09 ± 0.005; $P < 0.025$). Also in compartment A, significantly more dapsone was recovered from group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma: 0.16 ± 0.02 vs 0.09 ± 0.005). In compartment B, dapsone recovery was significantly greater in group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells; slope of regression line: 0.59 ± 0.05) compared with group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells; slope of line: 0.28 ± 0.02, $P < 0.005$). In addition, dapsone recovery was significantly greater in group 1 (0.59 ± 0.05) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma; 0.21 ± 0.02, $P < 0.005$). Dialysis of erythrocytes with dapsone itself over 120 min caused no detectable methaemoglobin formation. The process of erythrocyte-mediated dapsone formation from its hydroxylamine may feasibly occur *in vivo* and contribute to the systemic persistence and therapeutic effect of dapsone.

Dapsone is used in a variety of disease states, such as *Pneumocystis carinii* pneumonia, malaria and leprosy [1–3]. It is also a potent and rapid acting anti-inflammatory agent [4, 5]. The primary route of clearance of dapsone is mediated via hepatic cytochrome P450 isozyme CYP3A4 [6]. The resultant hydroxylamines are eliminated in urine as glucuronides although a considerable proportion of the hydroxylamine metabolites escape the liver and cause the well-known haematological toxicity of the drug [7]. Thus, methaemoglobinaemia, haemolysis and accelerated erythrocyte destruction [8] may lead to acute toxicity in some patients [9, 10] and anaemia in others [11]. Although both normal and abnormal erythrocytes are vulnerable to damage inflicted by aromatic amine metabolites, they have also been shown to detoxify these metabolites. In the rat, the hydroxylamine of 4-amino biphenyl, which structurally resembles dapsone, is reduced by erythrocytes to the parent amine [12]. In a previous report *in vitro*, we demonstrated that during the process of methaemoglobin formation, human erythrocytes could convert the hydroxylamine of dapsone to the parent compound [13]. The present *in vitro* study is intended to determine if dapsone thus formed is able to escape the erythrocyte and traverse a semi-permeable membrane into untreated human erythrocytes or plasma.

MATERIALS AND METHODS

Dapsone hydroxylamine was provided by the Jacobus Pharmaceutical Co. Inc. (Princeton, NJ, U.S.A.), and was found to be 97% pure by HPLC. Dapsone and the internal standard for the HPLC assay (3,3′-diaminodiphenyl sulphone was obtained from the Aldrich Chemical Co. (Poole, U.K.). All HPLC solvents were supplied by Fisons Ltd

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(Loughborough, U.K.). Whole human blood was drawn from healthy volunteers and anti-coagulated with sodium heparin and placed on ice. The erythrocytes were separated from the plasma and the buffy coat and top layer of cells removed. The cells were then washed twice in equal volumes of PBGS* and resuspended to a 50% haematocrit also in PBGS. Three experimental groups (1–3) were set up. Group 1 consisted of 20 600 μL aliquots of erythrocytes resuspended in PBGS, which were placed in 1.5 mL microcap tubes and placed in a waterbath at 37°. DDC was added in PBGS to give a final concentration of 5 mM to 18 of the 20 aliquots; the remaining two aliquots were untreated controls. After 2 min, dapsone hydroxylamine (0.075 μmol in 5 μL of acetone) was added to 15 of the 20 aliquots. The samples were then gently shaken and incubated at 37° for 1 min. All the samples (including the control incubations) were then centrifuged at 10,000 rpm for 2 min then washed twice with 400 μL PBGS to remove excess dapsone hydroxylamine. The aliquots were transferred to a Dianorm equilibrium dialysis system (Dianorm-Gerate, Maierhofer, Munich, F.R.G.). The apparatus consisted of a series of 20 bicompartmental teflon cells, where each cell was divided into two compartments, A and B, by a cellulose dialysis membrane (molecular weight cut-off, 5000). The washed samples were inserted into compartment A of the 20 Dianorm cells. DDC was again added to the samples to give a final concentration of 5 mM to 18 of the 20 samples. An equal volume of untreated resuspended erythrocytes was placed in each B compartment of all 20 cells. Thus, the apparatus comprised 15 test cells (N = 3 per time point, 30, 60, 90, 120 and 150 min) containing hydroxylamine, erythrocytes and DDC; three cells containing erythrocytes and DDC and the two remaining cells filled with erythrocytes alone. The apparatus was then incubated in a waterbath at 37°, each cell rotating at 8 rpm. At 30 min, the apparatus was removed from the waterbath and the entire contents of the first three cells, compartments A and B were expelled into individual microcap tubes and kept on ice. In the remainder of the cells, only the contents of compartment B were expelled and discarded, then refilled with fresh untreated erythrocyte suspension. The apparatus was then returned to the waterbath for a further 30 min. This process was repeated for each subsequent time point. Between time points, a 100 μL aliquot from each sample was immediately assayed for methaemoglobin formation using the spectrophotometric method of Harrison and Jollow [14]. The remainder of the samples were frozen at −20° until assayed by the HPLC method of Grossman and Jollow [15]. The whole process described above was repeated at 60, 90, 120 and 150 min. Hence, samples from both compartments A and B were derived for all five time points. Methaemoglobin levels were corrected for control (untreated erythrocytes) methaemoglobin values of 0.7% and incubations containing DDC and erythrocytes (1.2%). Experimental group 2 was treated as above except that DDC was not added to any incubations. Experimental group 3 was treated as for group 2, except all the samples in compartment A were dialysed against fresh human plasma in compartment B.

In a separate experiment, washed erythrocytes spiked with dapsone (delivered in 5 μL DMSO, N = 3) to give a final concentration of 500 μM in compartment A were dialysed against untreated washed cells (compartment B). This experiment was also carried out in the absence of dapsone (N = 3). Methaemoglobin levels were measured in both compartments at 120 min. In order to determine if DDC preserved the hydroxylamine, DDC was compared with ascorbate in a stability study involving dapsone hydroxylamine *in vitro*. Three sets (N = 4 per set) of uncapped tubes containing PBGS (500 μL) were incubated at 37°. DDC was added to the first set of tubes and ascorbate to the second set, to give a final concentration of 5 mM for both compounds. Dapsone hydroxylamine (20 μG) was added to all three sets. After a 30 min incubation, the samples were analysed for dapsone hydroxylamine by HPLC.

Recovery of dapsone from the compartments was measured by the HPLC assay in micrograms, then converted to micromoles and expressed as a percentage of the number of micromoles of dapsone hydroxylamine [13] originally added to the samples. Statistical comparisons were made using Student's *t*-test accepting $P < 0.05$ as significant. Where more than one comparison was made with the same data, the Bonferroni correction was employed, where the acceptable level of significance was reduced to $0.05/k$ (where k is the number of tests) to compensate for the increased likelihood of reaching $P < 0.05$ during multiple testing.

RESULTS

Dapsone hydroxylamine-dependent methaemoglobin levels in compartment A ranged between 50 and 70% over the total study period, did not significantly differ between the three experimental groups and greatly exceeded those levels measured in compartment B (Fig. 1). Also in compartment B, methaemoglobin levels at 30 and 60 min were significantly higher in group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) compared with group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells).

In compartment A dapsone recovery was not significantly different between groups 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) and 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) at all five time points (Fig. 2). However, significantly more dapsone was recovered from group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) at 30 ($P < 0.025$), 60 ($P < 0.01$) and 120 ($P < 0.025$) min, although there were no significant differences at 90 and 150 min. Significantly more dapsone was recovered from group 2 (erythrocytes and hydroxylamine dialysed against untreated red

* Abbreviations: PBGS, phosphate-buffered (pH 7.4) 0.9% saline containing 10 mM glucose; DDC, diethyl dithiocarbamate.

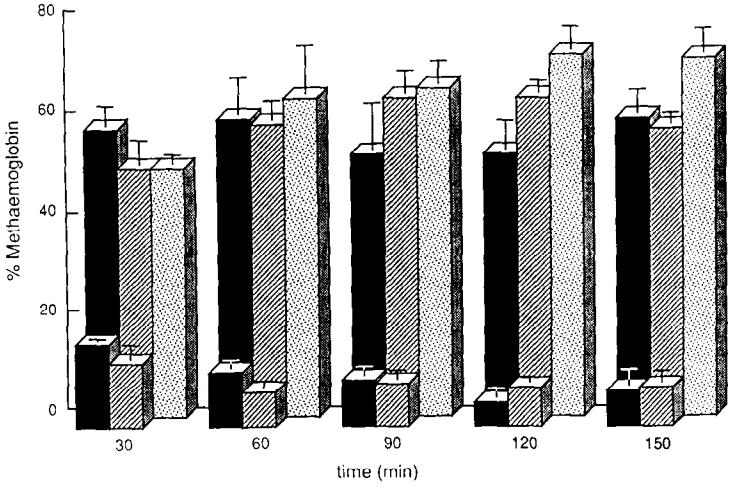

Fig. 1. Back row: methaemoglobin formation in compartment A against time after the addition of dapsone hydroxylamine (0.075 μmol) to human erythrocytes (N = 3 per group). Front row: methaemoglobin formation in compartment B against time after the addition of dapsone hydroxylamine (0.075 μmol) to human erythrocytes (N = 3 per group). Group 1: (■) erythrocytes, hydroxylamine and DDC dialysed against untreated red cells. Group 2: (▨) erythrocytes and hydroxylamine dialysed against untreated red cells. Group 3: (▩) erythrocytes and hydroxylamine dialysed against plasma.

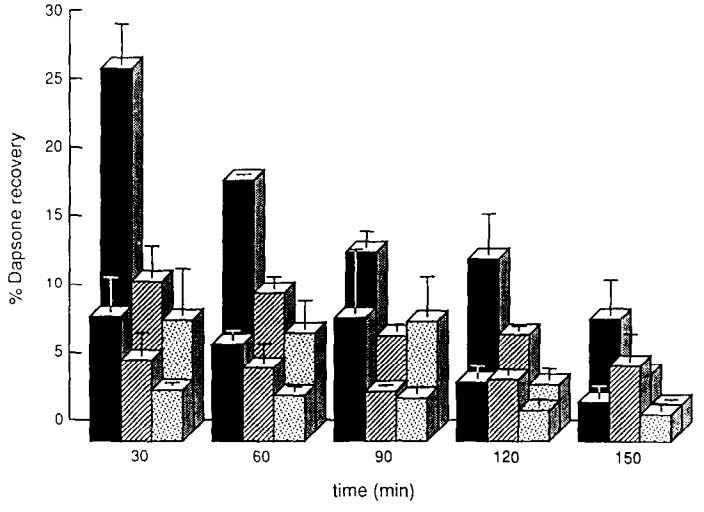

Fig. 2. Back row: dapsone recovery in compartment B against time after the addition of dapsone hydroxylamine (0.075 μmol) to human erythrocytes (N = 3 per group). Front row: dapsone recovery in compartment A against time after the addition of dapsone hydroxylamine (0.075 μmol) to human erythrocytes (N = 3 per group). Group 1: (■) erythrocytes, hydroxylamine and DDC dialysed against untreated red cells. Group 2: (▨) erythrocytes and hydroxylamine dialysed against untreated red cells. Group 3: (▩) erythrocytes and hydroxylamine dialysed against plasma.

cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) at 30 ($P < 0.01$), 120 ($P < 0.05$) and 150 ($P < 0.025$) min, although there were no significant changes at 60 and 90 min.

Overall recovery of dapsone was higher ($P < 0.05$) in compartment B compared with A for all three treatment groups at 30 and 60 min, but not at the remaining time points (Fig. 2). In compartment B significantly more dapsone was recovered from group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) in comparison with group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) at 30 ($P < 0.01$), 60 ($P < 0.01$), 90 ($P < 0.01$) and 150 ($P < 0.025$) min, although there was no significant difference at 120 min. Significantly more dapsone was recovered from group 1 (erythrocytes, hydroxylamine and DDC

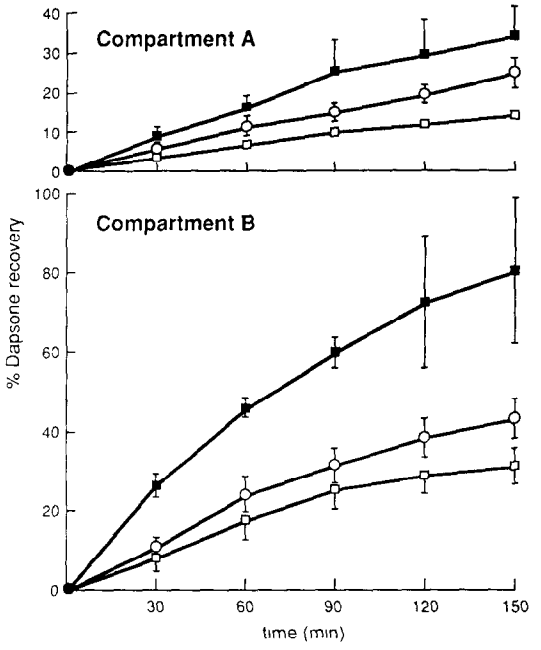

Fig. 3. Regression analysis of dapsone recovery in compartments A and B after the addition of dapsone hydroxylamine (0.075 μmol) to human erythrocytes (N = 3 per group). Group 1: (■) erythrocytes, hydroxylamine and DDC dialysed against untreated red cells. Group 2: (○) erythrocytes and hydroxylamine dialysed against untreated red cells. Group 3: (□) erythrocytes and hydroxylamine dialysed against plasma.

dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) at 30 ($P < 0.001$), 60 ($P < 0.005$) and 150 ($P < 0.01$) min. Dapsone levels in group 3 plasma (erythrocytes and hydroxylamine dialysed against plasma) were only significantly lower than in group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) at 120 and 150 min ($P < 0.05$).

Regression analysis of the cumulative recovery of dapsone over 150 min in all three treatment groups for both compartments A and B showed correlation coefficients close to unity (Fig. 3). In compartment A, correlation coefficients for groups 1–3 were 0.98 ± 0.01, 0.995 ± 0.003 and 0.97 ± 0.01, respectively. Analysis of the mean slopes of the regression lines indicated that, overall, significantly more dapsone was recovered from group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) (0.22 ± 0.05 vs 0.09 ± 0.005; $P < 0.025$). Also in compartment A, significantly more dapsone was recovered from group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) (0.16 ± 0.02 vs 0.09 ± 0.005). There was no significant difference between the slopes of groups 1 and 2 (0.22 ± 0.05 vs 0.16 ± 0.02).

In compartment B, correlation coefficients after regression analysis were 0.98 ± 0.001, 0.98 ± 0.009 and 0.97 ± 0.01 for groups 1–3, respectively. Overall, dapsone recovery was significantly greater in group 1 (erythrocytes, hydroxylamine and DDC dialysed against untreated red cells; slope of regression line: 0.59 ± 0.05) compared with group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells; slope of line: 0.28 ± 0.02, $P < 0.005$). In addition, dapsone recovery was significantly greater in group 1 (0.59 ± 0.05) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma; 0.21 ± 0.02, $P < 0.005$, Fig. 3). There was no significant difference in dapsone recovery between groups 2 and 3. In control dialysis studies, methaemoglobin levels in the presence of dapsone (compartment A: 1.6 ± 0.17; compartment B: 1.5 ± 0.35) were not significantly different from control (compartment A: 1.6 ± 0.8; compartment B: 1.0 ± 0.35). Although 94.2 ± 9.0% of the hydroxylamine was present after 30 min incubation with ascorbate, oxidation of the hydroxylamine was not significantly different in the presence of DDC compared with the metabolite alone (5.1 ± 1.5 vs 9.0 ± 3.2%).

DISCUSSION

There is considerable evidence that erythrocytes are capable of metabolizing a range of xenobiotic compounds such as 2-aminofluorine [16] and aniline [17]. These oxidative reactions normally associated with hepatic cytochrome P-450 are mediated by haemoglobin which readily undergoes redox reactions within the erythrocyte [18]. Aside from the ability of these cells to counteract the formation of unstable oxygen species routinely produced by haemoglobin, it is also clear that metabolites produced by the liver such as the hydroxylamines of 4-aminobiphenyl and dapsone may also be detoxified by erythrocytes [12, 13]. Hence, through the actions of haemoglobin, glutathione and other enzyme systems such as *N*-acetyl transferase [19], it appears that erythrocytes are not merely targets for hepatically derived toxins, but they resemble hepatocytes in their ability to activate and detoxify oxidative xenobiotic compounds. In the case of the hepatocyte, an imbalance in the formation and detoxification of oxidative species may lead to covalent binding and cell death; with erythrocytes, dapsone hydroxylamine is thought to cause attachment of haemoglobin to the skeletal protein meshwork of the cell [20], leading to exposure of senescent antigenic sites [21]. Thus, splenic sequestration is promoted, leading to reduced red cell lifespan. However, to date, the ability of erythrocytic detoxification processes to influence potentially the disposition and therapeutic effect of drugs such as dapsone has not been evaluated.

Using the two-compartment model in this study, the disposition of dapsone hydroxylamine-dependent methaemoglobin in the erythrocytes contrasted sharply with that of dapsone itself. It is likely that the hydroxylamine was converted to dapsone in the process of methaemoglobin formation in compartment A as described previously [13]. The

recovery of dapsone at the early time points in compartment B was greater than in compartment A in all experimental groups. The process of dapsone formation within erythrocytes is relatively rapid, linear and virtually complete at 30 min [13]. This probably led to a high local concentration of dapsone in compartment A, which quickly diffused down the concentration gradient across the semipermeable membrane where it was retained by the contents of compartment B long enough to be recovered before complete equilibration could occur.

Although at three time points, more dapsone was recovered from compartment A in group 2 (erythrocytes and hydroxylamine dialysed against untreated red cells) compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma), this trend did not continue in compartment B. Although at the early time points both erythrocytes and plasma in compartment B may have retained dapsone, erythrocytes did not appear to concentrate dapsone preferentially compared with plasma. The presence of DDC caused a marked increase in dapsone recovery compared with group 3 (erythrocytes and hydroxylamine dialysed against plasma) in both compartments, and compared with group 2 in compartment B. In our previous study, DDC, although a weak producer of methaemoglobin itself, was shown to accelerate hydroxylamine reduction to dapsone during methaemoglobin formation [13]. DDC is known to inhibit superoxide dismutase and deplete erythrocyte glutathione [22]; indeed, dapsone hydroxylamine caused significantly more rapid glutathione depletion in erythrocytes in the presence of DDC [13]. In order to account for the evident synergy between the two compounds it was suggested in our previous study that the thiol-containing DDC may have stabilized the hydroxylamine in the aqueous phase, thus allowing penetration of a greater proportion of the metabolite to enter the erythrocyte [13]. However, this study has indicated that this is not the case as DDC does not affect the stability of the hydroxylamine in buffer at pH 7.4. Therefore, it is probable that DDC in some way catalyses an acceleration in the redox cycling of dapsone hydroxylamine within the erythrocyte. Irrespective of its actual mode of action, DDC greatly increased the process of dapsone production from the hydroxylamine, which led to a net increase in dapsone movement across the membrane into compartment B compared with other groups.

Overall, it might be suggested that the process of dapsone formation through detoxification may occur *in vivo*, and due to the lack of an erythrocyte-mediated concentrating effect with dapsone in comparison with plasma, it is probable that the drug may leave the red cells and travel by simple diffusion into extracellular tissue fluid. Interestingly, the hydroxylamine is known to be rapidly taken up into erythrocytes [23] to the extent that mononuclear leucocytes are protected from hydroxylamine-mediated toxicity by the proximity of erythrocytes separated by a semipermeable membrane identical to that used in the present study [24]. The metabolite has never been detected in human plasma although it has been indirectly assayed in the rat [15]. It is conceivable that the whole process of rapid uptake of hydroxylamine from the liver by erythrocytes, conversion to dapsone and then release of the drug from the erythrocyte may make delivery of dapsone to tissues such as the skin slightly more efficient compared with other drugs which do not undergo these processes.

Evidence from this study also supports the hypothesis that dapsone and aromatic amines such as 4-aminobiphenyl undergo a form a systemic cycling, where hydroxylamines are formed in the liver by cytochrome P450, escape and are rapidly taken up by the erythrocytes, which reform a significant proportion of these species to the parent drug, which may return to the liver for re-hydroxylation [12, 13]. This cycle may account for the persistence of dapsone in blood and tissues, when it is comparatively rapidly metabolized in man [7].

The observation that methaemoglobin formation occurred in compartment B would indicate that the hydroxylamine travelled across the membrane from compartment A to B and its effect was thus promoted by the presence of DDC. Although the cells were washed twice in PBGS after the hydroxylamine was added, it is possible that some of the hydroxylamine was not taken up by the cells and caused methaemoglobin formation in compartment B by crossing the membrane. This may be unlikely, as this metabolite is avidly taken up by the erythrocytes into the methaemoglobin-forming redox cycling process [25]. In addition, the erythrocytes in compartment B could not have made the hydroxylamine out of dapsone arriving from compartment A, as in contrast to other arylamines [17], in this study, erythrocytes could not oxidize dapsone to the hydroxylamine. Hence, it is possible that dapsone hydroxylamine may enter an erythrocyte and the Kiese cycle, escape reduction to dapsone, as well as binding to glutathione or other thiols, and then leave the cell intact. If this were to occur, the hydroxylamine may be delivered by erythrocytes to tissues vulnerable to dapsone toxicity such as the bone marrow [26] *in vivo*. The inability of erythrocytes to oxidize dapsone is probably due to the electron withdrawing nature of the sulphone group, which leads to a lower electron density on the amine groups rendering them less reactive than aromatic amines such as aniline [27].

In summary, we have used a two-compartment *in vitro* system to illustrate the ability of dapsone formed by erythrocyte-mediated detoxification of dapsone hydroxylamine to escape the cell, cross a semipermeable membrane and enter untreated erythrocytes and plasma. It is possible that this process occurs *in vivo* and contributes to the systemic persistence and therapeutic effect of dapsone.

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