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Amino acid derivatives of dapsone as water-soluble prodrugs

Nancy L. Pochopin [a,1], William N. Charman [b], Valentino J. Stella [a,*]

[a] *Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, USA*
[b] *School of Pharmaceutics, Victorian College of Pharmacy, Parkville, Victoria 3052, Australia*

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Abstract

Amino acid amides of dapsone, a primary aromatic amine, have been synthesized as potential water-soluble, chemically stable prodrugs that target peptidase enzymes for cleavage to the parent drug in vivo. The alanine, glycine, leucine, lysine, and phenylalanine derivatives of dapsone all exhibited greater aqueous solubility, as their salts, than the parent compound. Solution half-lives ranged from 1 to 12 h at pH 7.4 and 73° C. In the presence of aminopeptidases and in whole blood, rapid enzymatic hydrolysis resulted in quantitative conversion to dapsone. The good chemical stability and rapid enzymatic hydrolysis in vitro suggest that these compounds would be good prodrug candidates.

Keywords: Prodrug; Dapsone; Stability; Amino acid; Aminopeptidase; Blood

1. Introduction

Amino acid prodrugs have been investigated in the past as promoieties to improve the aqueous solubility of various alcohol and amine drugs (Kovach et al., 1975; Amidon et al., 1980; Yalkowsky, 1981; Bundgaard et al., 1984a,b; Cho and Haynes, 1985; Johnson et al., 1985; Aggarwal et al., 1990; Upshall et al., 1990). Major advantages of these derivatives include good water solubility and rapid and quantitative bioreversion by the action of esterases and/or peptidases. Most previous studies have been carried out with amino acid esters, which have limited chemical stability. Amino acids have also been used as promoieties with drug containing carboxylate groups (Persico et al., 1988; Springer et al., 1990), but prodrugs of that type will not be addressed here.

This approach is also applicable to amine drugs. As a class, primary aromatic amines tend to have poor aqueous solubility and therefore would be good candidates for derivatization with amino acids. The resulting amide bond should be more chemically stable than an ester. A previous paper described amino acid prodrugs of prazosin which were of limited utility due to rapid non-hydrolytic degradation (Pochopin et al., 1994a). This was attributed to the nature of the quinazoline ring of prazosin. Dapsone (Fig. 1) was used as a model compound to explore the feasibility of a soluble amino acid amide prodrug with good

* Corresponding author.
[1] Present address: Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4755, Syracuse, NY 13221, USA.

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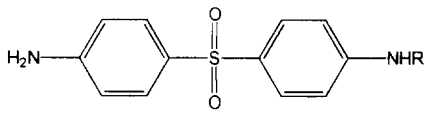

Fig. 1. Structures of dapsone and prodrugs.

R =	
-H	dapsone
-L-alanine	L-Ala-dapsone
-L-leucine	L-Leu-dapsone
-L-phenylalanine	L-Phe-dapsone
-glycine	Gly-dapsone
-L-lysine	L-Lys-dapsone
-D-alanine	D-Ala-dapsone
-D-leucine	D-Leu-dapsone
-D-phenylalanine	D-Phe-dapsone
-DL-alanine	DL-Ala-dapsone
-DL-leucine	DL-Leu-dapsone
-DL-phenylalanine	DL-Phe-dapsone
$-COCH_2CH_3$	monopropionyldapsone
$-COCH_3$	monoacetyldapsone

chemical stability and rapid in vivo conversion to the parent drug. Dapsone was chosen because the only derivatizable functional group is an aromatic amine, and the potential for alternate routes of degradation was limited. The synthesis, physical-chemical properties and in vitro chemical and enzymatic stability of prodrugs of dapsone are discussed. The in vivo conversion is the subject of another paper (Pochopin et al., 1994b).

2. Materials and methods

2.1. Materials

Dapsone, dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were obtained from Aldrich Chemical Co., Milwaukee, WI. Amino acids, leucine aminopeptidase, type IV-S from porcine kidney microsomes as a suspension in 3.5 M $(NH_4)_2SO_4$ pH 7.7 containing 10 mM $MgCl_2$, leucine *p*-nitroanilide, trypsin type IX from porcine pancreas, benzoyl-DL-arginine-*p*-nitroanilide, α-chymotrypsin type II from bovine pancreas, benzoyl-L-tyrosine-*p*-nitroanilide, *p*-nitroaniline, 1,10-phenanthroline, EDTA and bestatin were obtained from Sigma Chemical Co., St. Louis, MO. Solvents were HPLC grade and were obtained from Mallinckrodt Australia Pty Ltd, Clayton, Victoria, Australia. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories, UK. All other reagents were analytical grade.

Fresh blood was obtained in sodium heparin (15 U/ml) from female New Zealand white rabbits, male and female Wistar rats, and human volunteers. After centrifugation, the plasma was removed.

Reversed-phase HPLC was carried out using a Waters 510 HPLC pump (Waters Corporation, Milford, MA), a Perkin-Elmer LC-75 variable-wavelength ultraviolet detector set at 295 nm (Perkin-Elmer Corp., Norwalk, CT), a Shimadzu C-R3A integrator (Shimadzu Corp., Kyoto, Japan) and a Rheodyne injector (Rheodyne, Berkeley, CA) fitted with a 20 μl loop. The analytical column was a Spherisorb ODS-2, 5 μm particle size, 25 cm × 4 mm i.d (Deeside Ind. Est, Queensferry, Clwyd, UK), with a disposable Brownlee MPLC NewGuard precolumn cartridge, 1.5 cm × 3.2 mm i.d., packed with RP-18 silica, 300 Å pore size, 7 μm diameter, in a reusable holder (Applied Biosystems, Foster City, CA).

Ultraviolet spectra were determined using a Cary 2290 UV spectrophotometer (Varian Techtron Pty, Ltd, Australia). NMR spectra were recorded on a Bruker 300 spectrometer. Chemical shifts were referenced to the following internal standards: TMS (tetramethylsilane) in $CDCl_3$, TSP (trimethylsilyl propionic acid) in D_2O, and acetone in acetone-d_6 or acetone-d_6/D_2O. Mass spectra were obtained with a Varian CH-5 or a Ribermag R-10-10 quadrupole mass spectrometer operated in the fast atom bombardment (FAB) mode.

Melting points were determined on a Mel-Temp melting point apparatus (Laboratory Devices, Cambridge) and were uncorrected. Controlled temperatures were maintained at 25 ± 0.5, 37 ± 0.5 and 73 ± 1°C in an AquaTherm Water Bath Shaker (New Brunswick Scientific, New Brunswick, NJ). pH measurements were obtained using a Metrohm 632 pH-meter (Metrohm Herisau, Switzerland) equipped with a combination electrode. Optical rotations were measured

using a Model D Polarimeter and Sodium Lamp Unit, (Bellingham and Stanley Ltd, Kent, UK) with a 20 cm × 3 mm i.d. cell.

2.2. Analytical methods

The mobile phase for HPLC analysis consisted of 25% v/v acetonitrile/75% v/v 0.05 M phosphate buffer at pH 3.50 with 5 mM triethylamine (TEA) at a flow rate of 2.0 ml/min. The amounts of prodrug and dapsone were determined from a calibration curve of peak area response.

Leucine aminopeptidase, chymotrypsin and trypsin were standardized for activity by measuring the release rates of *p*-nitroaniline from leucine *p*-nitroanilide (LpNA), benzoyl-L-tyrosine *p*-nitroanilide (BTpNA) and benzoyl-DL-arginine *p*-nitroanilide (BApNA), respectively, by UV spectroscopy at 410 nm at 37° C.

2.3. Synthesis of prodrugs

4 mmol of the appropriate *t*-butyloxycarbonyl (t-BOC) protected D- or L-amino acid, 4 mmol dapsone, and 4 mmol DCC were stirred in 40 ml of ethyl acetate in an ice bath for 0.5 h, then at room temperature for 5 h. The byproduct dicyclohexylurea (DCU) was removed by filtration, and the ethyl acetate was washed with 2 × 50 ml 1% $KHCO_3$, 50 ml brine, dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified using flash chromatography on 80 g silica with ethyl acetate/toluene 2:1 as the eluent. Products were crystallized from ethyl acetate/toluene. The same process was also carried out in the presence of 10 mg dimethylaminopyridine (DMAP) as a base catalyst.

The t-BOC groups were removed by stirring t-BOC-aminoacyl-dapsone in TFA at room temperature for 2–4 h. The excess TFA was removed under vacuum, the residue dissolved in water and washed with ethyl acetate. The HCl salts were prepared with an anion-exchange column which had been prepared with HCl. The free base was obtained by dissolving the HCl salt in water, adjusting the pH to 9–10 with NaOH and extracting with ethyl acetate. The ethyl acetate was evaporated to dryness and the residue recrystallized as summarized in Table 1. FAB mass spectra exhibited $M + 1$ peaks as expected for each compound.

Monopropionyldapsone was synthesized by stirring 4 mmol each of dapsone and propionic anhydride in 50 ml ethyl acetate at room temperature for 3 h. The reaction was quenched by shaking with 50 ml of 1% $KHCO_3$. The ethyl acetate was then washed with water and brine, dried over $MgSO_4$, and evaporated to dryness.

Table 1
NMR chemical shifts, splitting patterns, and peak integration

Compound	MP (° C)	NMR chemical shifts
L-Ala · HCl [a]	130 (dec)	[f] δ 1.585 (d, 3, J = 7.20); 4.53 (q, 1, J = 7.1); 7.15 (m, 4); 7.74 (m, 4)
L-Leu · HCl [a]	> 215 (dec)	[g] δ 0.942 (d, 3, J = 5.64); 0.963 (d, 3, J = 5.62); 1.4–1.6 (m, 1, J = 9.99, 5.35, 13.7); 1.735 (m, 1, J = 3.84, 8.85, 13.7); 1.85–2.0 (m, 1); 3.62 (m, 1, J = 3.84, 9.99); 7.20 (m, 4); 7.69 (m, 4)
L-Phe base [b]	213–215	[g] δ 2.93 (m, 1, J = 14.2, 8.2); 3.17 (m, 1, J = 3.9, 14.2); 3.93 (m, 1, J = 3.9, 8.2); 7.32 (s, 5); 7.20 (m, 4); 7.77 (m, 4)
Gly base [c]	195 (dec)	[f] δ 4.01 (s, 2); 7.11 (m, 4); 7.73 (m, 4)
L-Lys · HCl [a]	n.d.	[h] δ 1.2–2.1 (m, 6); 2.8–3.0 (m, 2); 3.79 (t, 1, J = 6.16); 7.27 (m, 4); 7.74 (m, 4)
D-Ala · HCl [d]	196	[f] δ 1.62 (d, 3, J = 7.08); 4.33 (q, 1, J = 7.1); 7.12 (m, 4); 7.78 (m, 4)
D-Leu · HCl [a]	140 (dec)	[f] δ 0.915 (d, 6, J = 6.41); 1.6–2.0 (m, 3); 4.40 (t, 1, J = 7.0); 7.41 (m, 4); 7.86 (m, 4)
D-Phe · HCl [a]	185 (dec)	[f] δ 3.25–3.5 (m, 2); 4.63 (m, 1, J = 6.8, 7.3); 6.7–7.9 (m, 13)
DL-Ala base [e]	166–169	[g] δ 1.29 (d, 3, J = 6.8); 3.62 (q, 1, J = 6.8); 7.18 (m, 4); 7.68 (m, 4)
DL-Leu base [b]	> 141 (dec)	[g] δ 0.94 (m, 6); 1.3–1.8 (m, 2); 1.8–2.0 (m, 1); 3.6 (m, 1); 7.19 (m, 4); 7.68 (m, 4)
DL-Phe base [b]	> 211 (dec)	[g] δ 2.93 (m, 1, J = 14.1, 8.3); 3.17 (m, 1, J = 3.9, 14.1); 3.92 (m, 1, J = 3.9, 8.2); 2.8–3.2 (m, 2); 7.33 (s, 5); 7.21 (m, 4); 7.67 (m, 4)

Recrystallization solvent: [a] lyophilized, [b] ethyl acetate, [c] ethyl acetate/toluene, [d] methanol, [e] ethyl acetate/ether; NMR solvent: [f] acetone-d_6; [g] acetone-d_6; [h] D_2O.

White crystals with a melting point of 213.5–215° C were collected from ethyl acetate/toluene. NMR in $CDCl_3$: δ 1.23 (t, 3, $J = 7.6$; propionyl CH_3); δ 2.41 (q, 2, $J = 7.5$; propionyl CH_2); δ 6.4–7.9 (m, 8; dapsone aromatic CH's). FAB mass spectral analysis (Xe-methanol/glycerol/thioglycerol) indicated peaks at m/e 305 ($M + 1$) and 185.

Monoacetyldapsone, a metabolite of dapsone, was prepared by stirring 8 mmol each of dapsone and acetic anhydride in 125 ml of ethyl acetate at room temperature for 24 h. A white precipitate had formed which was filtered and recrystallized twice from methanol to give white crystals with a melting point of 228° C. NMR in $CDCl_3$: δ 2.19 (s, 3; acetyl CH_3); δ 6.6–7.9 (m, 8; aromatic CH's). FAB mass spectral analysis (Xe-methanol/DMSO/thioglycerol/glycerol) indicated peaks at $m/e = 291$ ($M + 1$) and 185.

The optical rotation of each prodrug was measured in HPLC methanol at room temperature (23° C) relative to the sodium D-line.

2.4. Solubility

Visual solubilities of the HCl salts of L-Ala-dapsone, L-Leu-dapsone, Gly-dapsone, and L-Lys-dapsone were measured at room temperature adding water in 100 μl aliquots to a known amount of prodrug until dissolution was complete. The resulting pH of each solution was approx. 3. Equilibrium solubilities in water and pH 7.4 phosphate buffer were measured by adding excess quantities of the prodrug or dapsone to water and shaking in a water bath maintained at 25° C for 1 week. The solutions were then filtered through 0.8 μm filters, appropriately diluted and assayed by HPLC for prodrug or dapsone. No chemical degradation was observed over this time period.

2.5. Solution stability

The solution stability of the prodrugs was assessed at 73° C. Solutions of the HCl salt of L-Leu-dapsone (approx. 20 μg/ml) were prepared in buffers at an ionic strength of 0.15 M with NaCl. The buffers used for each pH were as follows: HCl/NaCl at pH 2.0, sodium acetate at pH 4.0, sodium phosphate at pH 6.0 and 7.4, and sodium borate at pH 10.0. Corrections for temperature were made using a method described previously (Perrin and Dempsey, 1974). In order to check for buffer catalysis, three different buffer concentrations were used (0.0125, 0.025 and 0.05 M). Aliquots were placed in ampules, flame-sealed and maintained at 73° C. Periodically ampules were removed and analyzed by HPLC for dapsone and prodrug. For data collected at pH 2.0 and 4.0, rate constants were calculated using the method of initial rates. For other pH values, rate constants were determined from first-order plots of prodrug concentration vs time.

For the HCl salts of L-Ala-, L-Phe-, Gly- and L-Lys-dapsone degradation was followed at pH 4.0, 6.0, and 7.4 in the same buffers as above. Three buffer concentrations were studied at pH 6.0 and 7.4, but at pH 4.0 degradation was only measured at a buffer concentration of 0.05 M. Solutions were maintained at 73° C in a controlled temperature water bath in sealed vials and periodically 200 μl were removed and the solution acidified with 5 μl 70% $HClO_4$. Samples were stored in the refrigerator until analysis by HPLC for dapsone and prodrug. Rate constants were calculated using the method of initial rates.

2.6. In vitro prodrug reversion

Stability in the presence of peptidase enzymes was determined by the addition of an enzyme stock solution (1.1 μg (0.025 U) leucine aminopeptidase, 60 μg (2.8 U) α-chymotrypsin or 4.9 μg (750 U) trypsin) in 0.05 M phosphate buffer pH 7.40 ($\mu = 0.15$ M with NaCl) to a solution of prodrug in the same buffer and incubated at 37.0° C. The final enzyme concentration was 7.5×10^{-10} M, 4.9×10^{-7} M, or 2.1×10^{-7} M for leucine aminopeptidase, chymotrypsin or trypsin, respectively. The L-isomers were examined at five different prodrug concentrations and all others at 20 μg/ml. At appropriate time intervals an aliquot (200 or 100 μl) was removed and the reaction quenched with 5 μl of 70% $HClO_4$. Samples were assayed by HPLC for dapsone and prodrug. Initial rates of reaction were

determined from the initial linear portion of the dapsone concentration vs time curve.

The stability of the prodrugs in whole blood and plasma was determined by spiking human plasma or rabbit, dog or human blood (5 ml), which had been equilibrated to 37° C in a water bath, with approx. 5 μg/ml of the appropriate derivative. Periodically, 200 μl aliquots were removed and immediately extracted. Whole blood or plasma (200 μl) was extracted with 500 μl ethyl acetate containing 1 μg/ml monopropionyldapsone as an internal standard by vortexing for 1 min. The ethyl acetate (400 μl) was decanted, evaporated to dryness under a stream of N_2, reconstituted in 100 μl of mobile phase and analyzed by HPLC for dapsone, prodrug, and monopropionyldapsone. Standard curves for each species were prepared by spiking blank blood or plasma and extracting in the same manner.

The effects of peptidase inhibitors on the stability of L-Leu-dapsone were assessed using 0.05, 0.10, 0.50 and 1.0 μg/ml bestatin, 4 and 12 mM EDTA, and 1 and 10 mM 1,10-phenanthroline in rabbit blood.

Rabbit blood containing dapsone (5 μg/ml) was incubated at 37° C. Periodically 200 μl aliquots were removed and extracted and analyzed for dapsone, monoacetyldapsone and internal standard (monopropionyldapsone).

3. Results and discussion

Dapsone was used as a model compound to explore the feasibility of amino acid derivatives as soluble, chemically stable, biologically labile prodrugs. Various amino acids were compared in order to assess possible structure-activity relationships which could affect the properties of a prodrug. Alanine (Ala) is the simplest chiral amino acid, glycine (Gly) is achiral, leucine (Leu) is hydrophobic, lysine (Lys) is a basic amino acid which would be primarily cationic at physiological pH, and phenylalanine (Phe) has an aromatic side chain.

Table 2
Specific Rotations of L- and D-aminoacyl prodrugs of dapsone in methanol at 25° C and extent of racemization as measured by hydrolysis by leucine aminopeptidase (LAP) at pH 7.4 at 37° C and polarimetry at 23° C

Compound	$[\alpha]_D$ (°)		Percent D-isomer in racemate	
	L-isomer	D-isomer	Polarimetry	LAP
Ala-dapsone	37.2	−40.5	47.9	52
Leu-dapsone	56.9	−55.8	29.6	34
Phe-dapsone	116.5	−124.2	49.4	56

3.1. Synthesis

A number of amino acid derivatives of dapsone were synthesized in order to evaluate their potential as bioreversible prodrugs. Dapsone contains two reactive aromatic amine functional groups, but only the mono-amino acid derivative was desired. Because dapsone is a symmetrical molecule, derivatization at either amine would give the same product. In order to minimize formation of the bis product, only one molar equivalent of protected amino acid was used. Any undesired bis product was easily removed during column chromatography. The symmetry of the molecule precluded a reaction scheme involving protection of one group followed by derivatization and deprotection of the other since selectivity was not possible.

The products isolated each exhibited a single peak by HPLC and were characterized by NMR and mass spectral analysis to confirm monosubstitution. The NMR chemical shifts, splitting patterns, and peak integration (Table 1) were consistent with the desired product. The mass spectra of each derivative indicated the molecular ion at $M+1$ as well as primary peaks at m/e 185 and 110. The fragment at m/e 185 is consistent with loss of the amine, amino acid amide and sulfone oxygens. Subsequent loss of a single aromatic ring would result in a fragment with m/e 110. Another common fragmentation pattern observed was appearance of peaks at $M-122$ and/or $M-154$, which suggests loss of both sulfone oxygens and cleavage between the sulfur and either aromatic ring.

Synthesis with L- and D-amino acids in the absence of DMAP as a catalyst resulted in reten-

tion of stereochemistry as determined by optical rotation. Values for $[\alpha]_D$, as expected, were of equal magnitude and opposite direction (Table 2). The products were also subjected to hydrolysis by leucine aminopeptidase, which is specific for L-amino acids (Hofmann et al., 1958). The L-isomers were completely hydrolyzed while the D-isomers remained intact, supporting the optical activity results. Coupling of dapsone and N-protected L-amino acids in the presence of DMAP resulted in racemization. The amount of D-isomer (Table 2) was calculated from the $[\alpha]_D$ values in Table 2 and by measuring the fraction not hydrolyzed by leucine aminopeptidase (Hofmann et al., 1958). The results from each method are in good agreement and reflect a significant degree of racemization. Therefore, routine synthesis was performed without DMAP.

3.2. Chemical stability of prodrugs

The kinetics of degradation of L-Leu-dapsone at 73° C at pH 6.0–10.0 were followed for at least four half-lives and degradation rate constants (k_{obs}) determined from the slopes of pseudo-first order plots. Rate constants at pH 2.0 and 4.0 and for L-Ala-, Gly-, L-Lys-, and L-Phe-dapsone at all pH values were determined using the method of initial rates, i.e., k_{obs} was calculated by following the initial rate of appearance of dapsone. Dapsone was the only degradation product and was stable at these pH values. Rate constants at pH 2.0 were not determined for all compounds because of the slow rate of degradation. Buffer catalysis was evident due to phosphate buffer at pH 6.0 and 7.4 and borate buffer at pH 10.0, but not for acetate buffer at pH 4.0. The rate constants were extrapolated to zero buffer concentration and the results are presented in Fig. 2. The plots of k_{obs} vs buffer concentration for L-Phe-dapsone at pH 6 and 7.4 were not linear and the rate constants in Fig. 2 were calculated from the k_{obs} at a buffer concentration of 0.0125 M.

The stability results are consistent with pH-rate profiles for many amides, which generally exhibit maximum stability in the acidic pH range and undergo base catalyzed hydrolysis. The shape of

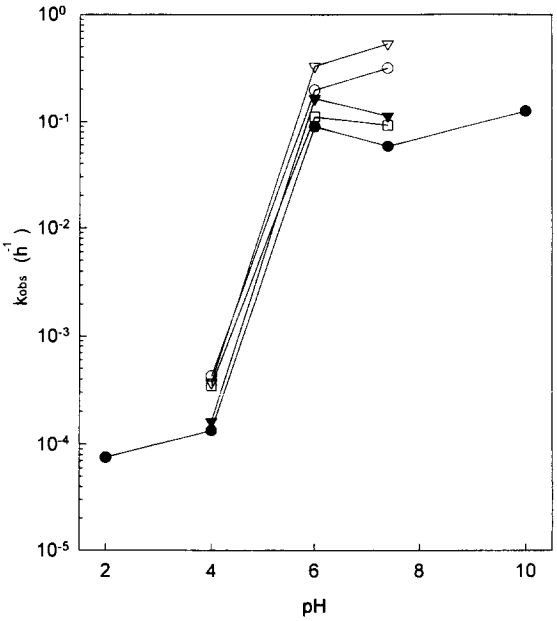

Fig. 2. pH-rate profile for Gly- (▽), L-Ala- (○), L-Phe- (▼), L-Lys- (□), and L-Leu-dapsone (●) at 73° C in HCl/NaCl (pH 2.0), sodium acetate (pH 4.0), sodium phosphate (pH 6.0 and 7.4) or sodium borate (pH 10). Values at pH 6.0 and 7.4 were corrected for buffer catalysis.

the curves suggests specific base-catalyzed hydrolysis of the protonated prodrugs, although this cannot be distinguished from the kinetically equivalent uncatalyzed degradation of the unionized species. The only degradation product observed at any pH value was dapsone, indicating no other routes of degradation apart from hydrolysis of the amide bond.

Examination of the rate constants in Fig. 2 reveals differences in relative rate constants for the degradation of the L-aminoacyl prodrugs at pH 6.0 and 7.4. In order of decreasing rates of hydrolysis, Gly > Ala > Phe > Lys > Leu. Possible contributing factors to the differences are small variations in the pK_a values and steric hindrance at the reactive site. Variability of the pK_a does not adequately explain the differences as predictions of the relative pK_a values (Albert and Serjeant, 1971; Weast, 1982) would result in a theoretical rank order of Ala > Gly > Leu > Phe > Lys. Prediction of the steric contributions using Newman's rule of six (Newman, 1956) and

Table 3
Solubilities (in mg/ml dapsone equivalents) of dapsone and the HCl salts of the L-aminoacyl prodrugs of dapsone at 25° C in water and pH 7.4 sodium phosphate buffer

Compound	Solubility (mg/ml dapsone)	
	Water	pH 7.4 phosphate buffer
Dapsone	0.16	0.14
L-Ala-dapsone	> 30	6.6
L-Leu-dapsone	> 25	0.31
L-Phe-dapsone	1.3	0.002
Gly-dapsone	> 15	0.87
L-Lys-dapsone	> 65	> 65

free energy relationships (Charton, 1977; Hansch and Leo, 1979; Cho and Haynes, 1985) would predict rank orders of Gly ≈ Ala > Lys > Phe > Leu and Gly > Ala > Phe = Lys > Leu, respectively, suggesting that steric contributions significantly influence the amide hydrolysis.

Assuming Arrhenius behavior and a reasonable activation energy for amide hydrolysis (Connors et al., 1986), projected shelf-lives ($t_{90\%}$) for the amino acid amides of dapsone are greater than 2 years at a pH of approx. 4. Unfortunately, studies to confirm this were not performed due to the very slow rates of degradation. The results indicate that chemical stability is not a limiting factor. This is contrary to results found for similar derivatives of prazosin, in which a non-hydrolytic degradation route was responsible for half-lives in the range of 5 min to 36 h at 25° C (Pochopin et al., 1994a).

3.3. Solubility of prodrugs

Solubilities of dapsone and the HCl salts of the prodrugs in water at 25° C are reported in Table 3. Except for L-Phe-dapsone and dapsone, the solubility limits were not reached due to limited quantities of the compounds available. The pH of the resultant solutions was approx. 3. In phosphate buffer at pH 7.4, saturated solubilities were determined at 25° C and the results are summarized in Table 3.

The addition of the amino acid residues to dapsone resulted in at least 2–3 orders of magnitude improvement in water solubility for the HCl salts. Lysine imparted the greatest solubility over the widest pH range due to the ionizable γ-amine which is protonated at physiological pH. All compounds except L-Phe-dapsone provided good water solubility, and the improvement compares well to reported values in the literature on solubilities of amino acid esters and amides (Hirai et al., 1978; Amidon, 1981).

3.4. Enzymic reversion of the prodrugs to dapsone by peptidases

L-Ala-, L-Leu-, L-Phe-, Gly- and L-Lys-dapsone were all substrates for leucine aminopeptidase. The rates of dapsone production were determined as a function of prodrug concentration, and K_m and V_{max} values were calculated from double-reciprocal plots (Dixon and Webb, 1979). The specificity constants (k_{cat}/K_m) were calculated from $V_{max}/([E] \cdot K_m)$ where [E] is the molar enzyme concentration. A summary of the results is presented in Table 4. The corresponding D-amino acid derivatives were completely stable in the presence of leucine aminopeptidase over 8 h, which is consistent with the specificity of leucine aminopeptidase for amino acids in the L-configuration. This of course excludes glycine which

Table 4
Enzyme parameters calculated from double-reciprocal plots for L-aminoacyl prodrugs of dapsone as substrates of leucine aminopeptidase in pH 7.40 sodium phosphate buffer at 37° C

Compound	K_m ($\times 10^5$) (M)	V_{max} ($\times 10^8$) (M s^{-1})	k_{cat}/K_m ($\times 10^{-5}$) (M^{-1} s^{-1})
L-Ala-dapsone	59.1	23.0	5.19
L-Leu-dapsone	8.96	3.83	5.70
L-Phe-dapsone	22.0	3.20	1.94
Gly-dapsone	37.6	2.58	0.92
L-Lys-dapsone	6.09	0.52	1.13

does not have a chiral center. Comparison of the values for k_{cat}/K_m in Table 4 indicates that L-Ala- and L-Leu-dapsone were the best substrates for leucine aminopeptidase, although the other derivatives have specificity constants of similar magnitude (Fersht, 1985).

In the presence of chymotrypsin L-Ala-, L-Leu-, Gly- and L-Lys-dapsone were completely stable over a 4 h time period and, as expected, only L-Phe-dapsone was a substrate. Similarly, L-Ala-, L-Leu-, L-Phe- and Gly-dapsone were completely stable over a 4 h time period in the presence of trypsin while only L-Lys-dapsone was a substrate. Enzymatic hydrolysis of the prodrugs by trypsin and chymotrypsin followed expected behavior. Trypsin has greater affinity for Lys and Arg residues while chymotrypsin preferentially hydrolyzes aromatic amino acids (Dixon and Webb, 1979). These results have implications for oral delivery of amino acid prodrugs (Johnson et al., 1985).

3.5. Stability of dapsone in rabbit blood

Dapsone was unstable in rabbit blood due to acetylation to give monoacetyldapsone with an in vitro t_{90} at 37° C of 48 min. The ability of blood to acetylate dapsone has been observed previously (Cossum, 1988) although a rate was not reported. The presence of *N*-acetyltransferase in blood has been confirmed (Drayer et al., 1974; Cossum, 1988) but the importance of this phenomenon in vivo would most likely be negligible since the liver and other tissues contain far greater acetylation capacity, and the overall elimination of dapsone and monoacetyldapsone is more efficient. However, due to the analytical implications, extraction of blood samples was performed as quickly as possible to minimize any conversion of dapsone to monoacetyldapsone during sample handling.

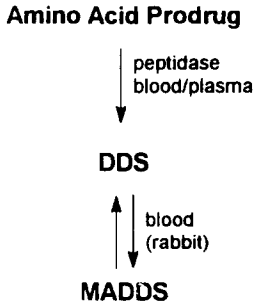

Fig. 3. Scheme for the fate of amino acid prodrugs of dapsone in whole blood.

3.6. Stability of prodrugs in blood and plasma

Hydrolysis of L-Ala-, L-Leu-, L-Phe-, Gly-, and L-Lys-dapsone to dapsone occurred in human plasma and human, rat and rabbit blood, and half-lives are presented in Table 5. As discussed previously, the dapsone produced from hydrolysis could be subsequently acetylated in rabbit blood as illustrated in the reaction scheme in Fig. 3. Hydrolysis of D-Ala-dapsone, D-Leu-dapsone and D-Phe-dapsone was limited in rabbit blood at 37° C, with dapsone production of only 3.76, 0.74, and 1.5%, respectively, after one hour. These results are consistent with the enzymatic specificity of aminopeptidases. The $t_{1/2}$ values of the dapsone prodrugs in plasma and blood in Table 5 represent a fairly wide range of hydrolytic lability,

Table 5
Half-lives (in min; ±SE, $n = 3$) of L-aminoacyl prodrugs of dapsone in blood and plasma of different animal species at 37° C

Compound	Rabbit blood	Rat blood	Human blood	Human plasma
L-Ala-dapsone	29.1 ± 1.1	28.9 [a]	20.5 ± 0.7	39.6 ± 6.7
L-Leu-dapsone	2.3 ± 0.1	1.7 ± 0.02	1.7 ± 0.2	34.4 ± 9.6
L-Phe-dapsone	21.9 ± 1.8	19.2 [a]	8.8 ± 0.7	190 ± 56
Gly-dapsone	28.9 ± 1.1	15.3 [a]	14.6 ± 0.9	107 ± 5.6
L-Lys-dapsone	14.9 ± 0.4	10.9 ± 0.7	10.9 ± 0.7	29.7 ± 4.8

[a] $n = 1$ for these samples.

but there appeared to be no obvious species dependence as the relative magnitudes of half-lives were similar among rat, rabbit and human blood.

The half-lives for amino acid amides of open-ring benzodiazepines in human serum reported in the literature (Cho et al., 1986) are of the same order of magnitude as the dapsone prodrugs suggesting that the amino acid affects the hydrolysis rate. However, amino acid amides of adamantanine are very stable to aminopeptidases, presumably due to steric hindrance (Nagasawa et al., 1975). Therefore, the hydrolytic lability of amino acid amides also is dependent on the structure of the drug molecule.

Comparison of the plasma to whole blood results in Table 5 indicates that whole blood has greater catalytic activity on prodrug reversion. Aminopeptidases have been identified with the erythrocytes and leukocytes of different species (Drayer et al., 1974; Cossum, 1988; Lochs et al., 1990), and therefore hydrolysis of amino acid amides would be expected to be greater in whole blood than in plasma. This activity may be dependent on the specific compound since catalysis in whole blood was not observed for a lysine derivative of an open-ring benzodiazepine (Upshall et al., 1990).

Attempts to correlate $t_{1/2}$ in blood to steric, electrical or hydrophobic parameters of the amino acid were unsuccessful, although in general those compounds which were most chemically stable were also the most enzymatically labile. This trend may reflect the presence of a hydrophobic binding site at the active site of the enzymes involved.

In vivo, the blood would be a major site of bioreversion after i.v. administration, particularly for L-Leu-dapsone which has a very short $t_{1/2}$. This could possibly be reflected in faster biological conversion of L-Leu-dapsone to dapsone as compared to the other derivatives, but all should undergo fairly rapid bioreversion. On the other hand, administration of a D-aminoacyl derivative, whether as a pure compound or a racemate, would most likely result in a distinctly different pharmacokinetic profile since these derivatives are much more stable to peptidase enzymes.

A variety of peptidase inhibitors were examined to understand the nature of the enzymes which hydrolyze the prodrugs in plasma and whole blood and to possibly identify a means for quenching the reaction for sample handling. Bestatin is a competitive inhibitor of leucine aminopeptidase, and 1,10-phenanthroline and EDTA are metal ion chelators which could potentially affect the activity of metalloenzymes. The results for L-Leu-dapsone in rabbit blood are summarized in Fig. 4. Although inhibition was observed, none of the inhibitors was completely effective in preventing the hydrolysis of L-Leu-dapsone in rabbit blood. The inhibitory action of phenanthroline, an efficient metal chelator, supports the involvement of metallopeptidases, of which leucine aminopeptidase is one, as part of the enzyme pool in whole blood. Also, the slight inhibitory effect of bestatin, a competitive inhibitor of leucine aminopeptidase, suggests the involvement of this enzyme or one of a similar nature in the blood hydrolysis.

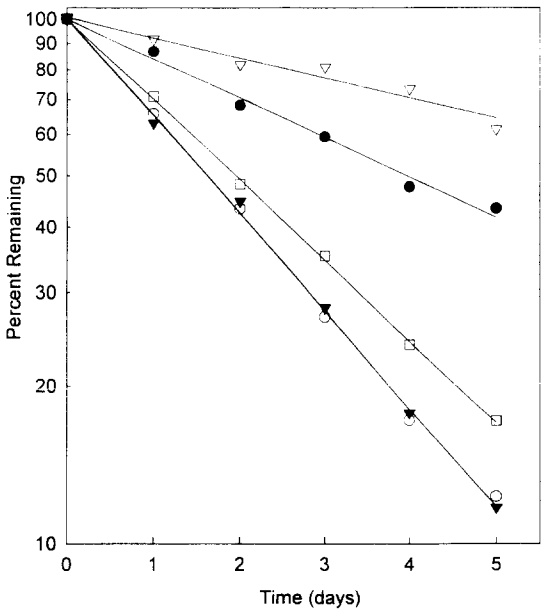

Fig. 4. Effect of potential aminopeptidase inhibitors on the stability of L-Leu-dapsone in rabbit blood at 37°C. 1,10-Phenanthroline, 10 mM (▽); bestatin, 1 μg/ml (●); 1,10-phenanthroline, 1 mM (□); EDTA, 12 mM (▼); control (○).

4. Conclusions

Amino acid amide prodrugs appear to be a feasible approach to deliver dapsone, and potentially other primary aromatic amines, parenterally in an aqueous vehicle. Good chemical stability and water solubility were accompanied by rapid and quantitative enzymatic hydrolysis in vitro. The solubility was dependent on the specific amino acid and pH of the solution. Lysine conferred the greatest solubility due to a second ionizable amine in the side chain. The various derivatives exhibited similar chemical stability, but had differing affinities for enzymatic hydrolysis. The in vivo bioreversion is the subject of another publication (Pochopin et al., 1994b).

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