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Studies on the Inhibitory Effects of Analogues of Dapsone on Neutrophil Function In-vitro*

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Abstract

We have compared twelve sulphone analogues of dapsone in terms of inhibition both of zymosan-mediated human neutrophil respiratory burst and inhibition of interleukin-1-stimulated neutrophil adhesion to transformed human umbilical vein endothelial cells.

Overall, there was a good correlation between the respective rank orders of compound potency in the two test systems. The most effective compounds in terms of respiratory burst and adherence inhibition were the 2-nitro-4-amino-, 2-hydroxy-4-aminopropyl-, and 2-methoxy-4-aminoethyl- derivatives. In general, potency was inversely associated with lipophilicity; compounds with bulky side-chains, e.g. the 2-methyl-4-aminopentyl, 2-methyl-4-aminohexyl and the 2-hydroxymethyl-4-aminoethyl derivatives, were less potent. A 2-hydroxy-4-amino- derivative was the exception, however, with low lipophilicity and relatively low potency.

All of the compounds tested showed comparable or greater inhibition in both the neutrophil-mediated assays compared with dapsone. Some of the compounds might, because of their good tissue penetration and lower toxicity than dapsone, have the potential to undergo further development.

Dapsone is the drug of choice for the treatment of dermatitis herpetiformis and other similar inflammatory conditions, which are characterized by neutrophil infiltration (Zone 1991; Uetrecht 1992). Dapsone inhibits neutrophil adhesion, chemotaxis, lipoxygenase activity and the cells' ability to generate reactive oxidative species (Kettle & Winterbourne 1991; van Zyl et al 1991; Booth et al 1992; Thuong-Nguyen et al 1993; Wozel & Lehmann, 1995). Clinically, dermatitis herpetiformis patient responses vary considerably and dosages can range from 50–300 mg day^{-1} (Coleman et al 1992), although at higher dosages the hydroxylamine metabolites of dapsone might cause significant methaemoglobinaemia and haemolysis (Coleman et al 1990; Manfredi et al 1979). Patient tolerance to dapsone has been improved by co-administration of cimetidine, which partially inhibits the cytochrome P-450 enzymes responsible for dapsone's toxicity (Coleman et al 1992; Rhodes et al 1995). In the long term, however, it would be beneficial to design potent analogues of dapsone which are less toxic, better tolerated and yet more effective as anti-inflammatory agents. A number of more intrinsically active derivatives of dapsone have been synthesized, originally with the aim of maximizing the anti-parasitic potential of the drug and reducing its cytochrome P-450 mediated haematological toxicity (Pieper et al 1989; Kansy et al 1992). Recent work has shown some of these compounds are substantially less potent methaemoglobin formers in-vitro compared with dapsone when tested with rat and human liver tissue (Coleman et al 1996).

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In the light of their low in-vitro toxicity and their close structural resemblance to dapsone, we wished to evaluate the possible application of these analogues to the control of neutrophil-mediated disease such as dermatitis herpetiformis. We have therefore studied the inhibitory effects of the twelve derivatives in two in-vitro models of neutrophil activation in man: zymosan-mediated respiratory burst and adhesion to a human endothelial cell line.

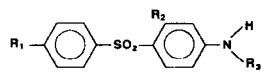
Materials and Methods

Chemicals

The dapsone (4,4′-diaminodiphenyl sulphone) analogues evaluated were: K-130 (2,4-diamino-5-{4-[3-(4″-aminophenyl-4′-sulphonylphenylamino)propoxy]-3,5-dimethoxybenzyl} pyrimidine; Kansy et al 1992); WFC 1 (2-methyl-4-acetylamino-4′nitrodiphenyl sulphone); AXDD17 (2-methyl-4-cyclopentylamino-4′-aminodiphenyl sulphone); ABDD5 (2-hydroxy-4,4′-aminodiphenyl sulphone); ABDD39 (2-hydroxymethyl-4′-ethylaminodiphenyl sulphone); ABDD1 (2-hydroxy-4-propylamino-4′-aminodiphenyl sulphone); AXDD16 (2-methyl-4,4′-hexylamino diphenyl sulphone); W10 (2-methoxy-4-ethylamino-4′-aminodiphenyl sulphone); PXDD19 (2-trifluoromethyl-4-ethylamino-4′-aminodiphenyl sulphone); PXDD4 (2-trifluoromethyl-4′-aminodiphenyl sulphone; and W25 (2-amino-4′-aminodiphenyl sulphone); W13 (2-nitro-4-amino-4′-aminodiphenyl sulphone); Pieper et al 1989). The structures are shown in Fig. 1; the relative lipophilicities (log k values) of the analogues given in Table 1, are derived from Pieper et al (1989). Dapsone was obtained from Aldrich (Poole, UK).

Experimental

Whole blood was obtained by venepuncture from seven healthy volunteers, age range 23–55 years. The ability of neutrophils to generate a respiratory burst was measured using



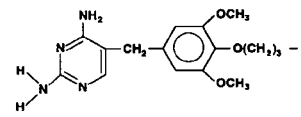
	R_1	R_2	R_3
K-130	NH_2	H	
WFC1	NO_2	CH_3	$COCH_3$
AXDD17	NH_2	CH_3	
ABDD39	NH_2	CH_2OH	C_2H_5
ABDD5	NH_2	OH	H
ABDD1	NH_2	OH	C_3H_7
AXDD16	NH_2	CH_3	C_6H_{13}
W10	NH_2	OCH_3	C_2H_5
PXDD19	NH_2	CF_3	C_2H_5
PXDD4	NH_2	CF_3	H
W25	NH_2	NH_2	H
W13	NH_2	NO_2	H

FIG. 1. The structures of twelve analogues of dapsone; R_1 and R_3 are hydrogen atoms in the parent drug.

whole blood, whereas adhesion to endothelial cells was measured using a purified neutrophil preparation. Neutrophil respiratory burst was elicited by means of an opsonized zymosan preparation. Zymosan (Sigma) was suspended in guinea pig serum (Gibco, UK) at 5 mg mL^{-1} and incubated at 37°C for 20 min in a 95:5% air-CO_2 atmosphere. The particles were washed three times with RPMI 1640 medium and resuspended in the RPMI medium at 5 mg mL^{-1} and stored at 4°C until required. The respiratory burst was assessed by means of lucigenin-enhanced chemiluminescence in a Bio-Orbit 1253 luminometer (Labtech International, Sussex, UK). Lucigenin (Sigma; 5×10^{-4} M; 100 mL) and whole blood (100 mL) were added to opsonized zymosan (300 mL); chemiluminescence readings were taken at intervals thereafter. All reactions took place in the dark at 37°C. When pre-treatment of neutrophils with dapsone or its analogues was required, before analysis samples of purified neutrophils or whole blood were incubated with the compounds (0·5, 1·0 or 1·5 mM) for 30 min at 37°C.

Isolation of neutrophils before measurement of adhesion was performed by the method of Afford et al (1992) with some modifications. Briefly Hespan (AAH Pharmaceuticals, Kingswinford, West Midlands, UK; 1·5 mL) was added to blood (10 mL) and the resulting mixture left at room temperature for 45 min to enable sedimentation of the erythrocytes. The resulting upper leucocyte-rich fraction was then removed and diluted with an equal volume of Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS; Sigma, Dorset, UK). This cell suspension (2 mL) was then layered on to isotonic Percoll (Sigma) gradient consisting of 3 mL of 60% Percoll above 2 mL of 80% Percoll; the mixture was centrifuged at 200 g for 20 min. Neutrophils were then collected from the interface between the two Percoll layers and washed twice in PBS before re-suspension at a final concentration of 1×10^6 cells mL^{-1}. Cell purity was routinely greater than 95%.

Neutrophil adherence studies were performed using transformed human umbilical vein cells (ECV 304) grown to confluence in 96-well plates. The growth medium (DMEM; streptomycin IU mL^{-1}; penicillin 100 mg mL^{-1}; 10% foetal calf serum) was then aspirated away and replaced with unsupplemented medium (100 mL) containing human interleukin-1a (h-IL-1a; 10 ng mL^{-1}). The cells were then incubated in a 95:5% air-CO_2 mixture at 37°C for 4 h during which time up-regulation of adhesion molecules occurs on the endothelial cell surface. The medium was then removed and the purified isolated neutrophils (suspensions pretreated with dapsone or its analogues; 100 mL) were added. The neutrophils were left for 30 min to adhere, then unadhered neutrophils were removed by washing with PBS (2×100 mL). The adherent neutrophils were then assessed by means of the method of Junger et al (1993). The neutrophils were lysed with triton X-100 in PBS (0·2% v/v; 40 mL) and the liberated myeloperoxidase measured using hydrogen peroxide (0·02%) as substrate and *O*-dianisidine dihydrochloride (0·34 mM) as the chromogen at pH 5. Absorbance values were measured at 405 nM. The concentration range 0·5–1·5 mM was chosen on the basis of in-vitro pilot studies which showed complete inhibition of both neutrophil responses by dapsone at 1 mM. The twelve candidate compounds were compared with the parent drug for their ability to inhibit adhesion and respiratory burst completely in this concentration range.

All statistical analysis was by Student's *t*-test and all data are represented graphically as means ± s.d. 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 (k is the number of tests) to compensate for the increased likelihood of reaching $P < 0.05$ during multiple testing (Elashoff 1981)

Results

With the exception of AXDD17 at the lowest concentration, all the compounds tested (including dapsone) significantly ($P < 0.01$) attenuated the stimulated respiratory burst at all three concentrations (Table 2). Compared with dapsone, WFC2, ABDD39, ABDD1, PXDD19, PXDD4, W25 and W13 were all significantly more potent at the 0·5 mM concentration (Table 2). At 1 mM, only AXDD17 and AXDD16 were not significantly more potent than dapsone. At the highest concentration (1·5 mM), all the compounds tested were significantly more potent than dapsone (1 mM). Overall, the most potent derivative was W13, which had 4·25 times the effect of dapsone (Tables 1 and 2). When the rank order of potency was plotted against the respective log k′ value, the relationship between lipophilicity and inhibitory effect was poor ($r = 0.41$) for all 12 compounds. Regression of the ranks with their respective lipophilicities (starting with the most potent) from 1–6, showed a strong correlation ($r = 0.85$), however, and regression of ranks 7–12 with the log k′ values showed a similar correlation ($r = 0.83$).

Table 1. Rank orders of inhibitory effects of twelve sulphone analogues in in-vitro assays for neutrophil respiratory burst and inhibition of neutrophil adhesion to umbilical cord cells (rank orders of potency compared between dapsone and each compound at 1 mM); relative lipophilicity (log k'; Pieper et al 1989) is also listed.

Compound	Lipophilicity (log k')	Respiratory burst inhibition		Adhesion inhibition	
		Potency	Rank	Potency	Rank
K-130	2·09	1·2	10	1·5	4
WFC1	–	2	5	1·42	5
AXDD17	3·288	1·04	12	1·14	9
ABDD39	0·616	1·53	7	0·75	11
ABDD5	−0·0091	1·45	9	1·0	10
ABDD1	1·933	2·63	2	1·9	2
AXDD16	4·04	1·08	11	1·2	7
W10	1·27	2·16	4	3·2	1
PXDD19	2·45	1·82	6	1·15	8=
PXDD4	1·409	2·60	3	1·15	8=
W25	−0·096	1·5	8	1·34	6
W13	0·8543	4·25	1	1·8	3

Table 2. The ability of twelve analogues of dapsone to inhibit the neutrophil zymosan-mediated respiratory burst in-vitro.

Assay	Analogue	Drug-free unstimulated	Drug-free stimulated	0·5 mM stimulated	1 mM stimulated	1·5 mM stimulated	Dapsone (1 mM) stimulated
1	K-130	20 ± 6	205 ± 10	111 ± 6	86 ± 4*	81 ± 7*	104 ± 6
2	WFC1	20 ± 2	287 ± 24	189 ± 13*	106 ± 7†	32 ± 4‡	211 ± 8
3	AXDD17	43 ± 3	238 ± 26	239 ± 13	164 ± 20	119 ± 31*	170 ± 20
4	ABDD39	15 ± 2	200 ± 20	96 ± 4†	95 ± 5†	64 ± 2‡	146 ± 5
5	ABDD5	21 ± 3	172 ± 6	46 ± 4	33 ± 5*	32 ± 2*	48 ± 4
6	ABDD1	26 ± 1	113 ± 2	47 ± 3*	24 ± 3†	24 ± 2†	63 ± 9
7	AXDD16	31 ± 2	245 ± 4	189 ± 4	182 ± 6	65 ± 3‡	197 ± 22
8	W10	29 ± 2	116 ± 6	63 ± 16	37 ± 5†	21 ± 5†	80 ± 7
9	PXDD19	33 ± 5	299 ± 19	167 ± 43*	123 ± 6†	32 ± 3‡	224 ± 4
10	PXDD4	18 ± 5	164 ± 5	28 ± 2†	25 ± 2†	16 ± 2†	65 ± 5
11	W25	26 ± 5	375 ± 5	205 ± 12†	170 ± 6‡	160 ± 8‡	255 ± 2
12	W13	73 ± 9	286 ± 31	103 ± 9†	55 ± 6‡	35 ± 3‡	234 ± 15

Each assay contained dapsone as a reference (n = 4 per incubation, mean ± SD data tabulated as luminescence units). In all assays, dapsone (1 mM; final column) significantly reduced the zymosan-mediated respiratory burst ($P < 0.05$) compared with drug-free stimulated cells (4th column). Assay comparisons: all analogue respiratory burst inhibition values were compared with those of dapsone (final column); *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

Studies on the inhibition of adhesion revealed that all the analogues caused a significant reduction compared with stimulated control at all three concentrations with the exception of PXDD4 at 0·5 mM (Table 3). At a concentration of 0·5 mM, ABDD5, ABDD1, W10 WFC1, AXDD17 and W13 exerted inhibitory effects which were significantly lower than that of dapsone (1 mM). At concentrations of 1 and 1·5 mM, however, ABDD5 was not significantly different from dapsone. At 1 mM, K-130, WFC1, ABDD1, W10 and W13 showed an effect which was significantly greater than that of dapsone. At the highest concentration, K-130, WFC1, AXDD17, ABDD1, W10 and W13 caused significantly greater inhibition than dapsone. The effects of ABDD39, PXDD19, PXDD4 and W25 were not significantly different from that of dapsone at all three concentrations. Only W10, ABDD1 and W13 were significantly more potent than dapsone at all three concentrations. For the first six ranked compounds and the correlation with lipophilicity was poor (0·19), as was that for 7–12 (0·44) and for 1–12 (0·2). The correlation of the analogue ranks between the two test systems, i.e. adhesion and respiratory burst inhibition (at 1 mM), was, however, 0·7.

Discussion

At the molecular level, many of the functions of neutrophils are not fully understood, so it is not surprising that the mechanisms of the inhibitory effects of dapsone on activated neutrophils are also unclear. Inflammatory diseases which respond to dapsone often feature extensive skin disposition of immunoglobulin A; in certain skin areas, accumulation of activated neutrophils is associated with tissue damage. In the most common condition of this type, dermatitis herpetiformis, neutrophil-induced skin lesions cause intense and often unbearable pruritis (Zone 1991). There are several stages in neutrophil migration to areas of tissue inflammation. Neutrophils might normally roll along the surface of vascular endothelial cells by a process which involves loose binding between selectins and carbohydrate ligands. In response to inflammatory mediators, much firmer tethering as achieved by activation of integrin molecules on the neutrophil surface; these enable binding to the endothelial adhesion molecules, the necessary prelude to extravasation and migration towards the site of the inflammatory lesion (Osborn 1990; Springer 1990; Lawrence & Springer 1991). During treatment of dermatitis

Table 3. The ability of twelve analogues of dapsone to inhibit neutrophil interleukin-1-mediated stimulated adhesion in-vitro.

Assay	Analogue	Drug-free unstimulated	Drug-free stimulated	0·5 mM stimulated	1 mM stimulated	1·5 mM stimulated	Dapsone (1 mM) stimulated
1	K-130	32·1 ± 1·7	50·1 ± 7·1	32·1 ± 7·0	25·0 ± 0·7*	26·7 ± 5·0*	37·5 ± 3·5
2	WFC1	22·4 ± 11·2	56·1 ± 5·6	22·4 ± 5·6*	22·4 ± 3·3*	21·9 ± 4·4*	32·0 ± 2·8
3	AXDD17	22·4 ± 11·2	56·1 ± 5·6	20·1 ± 2·0†	28·0 ± 3·9	19·6 ± 0·5†	32·0 ± 2·8
4	ABDD39	11·9 ± 2·3	28·5 ± 2·1	19·2 ± 5·9	28·5 ± 1·0	29·1 ± 3·0	21·4 ± 4·7
5	ABDD5	22·4 ± 11·2	56·1 ± 5·6	25·2 ± 3·9*	32·0 ± 2·8	29·7 ± 0·5	32·0 ± 2·8
6	ABDD1	17·4 ± 4·3	31·5 ± 2·1	8·6 ± 3·1†	10·8 ± 5·4*	10·8 ± 5·4*	20·6 ± 1·0
7	AXDD16	22·4 ± 11·2	56·1 ± 5·7	22·4 ± 5·6	26·4 ± 0·5*	26·3 ± 5·0	32·0 ± 2·8
8	W10	17·4 ± 4·0	31·5 ± 2·1	7·6 ± 2·0†	6·5 ± 3·5†	13·1 ± 5·4*	20·6 ± 1·0
9	PXDD19	11·9 ± 2·3	28·5 ± 2·1	16·6 ± 1·1	18·5 ± 2·1	17·6 ± 1·4	21·4 ± 4·7
10	PXDD4	11·0 ± 2·0	28·0 ± 2·2	27·3 ± 9·5	18·5 ± 2·1	17·6 ± 1·4	21·4 ± 4·7
11	W25	11·6 ± 2·3	28·5 ± 2·1	16·6 ± 3·3	15·9 ± 4·0	16·6 ± 1·1	21·4 ± 4·7
12	W13	32·8 ± 1·7	52·6 ± 5·5	28·5 ± 3·5†	20·3 ± 10·7*	15·7 ± 7·1†	37·5 ± 3·5

Each assay contained dapsone as a reference. Data are expressed as percentage adhesion (n = 3 per incubation. mean ± s.d.). In all assays, dapsone (1 mM; final column) significantly ($P < 0.05$) reduced neutrophil adhesion compared with drug-free stimulated cells (4th column). Assay comparisons: all analogue adhesion inhibition values were compared with those of dapsone (final column). *$P < 0.05$; †$P < 0.01$.

herpetiformis with dapsone the characteristic neutrophil accumulation in skin does not occur. Dapsone has been shown to disrupt the integrin-mediated adhesion process and this might be the main explanation of its ability to inhibit the migration process (Booth et al 1992).

It seems that after extravasation, stimulated neutrophils must then reach the tissue fixed immunoglobulin A and bind before initiation of a destructive respiratory burst (Thuong-Nguyen et al 1993). It has been suggested that Dapsone interferes with this process also, although the drug does not affect antibody disposition. Finally, the actual process of tissue damage is mediated by the release of neutrophil myeloperoxidase, which mediates the respiratory burst by combining with hydrogen peroxide (formed from NADPH oxidase) to form 'Compound I'. This activated form of the enzyme, together with chloride ions, form large quantities of hypochlorous acid, the most potent oxidant produced by neutrophils (Kettle & Winterbourne 1991; van Zyl et al 1991; Zimmermann & Granger 1994). Dapsone is believed to convert the enzyme to the Compound II stage, which is an inactive redox intermediate (reversible by superoxide), which was thus believed to prevent the hypochlorous acid-mediated tissue destruction which is associated with neutrophil infiltration (Kettle & Winterbourne 1991; van Zyl et al 1991). More recent work, however, suggests that the effects of dapsone on myeloperoxidase are a much smaller component of its anti-inflammatory action than was previously thought (Kettle et al 1993). It is likely that dapsone's major action in diseases such as dermatitis herpetiformis is to suppress adhesion, probably by inhibiting a chemoattractant receptor-coupled G protein, which is the chief mediator of the signal transduction cascade which controls the adhesive, migratory, respiratory and secretory functions of neutrophils (Debol et al 1995). The respiratory burst and adhesion assays both revealed substantial inhibitory activity associated with the twelve dapsone derivatives tested. There was reasonable agreement in the potency rankings between the two assays, which lends support to the hypothesis of Debol et al (1995) that a common mode of action of the drug might apply for a number of neutrophil-mediated activities. In general, the most potent compounds (W13, W10 and ABDD1) tended to be of lower lipophilicity than the less effective compounds (AXDD17, ABDD39 and AXDD16) although there were exceptions such as ABDD5, which was low in potency and lipophilicity, and K-130, which was less effective in the respiratory burst assay, but much more effective in inhibition of adhesion. K-130 binds strongly to lipopolysaccharides in bacteria (Kansy et al 1992) and it is possible that the structure of this analogue favours binding to the integrins and selectins which are known to mediate neutrophil adhesion (Booth et al 1992). K-130 was less effective in the suppression of respiratory burst, possibly because of its relatively high lipophilicity, which could have caused it to become 'trapped' in the neutrophil cell-wall lipids, thus reducing available compound for binding to myeloperoxidase. With regard to the respiratory burst assay, there appeared to be an apparently biphasic relationship between log k values and the anti-inflammatory effect of the analogues. The structures fell into two broad categories with regard to their effectiveness. It could be speculated that there is more than one inhibitory binding site in the myeloperoxidase system, which could account for these results, although no such relationship could be shown to exist in the adhesion assay potency ranks.

The in-vitro drug concentrations employed in this study were more than one order of magnitude greater than dapsone plasma concentrations previously reported in dermatitis herpetiformis patients (up to 40 mM; Harvath et al 1986; Moncrieff 1994). Previous studies have, however, observed a discrepancy between in-vitro inhibitory concentrations of dapsone and its actual therapeutic effect. Neutrophils isolated from patients treated with therapeutic dapsone dosages that would yield drug levels up to 100 times lower than concentrations used in-vitro showed similar inhibition of adhesion compared with normal neutrophils incubated with dapsone for 60 min (Booth et al 1992). It is probable that the length of time the cells are in contact with dapsone might well be crucial, as neutrophils in-vivo will have spent their entire life-span in the presence of the drug. In addition, because dapsone's in-vitro efficacy varies with the method of neutrophil stimulation (Kettle et al 1993), it is likely that the discrepancy between in-vitro and in-vivo results arises from a combination of duration

of drug exposure and the actual nature of the neutrophil stimuli.

The clinical potency of these compounds would depend partly on their physicochemical properties, such as lipophilicity, ionization at physiological pH and partitioning in cellular fluids. These factors would, when these compounds are administered orally, influence their ability to penetrate skin tissues in sufficiently effective concentrations and their lack of non-specific binding or trapping in cell membranes. The propensity of sulphone metabolites to cause methaemoglobinaemia and cytotoxicity will, however, have a major influence on their future application. In previous in-vitro studies with rat as well as human liver microsomes, the least toxic of the derivatives compared with dapsone included WFC1, K-130, ABDD1 and ABDD5 (Coleman et al 1996). WFC1, K-130 and ABDD1 have been shown to be effective inhibitors of neutrophil adhesion (Tables 1 and 3), a process which is regarded as crucial to the initiation of the neutrophil response (Booth et al 1992).

There is a dearth of effective compounds which suppress diseases related to dermatitis herpetiformis. If patients do not respond to dapsone, cannot tolerate it, or do not respond well to the gluten-free diet, there are few alternatives (Zone 1991). The combination of efficacy superior to that of dapsone, potentially good tissue penetration and, above all, lower toxicity compared with the parent drug, all indicate that AXDD14 and K-130 might have clinical potential in the therapy of dermatitis herpetiformis.

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