The effects in volunteers of BW12C, a compound designed to left-shift the blood-oxygen saturation curve

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- 1 BW12C, a potent left-shifting anti-sickling compound *in vitro*, was administered to normal healthy male Caucasian volunteers.
- 2 Doses of 2–20 mg kg⁻¹ given by intravenous infusion over 1 h caused a dose-dependent left-shift of the blood-oxygen saturation curve and at the highest dose some 16% of the haemoglobin existed in a high affinity form.
- 3 Peak left-shift was observed at the end of infusion and decayed thereafter with a mean half-life of approximately 3 h.
- 4 There were no adverse systemic effects, either clinical, biochemical or haematological, but there was some local irritation at the intravenous infusion site if the infusion was too concentrated.
- 5 Pharmacokinetic measurements indicated uptake into erythrocytes, low levels in plasma and a volume of distribution not appreciably greater than the blood volume. A pilot radiolabel study indicated extensive metabolism with elimination into the urine.

Keywords BW12C anti-sickling compound blood-oxygen saturation curve

Introduction

The symptoms of sickle cell disease are caused by the reduced solubility of deoxygenated, but not oxygenated sickle haemoglobin, so that at low partial pressures of oxygen the sickle haemoglobin polymerizes within the erythrocyte as long fibres (Dean & Schechter, 1978). These fibres physically distort the erythrocyte membrane to give the classical sickle morphology (Herrick, 1910) and reduce the erythrocyte deformability essential for capillary perfusion. Three strategies to delay the onset of intra-erythrocytic polymerization have been proposed (see Sunshine *et al.*, 1978). One is to directly inhibit polymerization with compounds that competitively bind to sites of intermolecular contact on sickle haemoglobin. A second is to

inhibit polymerization indirectly by increasing the haemoglobin oxygen affinity, thereby increasing the percentage oxygen saturation in the capillaries. A third strategy, suggested by kinetic studies, is to decrease the intracellular concentration of sickle haemoglobin. Following from the second strategy, experimental and clinical evidence suggests that the maintenance of a minimum of some 15–30% of haemoglobin in a high affinity form throughout the circulation might confer therapeutic benefit, either by inhibiting sickling *per se* or, at least, delaying onset beyond the circulation time and into a new cycle of oxygenation (Sunshine *et al.*, 1978; Franklin *et al.*, 1983). We have recently described a compound, BW12C, designed to bind

preferentially to the oxy conformation of human haemoglobin at a site between the amino terminal residues of the α -subunits. BW12C increases the oxygen affinity of haemoglobin and thereby left-shifts the oxygen saturation curve. The phenomenon has been observed, *in vitro*, in normal blood and also in sickle blood where it inhibits reversible sickling (Kneen & White, 1981; Beddell *et al.*, 1984) and preserves the deformability of sickle cells during progressive deoxygenation (Kenny & Stuart 1983). It is predicted from the design that the oxygen affinity of other human haemoglobins will be increased but the specific nature of the binding site means that binding to myoglobins and other proteins will be much less favoured. We present here the results of the first administration of BW12C to normal healthy male volunteers.

Methods

The study was approved by University College Hospital Ethics Committee before implementation. The subjects were seven healthy male Caucasian volunteers (mean age 31, range 24–39 years) from the Wellcome Research Laboratories. They were accepted on the basis of satisfactory clinical history and screening (including electrocardiography (ECG), urine analysis, haematology and clinical chemistry) and informed consent. The subjects were given a copy of the protocol and asked not to take medication or alcoholic beverages for 24 h before the study nor eat breakfast (one cup of tea or coffee permitted) on the day of the study, and were brought by car to the Clinical Pharmacology Laboratory at University College Hospital. The subjects were again interviewed and given opportunity to withdraw before proceeding with the study.

The subjects lay on a couch and intravenous cannulae were inserted, one in each arm, one for infusion and one for sampling. Initially, the infusion was made into a superficial forearm vein, but in later studies an antecubital vein was used. Clinical measurements of blood pressure, pulse, respiratory rate and continuous ECG monitoring were made for 20 min, with a saline infusion. After this the drug was introduced into the infusion line by means of a three-way tap, using a syringe pump (Perfusor: B. Braun & Co.). At the end of the 1 h drug infusion, the intensive rate of measurement was continued for a further hour, after which the subject got down from the couch and intermittent observation and samples were taken for the rest of the day (about 6 h more). Meals were provided from 2 h after the end of the infusion. The

subjects were then sent home by car and advised not to drive until the next day.

Animal toxicology and *in vitro* testing suggested that a total dose of around 20 mg kg⁻¹ body weight should give the level of haemoglobin modification needed to protect against the sickle effect (i.e. within the range 15–30%). It was decided to give initially 2 mg kg⁻¹ by slow intravenous infusion over 1 h to two subjects and if all went well to give increasing doses up to 20 mg kg⁻¹ to subsequent subjects. During and after the infusion, clinical state, haemoglobin modification and pharmacokinetics of the drug were to be observed. In one pilot study ¹⁴C-labelled drug was given.

Blood samples

Blood (9 ml) was taken into lithium-heparin before, and at various times during and after the infusion, and was processed immediately as follows: approximately 0.5 ml was reserved on ice for blood-oxygen saturation curve determination, 3 ml of the sample was haemolysed by the addition of an equal volume of distilled water, and plasma was recovered from the remainder of the sample following centrifugation. Haemolysate and plasma were frozen at –20°C and stored for assay of BW12C. In the 20 mg kg⁻¹ group, extra samples were taken for serum enzyme measurements. Overall, about 17 samples were taken, with slight variation to accommodate individual timetables, but the total amount of blood taken did not exceed 170 ml.

Other samples

Screening measurements were made for urinary proteins at the end of the study day for the subjects receiving 10 mg kg⁻¹ or more. In the radiolabel study, urine and faeces were collected up to 5 days post-dose. Collection times were noted and samples frozen at –20°C as soon as practicable.

Drug dosage

BW12C, made up in vials of 1000 mg in 10 ml (Pharmaceutical Development Laboratories, Wellcome Foundation Ltd), was filtered through a Sartorius micropore filter (0.2 μ m) into saline to make a solution containing 4 to 40 mg ml⁻¹ so as to contain the required dose in a volume of 20 to 45 ml. The calculated volume was given over 1 h into a saline infusion running at 5–15 ml min⁻¹. In the radiolabel study [¹⁴C-formyl]BW12C was aseptically dispensed into the BW12C dosing solution (total intended

dose 10 mg kg⁻¹ body weight) to give a total radioactive dose of 16.03 μ Ci.

Determination of BW12C in samples

Internal standard (an inactive analogue of BW12C) was added to samples of haemolysate, plasma and urine which were then acidified by the addition of 1 M HCl (100 μ l/2 ml sample) and extracted with toluene. The toluene was back-extracted into 0.1 M phosphate buffer pH 8.5 and the aqueous extract was analysed by h.p.l.c. using a 250 mm $\times$ 4.6 mm i.d. Zorbax C8 Column and methanol/0.1 M phosphate buffer, pH 6.8, 50/50 v/v as the mobile phase. Detection in the eluant was by u.v. absorption at 275 nm, and calibration standards of BW12C were incorporated.

Total radioactivity in dosing solutions, plasma and urine was measured directly by liquid scintillation counting. Samples of blood and homogenised faeces were analysed for total radioactivity by combustion in a Packard Tri-carb automatic sample oxidiser followed by liquid scintillation counting.

Pharmacokinetic analysis

The time-courses of whole blood concentrations of BW12C post-infusion were examined in terms of conventional biexponential elimination (Metzler *et al.*, 1974). Fits were weighted by reciprocal squared concentration and computed by non-linear regression analysis. The rate constants λ_1 (redistribution) and λ_2 (elimination) were determined, together with the appropriate half-lives, as were the volume of the central compartment (V_c), clearance (CL) and area under the curve (AUC). Calculations of erythrocyte concentrations of BW12C from data for whole blood and plasma were made for each subject using the individual haematocrits determined during haematological screening.

Blood-oxygen saturation curves

These were measured using a commercially available semi-automated apparatus (Hem-O-Scan; Aminco) in which relative percent oxygen saturation is plotted as a continuous function of oxygen tension. The method of operation used

Figure 1 Graph of measured concentrations of BW12C in blood and plasma and calculated concentrations in erythrocytes (μ g ml⁻¹) plotted against time (h) during and after the intravenous infusion of BW12C 20 mg kg⁻¹ over 1 h. The duration of the infusion is indicated by the horizontal bar. Results are the mean $\pm$ s.e. mean for five independent subjects.

was as recommended in the instrument handbook and gas mixtures containing 5% carbon dioxide were employed. Three characteristic features were determined for each curve: first, the P20 (oxygen tension in mm Hg producing an oxygen saturation of 20%); second, the S10 (the % oxygen saturation of the blood corresponding to an oxygen tension of 10 mm Hg) and, third, the % modification (the proportion of haemoglobin converted to a high affinity form by BW12C). The last calculation is possible because BW12C interacts stoichiometrically with haemoglobin and curves measured in the presence of compound have a biphasic appearance explicable in terms of a proportional summation of a fully left-shifted curve (haemoglobin reacted with BW12C) and a normal curve (Beddell *et al.*, 1984).

Results

Clinical observations

No significant systemic effects of BW12C were observed in any subject. There were no signifi-

cant changes in pulse, blood pressure, respiratory rate or ECG. There were no changes of consciousness or mood other than relief of anxiety sometimes present at the initial time, and some drowsiness after lunch. Neither of these seemed drug related since they were also observed in the staff of the laboratory.

Local discomfort was reported around and above the injection site even at the lowest doses and fleeting erythema was observed above the injection site in one subject, C. During the initial stages of the first 20 mg kg⁻¹ dose in subject E, the pain over the superficial forearm vein used for infusion became intense soon after the start of infusion of the drug. The pain stopped almost immediately when the drug infusion was stopped (the saline drip continued throughout). Pain was reduced by increasing blood flow in the vein by finger movement, and also by increasing the diluent saline infusion rate. The infusion was recommenced at half the calculated rate but pain became intense and infusion of BW12C was discontinued. The pain stopped at once and there were no after-effects. Results from subject E are not further discussed, since the infusion time-course was interrupted,

Figure 2 Graphs of (a) maximum measured concentration of BW12C in blood (µg ml⁻¹) and (b) area under the blood concentration-time curve for BW12C (µg ml⁻¹ h) plotted against dose administered (mg kg⁻¹). Two subjects at 2, 5, 10 and 15 mg kg⁻¹; five at 20 mg kg⁻¹.

dose-rate was varied and the intended dose was not administered. In none of the subjects was there any sign of damage to the veins, or of thrombosis and it was concluded that the pain was probably due to stimulation of nerve endings in the veins by free BW12C. Subsequent subjects were given the infusion into a large (antecubital) vein, where greater blood flow was expected to decrease the local transient concentration of BW12C and no further complaints of pain or irritation were made, even though the subjects were told of the incident of pain requiring cessation of infusion.

In subject D, the first of two infusions of BW12C was accompanied by a rise in diastolic blood pressure from 78 to 90 mm Hg, the dose being 15 mg kg^{-1} . However, the second infusion of 20 mg kg^{-1} did not produce any significant rise of blood pressure. Some atrial premature beats were seen on his ECG before and during the first infusion, but these died down in the course of the day. In the second infusion no ECG abnormalities were observed and the effects of the first infusion were thought to be related to anxiety rather than to BW12C.

No other adverse effects were noted on clinical observation or biochemical screening.

Pharmacokinetic observation

Blood and plasma concentrations of BW12C were determined for the complete time-course in each subject and erythrocyte concentrations were then calculated. Results for the five subjects who received 20 mg kg^{-1} are summarised in Figure 1. Peak concentrations were observed immediately post-infusion. Scrutiny of individual results revealed that plasma concentrations at later time points ($\geq 10.5 \text{ h}$) equalled or even exceeded those in whole blood. This may be reasonably interpreted as the appearance of a metabolite of BW12C which is restricted to the plasma but is acid-labile and appears as parent BW12C during the extraction procedure. This postulated metabolite was not apparent in previous animal studies but subsequent work has revealed that the rat may behave in a similar fashion. Pharmacokinetic analysis of the data was restricted to data for the measured whole blood levels (Table 1) and serves to give upper limits for the elimination half-life of the compound and a lower limit for the volume of distribution (V_c). At the end of the infusion, some 80% of the dose was sequestered within the erythrocytes and V_c was not appreciably greater than the blood volume.

Elimination from whole blood was apparently biphasic with a mean λ_1 -phase half-life of 2.2 h

(range 1.9–2.6 h) and a mean λ_2 -phase half-life of 8.5 h (range 5.4–10.3 h). The pattern of elimination was the same at the lower doses ($2\text{--}15 \text{ mg kg}^{-1}$) with similar values for λ_1 - and λ_2 -phase half-lives although the latter were more poorly defined because of the limit of detection of the assay and detailed results are not presented. The λ_1 - and λ_2 -phases probably do not represent re-distribution and elimination, respectively, in the conventional view. Rather, it is likely that the λ_1 -phase represents the loss of BW12C from haemoglobin (likely to be the rate-limiting step) since this phase parallels the loss of the left-shifting effect (see later) and the λ_2 -phase represents loss of the proposed labile metabolite.

The blood levels of BW12C immediately post-infusion showed a non-linearity with increasing dose (Figure 2a) which was reflected by a reduction in apparent blood clearance. It is implicit that there was a dose-related increase in exposure of red cells to BW12C and the dose/AUC relationship confirms this (Figure 2b). This probably reflects a saturation of elimination at the higher doses and should be taken into consideration if the compound is administered to anaemic individuals.

The results of the analysis of total radioactivity in the blood and plasma of the subject infused with [^{14}C -formyl]-BW12C 10 mg kg^{-1} are illustrated in Figure 3. Total recovery of radioactivity after 5 days collection was only 67%. The major route of excretion of radioactivity was in the urine which accounted for 65% of the dose. Only 1% of the dose was measured in the faeces and a further 1–2% in the blood samples removed. The half-life of the elimination of total radioactivity in blood was 22 h and measurable levels were still present 48 h following administration. However, at 24 h after administration unchanged drug was not detectable. Analysis of excreted urine for unchanged drug indicated that only a total of 1.4% of the dose (2.2% of the excreted total radioactivity) was present as BW12C. The slow elimination of radioactivity from blood did not therefore reflect elimination of BW12C, but metabolites which are stable to extraction and which do not possess left-shifting activity (see below).

Effects on the blood-oxygen saturation curve

BW12C administered by intravenous infusion over 1 h caused a left-shift of the blood-oxygen saturation curve and curves are illustrated (Figure 4) for subject (F) before (i) and immediately after (ii) a dose of 20 mg kg^{-1} . The control curve (i) has a normal, sigmoid shape

Figure 3 Graph of concentration of BW12C in blood (●) and plasma (■) measured as compound (—) ($\mu\text{g ml}^{-1}$) or radioactivity (....) ($\mu\text{g equivalents ml}^{-1}$) plotted against time (h) during and after the intravenous infusion of [^{14}C -formyl]-BW12C 10 mg kg^{-1} over 1 h in subject A. The duration of the infusion is indicated by the horizontal bar.

whilst curve (ii) has the biphasic appearance typical of the effect of BW12C. Characteristics of the two curves (P20, S10 and % modification) are given in the inset to Figure 4. The largest effect was observed at the end of the infusion and characteristics of the curves measured at this time have been plotted against the dose administered (Figure 5). Clearly, the left-shift is dose-dependent. As well as dose dependence, the curves reveal that the threshold for detection of a left-shift lies at a dose of between 2 and 5 mg kg^{-1} . Characteristics of the blood-oxygen saturation curves at the end of the infusion are summarised in Table 2. The finding that the largest effect is observed immediately post-infusion is illustrated in Figure 6, where the mean % modification for the five subjects who received 20 mg kg^{-1} has been plotted against time. The slight dip in % modification after the end of the infusion is not statistically significant and is not paralleled by changes in blood levels of BW12C. The loss of left-shift, as characterised by the proportion of modified haemoglobin, is monoexponential with a mean $\pm$ s.e. mean half-life of $3.1 \pm 0.5 \text{ h}$. After 24 h, a marginal effect of BW12C could be detected in the

oxygen saturation curves, but the resolution of the curve analysis is poor at low values of % modification and the significance of the finding is at present equivocal.

For each subject receiving BW12C 20 mg kg^{-1} the calculated % modification was converted to a value for the erythrocyte concentration of modified haemoglobin by reference to the erythrocyte haemoglobin concentration determined by routine haematology. The molar ratio of the calculations of erythrocyte BW12C to modified haemoglobin was determined for data during, and up to 2 h post-infusion and was 3.04 ± 0.67 (mean $\pm$ s.e. mean for $n = 5$ subjects, 5–6 determinations per subject). The figure is in agreement with observations made *in vitro* which indicated that there may be other binding sites for BW12C on haemoglobin in addition to the postulated site (Beddell *et al.*, 1984).

Discussion

BW12C is a novel compound designed to left-shift the blood-oxygen saturation curve (Beddell *et al.*, 1984). When administered to normal

Figure 4 Blood-oxygen saturation curves for subject F before (i) and immediately after (ii) the intravenous infusion of BW12C 20 mg kg⁻¹ over 1 h. The oxygen saturation of the blood (%) is plotted against the oxygen tension (mm Hg) and characteristics of the curves (P20 - mm Hg, S10 - % and % modified haemoglobin) (see text) are shown in the inset table.

healthy male volunteers in doses of 2–20 mg kg⁻¹ by intravenous infusion, the compound caused a dose-dependent left-shift of the blood-oxygen saturation curve. There were no adverse systemic effects, either clinical or biochemical. A transient irritation at the site of infusion did not cause damage and was circumvented by the simple expedient of ensuring an adequate flow of blood through the vessel. The pharmacokinetic measurements confirmed laboratory findings that BW12C has a high affinity for haemoglobin: the largest proportion of the dose was sequestered within the erythrocytes.

Within the erythrocyte, the effect of BW12C on haemoglobin is essentially stoichiometric and can be quantified by numerical analysis of the blood-oxygen saturation curves. The percentage of modified haemoglobin gives a direct indication of the effect of the compound but it is arguable that the two empirical measures, the P20 (chosen in preference to the more usual P50 because of the shape of the modified curve) and the percentage saturation at 10 mm Hg oxygen tension, show more sensitivity with small left-shifts. Peak effect of the compound was seen at the end of infusion and the pharmacological effect (as judged by the proportion of modified haemoglobin) decayed monoexponentially thereafter with a half-life of 3.1 h. This contrasts with the pharmacokinetic

Figure 5 Graph of P20 (mm Hg), S10 (%) and % modified haemoglobin at the end of the infusion of BW12C 2–20 mg kg⁻¹ over 1 h for individual subjects (●) plotted against the dose administered. Number of subjects as for Figure 2. Range in the median control values (◆) is indicated by the vertical bar.

Table 2 Summary of blood-oxygen saturation curve data for five subjects receiving BW12C 20 mg kg⁻¹ by intravenous infusion over 1 h

	<i>F</i>	<i>D</i>	<i>Subject C</i>	<i>B</i>	<i>G</i>	<i>Mean ± s.e. mean</i>
<i>P20 (mm Hg)*</i>						
Control	15.0	14.0	14.9	15.1	14.3	14.7 ± 0.2
End of infusion	8.0	8.5	9.4	8.6	9.5	8.8 ± 0.3
<i>S10 (%)*</i>						
Control	10.0	11.0	9.5	9.0	10.5	10.0 ± 0.4
End of infusion	24.0	24.0	21.5	22.0	21.0	22.5 ± 0.6
<i>% Modified* haemoglobin</i>						
End of infusion	17.5	15.2	14.2	18.2	12.3	15.5 ± 1.1

*For definitions of abbreviations, see text.

Figure 6 Graph of the calculated proportion of modified haemoglobin (%) plotted against time (h) during and after the intravenous of 20 mg kg^{-1} BW12C. Results are the mean $\pm$ s.e. mean for five subjects and the duration of the infusion is indicated by the horizontal bar. The post-infusion loss of modification is estimated to have a mean half-life of $3.1 \pm 0.5 \text{ h}$.

behaviour of the compound, based on blood concentrations, which indicated a biexponential elimination. However, it is probable that the so-called ' λ_z '-phase is an artefact and represents the elimination of an inactive metabolite which is labile to the extraction and assays as parent compound. The presence of one or more metabolite(s) of BW12C, other than the labile metabolite, is confirmed by the pilot radiolabel study because [^{14}C]-labelled material was being recovered in the urine long after the loss of the effect on the blood-oxygen saturation curve and less than 2% of the recovered dose was present as BW12C. However, only some 67% of the radiolabel was recovered within 5 days and the possibilities remain, outside experimental error, either that BW12C was incorporated into intermediary metabolism, or the [^{14}C]-formyl moiety was cleaved and excreted as carbon dioxide.

So far as the application of compounds such as BW12C to the therapy of sickle cell disease is concerned, BW12C, *in vitro*, does inhibit morphological sickling (Beddell *et al.*, 1984) and preserves the filterability of sickle cells during progressive deoxygenation (Kenny & Stuart, 1983). In our studies, BW12C caused effects in blood towards the lower end of the range predicted to be of use and it should be

remembered that a decrease in oxygen tension to maintain oxygen extraction may well buffer the inhibitory effect on sickling of increased oxygen affinity (Sunshine *et al.*, 1978). However, since the effect of the compound is stoichiometric, the same dose might be expected to give twice the effect (or more, depending on the dose/AUC relationship) in an anaemic sickle patient with a circulating haemoglobin of, say, $6\text{--}8 \text{ g } 100 \text{ ml}^{-1}$ and the consequent modification (30%) is predicted to be of therapeutic benefit. Furthermore, therapy would be expected to cause a rise in erythrocyte mass either through hypoxaemic stimulation or reduction in haemolysis. Whilst this should compensate for reduced oxygen extraction occasioned by increased affinity, it remains to be seen whether the possibly detrimental effects of a rise in whole blood viscosity might overcome the rheological benefit of increased erythrocyte deformability (Kenny & Stuart, 1983).

In conclusion, it is anticipated that the compound will be of value to test the hypothesis that a stoichiometric modification of a proportion of haemoglobin into a high affinity form will be of therapeutic benefit to the sickle cell patient and investigations in sickle cell patients have now commenced.

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