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The pharmacokinetics, tolerability and pharmacodynamics of tucaresol (589C80; 4[2-formyl-3-hydroxyphenoxymethyl] benzoic acid), a potential anti-sickling agent, following oral administration to healthy subjects

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1 Tucaresol (589C80; 4[2-formyl-3-hydroxyphenoxymethyl] benzoic acid) interacts stoichiometrically with haemoglobin to increase oxygen affinity. By decreasing the proportion of insoluble deoxy sickle haemoglobin at capillary oxygen concentrations, tucaresol may be of therapeutic benefit in sickle cell anaemia.

2 In this study, which involved the first administration to man, the pharmacokinetics and pharmacodynamics of tucaresol were studied in healthy male volunteers following oral doses of 200–3600 mg.

3 Peak drug concentrations in plasma and erythrocytes were linearly related to dose; mean (s.d.) values were 95.8 (26.1) and 1035 (67) $\mu g\ ml^{-1}$, respectively, at the highest dose. Median t_{max} in plasma was 6.5 h and in erythrocytes 24.5 h, when approximately 60% of the administered dose was in the target tissue. Plasma drug concentrations fell biexponentially with commencement of the apparent terminal elimination phase at approximately 24 h. The terminal elimination half-life from plasma increased with dose ($r = 0.77$; $P < 0.0001$) from 133–190 h at 400 mg to a mean (s.d.) of 289 (30) h at 3600 mg. Erythrocyte drug concentrations declined monoexponentially with a half-life that was always shorter than the apparent terminal half-life in plasma: overall mean (95% CI) of $t_{1/2}$ erythrocyte/$t_{1/2}$ plasma ratio was 0.57 (0.53, 0.61). The erythrocyte AUC/plasma AUC ratio increased with dose ($r = 0.67$; $P < 0.001$).

4 The proportion of haemoglobin modified to a form with high oxygen affinity (%MOD) increased in a dose-related manner above doses of 800 mg reaching 19–26% after the 3600 mg dose. The %MOD was directly proportional to erythrocyte drug concentrations and declined in parallel during the elimination phase.

5 The drug was well tolerated, with no clear effects on resting or exercise heart rates or blood pressures. Small increases in reticulocyte counts were seen following doses of 2800 and 3600 mg suggesting stimulation of erythropoiesis.

Keywords tucaresol anti-sickling agent pharmacodynamics pharmacokinetics tolerability

Introduction

Sickle cell disease represents a family of inherited haemoglobinopathies characterised by an abnormal haemoglobin, haemoglobin S, with polymerises when deoxygenated (Dean & Schechter, 1978a). Polymerisation of haemoglobin S results in rigid distorted red cells, with the characteristic 'sickle' shape and leads to the microvascular obstruction which is thought to be the pathological basis for the clinical course of the disease (Dean & Schechter, 1978a). At present there is no specific therapy and treatment is primarily supportive (Platt, 1988). As the rate of sickling is dependent on the concentration of insoluble deoxy-haemoglobin S,

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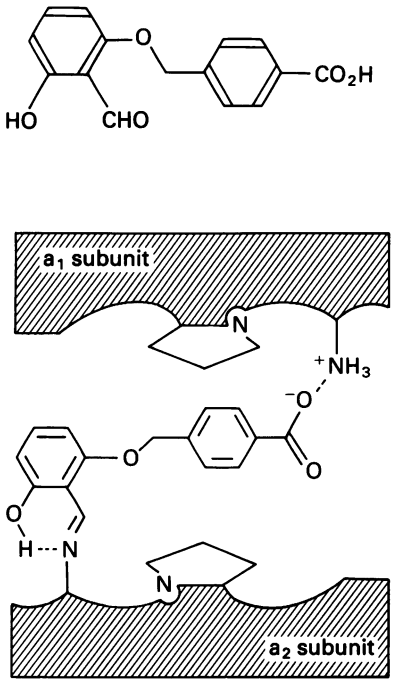

Figure 1 Structure of tucaresol and schematic representation of its binding to the oxy conformation of haemoglobin. The compound is postulated to bridge between the terminal amino groups of the α-chains forming a Schiff's base adduct with one and an ionic interaction with the other, with an important hydrophobic binding component from proline residues 77α.

decreasing the deoxy-haemoglobin S concentration relative to the oxy- conformation would be expected to confer therapeutic benefit. It has been postulated that this could be achieved by increasing the oxygen affinity of haemoglobin, i.e. 'left-shifting' the haemoglobin-oxygen saturation curve (OSC) (Dean & Schechter, 1978b).

Tucaresol, (589C80; 4[2-formyl-3-hydroxyphenoxymethyl] benzoic acid), is a substituted benzaldehyde which interacts stoichiometrically with haemoglobin to produce a haemoglobin tetramer with increased oxygen affinity (Figure 1) (Beddell *et al.*, 1984; White & Wootton, 1989). Evidence for an anti-sickling action of tucaresol *in vitro* comes from increased filterability of suspensions of sickle erythrocytes at low oxygen concentrations in a concentration-dependent manner (Keidan *et al.*, 1989). It is estimated, by analysis of the kinetics of sickle haemoglobin polymer formation, that for effective therapy it will be necessary to modify between 20 and 30% of a patient's haemoglobin to the high-affinity form (20–30%MOD) (Franklin *et al.*, 1983; Sunshine *et al.*, 1978).

In this study tucaresol was administered to man for the first time, as single oral doses to healthy male volunteers. The objectives were to describe the pharmacokinetics of tucaresol, to examine the effect on the haemoglobin-oxygen saturation curve (OSC) *ex vivo* and to investigate tolerability, including the heart rate response to moderate exercise in doses producing haemoglobin modification up to the predicted therapeutic range. The study was performed to determine whether tucaresol was suitable for further development as an antisickling agent.

Methods

Subjects

Nine healthy, male, non-smoking volunteers aged 23–38 years, weighing 65–84 kg, participated. They were assessed by physical examination, ECG, spirometry, urinalysis (Ames Multstix®), routine haematology and biochemical tests and haemoglobin electrophoresis before the study. All subjects were of haemoglobin A phenotype.

Protocol

The protocol was approved by an independent Ethics Committee. Subjects gave written informed consent and were paid an honorarium on completion of the study. The study was according to an open dose-escalating design. Pairs of subjects received 200, 400 and 800 mg, and groups of four subjects received 1200, 2000, 2800 and 3600 mg. All doses were given orally with 200 ml water at approximately 08.00 h following an overnight fast, except for the last two subjects at the highest dose (3600 mg—split dose group). In this pair, to avoid gastrointestinal symptoms (see Results—tolerability) 1600 mg was administered at approximately 01.00 h on the main study day with the remaining 2000 mg at the scheduled time of 08.00 h, which was still regarded as study time 0 h. Before and at intervals up to 24 h after dosage, blood samples were taken through an indwelling venous cannula, with an occluding stylet (Jelco), thus avoiding a heparin lock. Samples were taken for assay of tucaresol in lysed whole blood and plasma, estimation of %MOD, tests of coagulation and *ex vivo* platelet aggregation, blood counts and biochemistry tests. Urine samples were taken for urinalysis (Ames Multistix®). Heart rate and blood pressure were recorded at intervals and ECG was monitored continuously for the first 24 h. The heart rate response to moderate graded exercise performed on a cycle ergometer was assessed before drug administration and at 4 and 8 h and additionally at 24 h following doses of 2800 mg and above. Meals were provided 5, 10 and 24 h post-dose. Subjects remained in the laboratory for 24 h after dosing. The cannula was then removed and further blood and urine samples were taken at intervals up to 168 h (200 mg), 336 h (400 mg), and 504 h (800–3600 mg).

Tucaresol was supplied as 200 and 400 mg tablets by The Wellcome Foundation Ltd, Dartford, Kent.

Tucaresol assay in lysed whole blood (haemolysate)

Whole blood was lysed by the addition of 1 ml of blood to 4 ml of distilled water, and the sample was frozen pending assay.

A 1 ml aliquot of the haemolysate sample was acidified with 500 μl 1 M hydrochloric acid, to which was added 50 μl of 12.5 mg ml^{-1} internal standard (4[4-formyl-3-hydroxy phenoxymethyl] benzoic acid) (an inactive analogue). 50 μl dimethyl formamide: 0.1%

w/v sodium hydrogen carbonate solution (20:80 v/v) was added, followed by 5. 8 ml toluene and mixed for 15 min on a horizontal-bed shaker to extract the analytes. Following centrifugation at 500 *g*, the organic layer was removed and shaken with 1 ml of 0.067 M phosphate buffer (pH 8.5) to back-extract the tucaresol and internal standard. A 50 μl aliquot was subjected to h.p.l.c. with u.v. detection at 280 nm. The h.p.l.c. system used an octadecyl reversed-phase analytical column at 38° C with a flow rate of 1.4 ml min^{-1}. The mobile phase was 63% ammonium acetate (0.1 M):37% acetonitrile (v/v) with 25 ml l^{-1} glacial acetic acid and 1.5 ml l^{-1} di-*n*-butylamine.

The limit of quantification for tucaresol was 0.5 μg ml^{-1} in haemolysate, with a calibration range from 0.5 μg ml^{-1} to 50 μg ml^{-1}. The bias and precision at 0.5 μg ml^{-1} were 15% and 6% and at 50 μg ml^{-1} they were −1% and 2.5%, respectively.

Tucaresol assay in plasma

A 50 μl aliquot of 12.5 mg ml^{-1} internal standard was added to 200 μl plasma, and the sample was buffered with 300 μl of ammonium formate/formic acid buffer (0.5 M, pH 2.75). The sample was then microcentrifuged and the analytes adsorbed onto a preconditioned solid-phase extraction cartridge (C18, 100 mg) and washed with two consecutive 1 ml buffer washes and 1 ml of water. The analytes were then eluted with 250 μl of methanol and an aliquot was analysed by the same h.p.l.c. system as for haemolysate.

The limit of quantification was 0.5 μg ml^{-1} with a calibration range from 0.2 μg ml^{-1} to 40 μg ml^{-1}. Bias and precision were −4% and 7% at 0.5 μg ml^{-1} and 1% and 4% at 40 μg ml^{-1} respectively.

Haemoglobin modification—%MOD

Whole blood (0.5 ml) was kept at 4° C and the haemoglobin-oxygen saturation curve was determined within 24 h using a Hem-O-Scan apparatus. The Hem-O-Scan produces a continuous tracing of the OSC that is compared by eye with template curves constructed to show 0–100%MOD in 5% increments (Keidan *et al.*, 1986). Haemoglobin modification less than 5% could not be measured reliably and was recorded as less than 5%.

Haematology

Erythrocyte, white cell and platelet counts were determined with a Coulter Counter. Reticulocyte counts and differential white cell counts were performed by manual staining methods. Activated partial thromboplastin times and prothrombin times were determined using standard methods. Haemoglobin F was estimated by electrophoresis.

Platelet aggregation

Ex vivo platelet aggregation was assayed in citrated whole blood using an Ultraflo 100 Whole Blood Platelet Counter with adenosine diphosphate (2 μM) and collagen (0.5 μg ml^{-1}) as aggregating agents (Lumley & Humphrey, 1988).

Clinical measurements and exercise test

Heart rate at rest and systolic and diastolic blood pressures were determined with an automated oscillometric device (Hewlett Packard 78354A). Respiratory rate was measured by observation for 30 s. The exercise test was performed supine at 7.5° from the horizontal on an electrically-braked cycle ergometer (Lode). Exercise was performed at work rates of 40, 80 and 120 W for 3 min at each level, with an interval of 1 min between levels. Heart rate was measured at the end of each exercise period from a 5-beat portion of the monitored ECG tracing.

Data analysis

Pharmacokinetics Erythrocyte concentrations of tucaresol (C_e) were calculated using the following equations:

$$C_{wb} = C.\,(1 - \mathrm{Hct}) + C_e.\mathrm{Hct}$$

$$C_e = \frac{C_{wb} - (1 - \mathrm{Hct}).C}{\mathrm{Hct}}$$

where C_{wb} and C are tucaresol concentrations in whole blood and plasma respectively, and Hct is the haematocrit.

Drug concentration-time courses were analysed by standard non-compartmental methods using the Siphar program (Simed, Creteil, France). The terminal elimination rate constant (k) was determined by least-squares regression analysis of the terminal segment of the log drug concentration-time plot. AUC was calculated from AUC(0,t) + C_t/k, where AUC(0,t) was calculated by the linear trapezoidal rule, and C_t was the concentration at the last time point t. Oral clearance (CL_o) was calculated from Dose/AUC. C_{max} was the maximum observed concentration and t_{max} the time at which it occurred.

Statistics Means and standard deviations of pharmacokinetic and %MOD data are given for doses 1200–3600 mg. For the three lowest doses individual results are presented as only two subjects were studied at each dose. 95% confidence intervals were calculated for individual erythrocyte/plasma ratios for C_{max}, AUC, and $t_{½,z}$. The Pearson correlation coefficient was used to test for dose-dependency of pharmacokinetic variables with P values from the appropriate t statistic; linear regression was used to quantify the relationship. Medians were calculated for t_{max} and a 95% confidence interval was calculated for the difference in erythrocyte and plasma t_{max} using the Wilcoxon signed-rank test.

Results

Pharmacokinetics

Pharmacokinetic variables are summarised in Table 1. The mean drug concentration-time curves for the four subjects receiving 2800 mg are shown in Figure 2. C_{max}

Table 1 Individual pharmacokinetic and %MOD data

			Plasma					Erythrocyte				% Modification		
Subject	*Dose (mg)*	*Weight (kg)*	$t_{1/2,z}$ *(h)*	CL_o *(ml min^{-1})*	C_{max} *(μg ml^{-1})*	t_{max} *(h)*	*AUC (μg ml^{-1} h)*	$t_{1/2,z}$ *(h)*	C_{max} *(μg ml^{-1})*	t_{max} *(h)*	AUC *(μg ml^{-1} h)*	$t_{1/2}$ *(h)*	*Maximum %MOD*	t_{max} *(h)*
1	200	82.5			9.9	5.00			37.0	10.00				
2	200	77.8			11.1	5.00			48.5	10.00				
3	400	65.0	133	2.35	16.6	4.03	2829	91	82.2	12.00	13214			
4	400	69.0	190	1.86	19.6	3.00	3562	125	115.4	24.00	29863			
7	800	84.7	134	2.72	28.3	4.00	4889	85	172.8	24.00	29573			
8	800	68.0	126	2.31	36.5	4.08	5759	88	240.9	16.07	35011			
5	1200	80.5	252	1.65	40.4	8.00	12103	176	338.7	24.00	91181		7	12.0
6	1200	74.0	192	2.17	40.1	6.00	9180	115	306.4	48.25	72449		7	8.0
1	1200		123	3.33	39.3	4.00	5987	77	244.4	24.00	38739		5	6.0
3	1200		215	2.44	33.7	8.00	8161	100	305.7	50.75	55235		7	12.0
Mean			195	2.40	38.4	6.50	8858	117	298.8	36.75	64401		6.5	9.5
2	2000		278	2.04	60.8	6.00	16273	138	671.2	24.00	169144	215	17	49.3
4	2000		222	2.01	59.3	6.00	16487	108	640.7	24.00	191881	208	16	24.0
6	2000		207	2.37	57.1	6.00	14010	132	649.7	49.50	147352	89	12	24.0
9	2000	82.0	209	2.67	57.1	4.00	12455	97	601.1	24.00	123790	124	16	12.0
Mean			229	2.27	58.6	5.50	14806	119	640.7	30.38	158042	159	15.3	27.3
7	2800		232	3.61	56.6	6.00	12881	87	469.9	24.03	106316	310	12	24.0
8	2800		240	2.23	87.7	6.00	20871	146	853.6	24.00	210632	214	18	12.0
1	2800		178	2.97	91.4	6.00	15635	91	682.3	24.00	138053	161	17	16.0
3	2800		241	2.58	59.3	12.00	18018	156	671.7	24.00	174794	171	16	24.0
Mean			223	2.85	73.7	7.50	16851	120	669.4	24.01	157449	214	15.8	19.0
6	3600		291	2.36	81.7	6.08	25353	146	986.8	48.50	293607	166	22	16.0
2	3600		329	2.15	86.3	8.00	27818	158	1011.3	24.00	290871	161	24	16.0
7	3600*		265	2.48	80.3	8.00	24131	141	1007.1	73.50	274858	175	19	23.0
8	3600*		268	2.05	134.7	4.00	29116	161	1134.0	8.00	262505	165	26	23.0
Mean			289	2.26	95.8	10.02	26604	151	1034.8	42.00	280460	167	22.8	19.5

*Split dose.

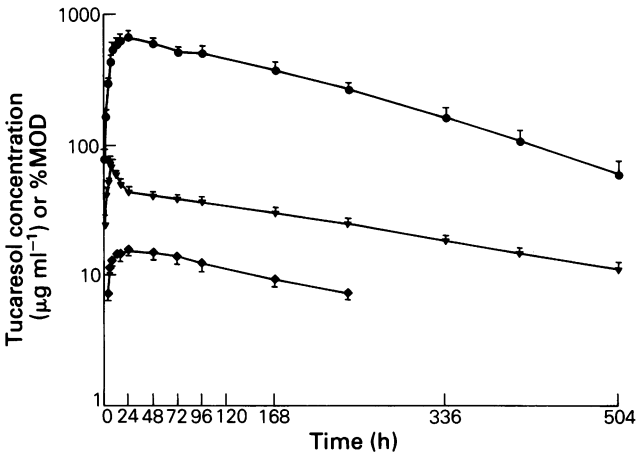

Figure 2 Mean concentration-time curves in plasma (▼) and erythrocytes (●) and time course of haemoglobin modification (◆) for four subjects receiving 2800 mg tucaresol as a single dose.

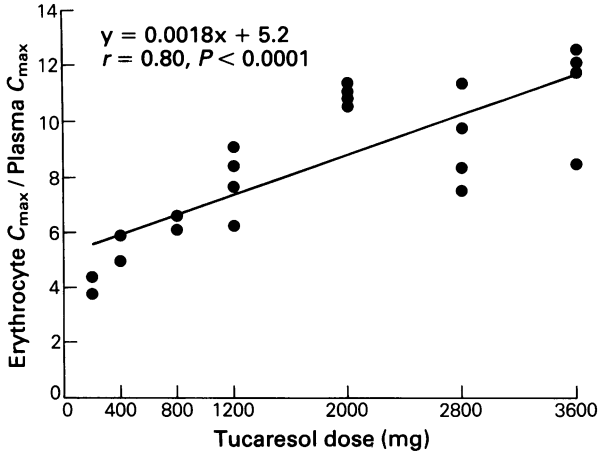

Figure 3 Relationship between the ratio of erythrocyte C_{max}/plasma C_{max} and dose of tucaresol.

values in plasma and erythrocytes were proportional to dose. The mean C_{max} in plasma at the highest dose of 3600 mg was 96 μg ml^{-1} and in erythrocytes it was 1035 μg ml^{-1}; there appeared to be no difference between the values for single dose and split dose pairs. The ratio of erythrocyte C_{max}/plasma C_{max} increased with dose (r = 0.80; P < 0.0001) from 3.8 and 4.4 at 200 mg to a mean (s.d.) of 11.2 (1.9) at 3600 mg (Figure 3). t_{max} was earlier in plasma (median 6.5 h, 3600 mg split dose pair not included) than in erythrocytes (median 24.5 h); (median and 95% non-parametric CI for difference: 18.5 h, 15–29 h). Plasma drug concentrations fell biexponentially with an initial fall from t_{max} to about 24 h followed by a slower terminal phase. Erythrocyte drug concentrations fell monoexponentially. The terminal half-life in erythrocytes (mean 151 h at 3600 mg) was shorter than that in plasma (mean 289 h); the overall mean ratio for erythrocyte $t_{½}$/plasma $t_{½}$ was 0.57 (95% CI; 0.53–0.61). Both half-lives increased with dose (r = 0.77, P < 0.0001 for plasma; r = 0.51, P < 0.05) for erythrocyte; Figure 4). The elimination rate constants and hence half-lives and values of AUC could not be calculated reliably following 200 mg because the sampling interval was only approximately one half-life.

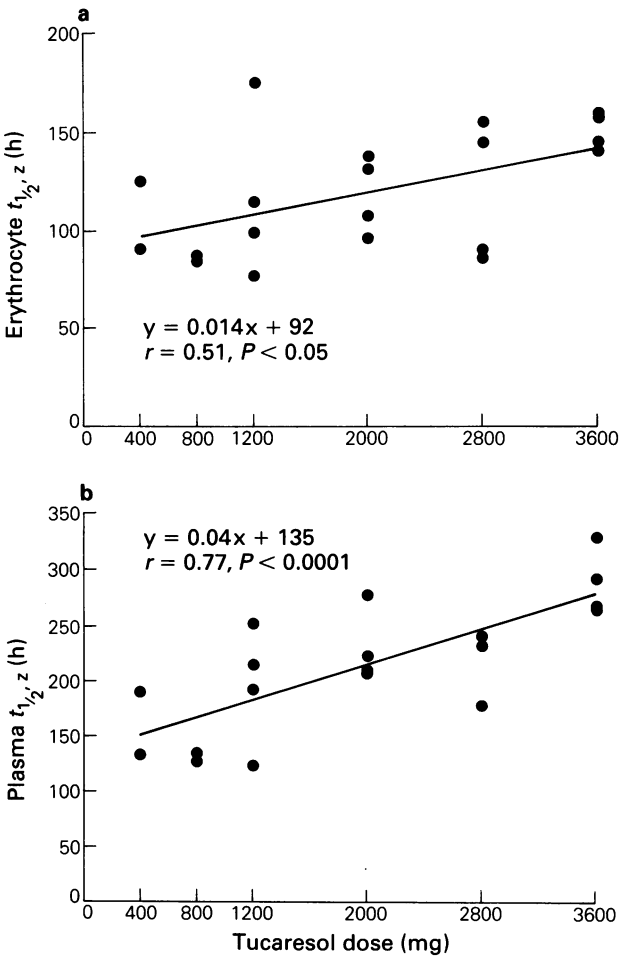

Figure 4 a) Relationship between erythrocyte $t_{½}$ and tucaresol dose. b) Relationship between plasma $t_{½,z}$ and tucaresol dose.

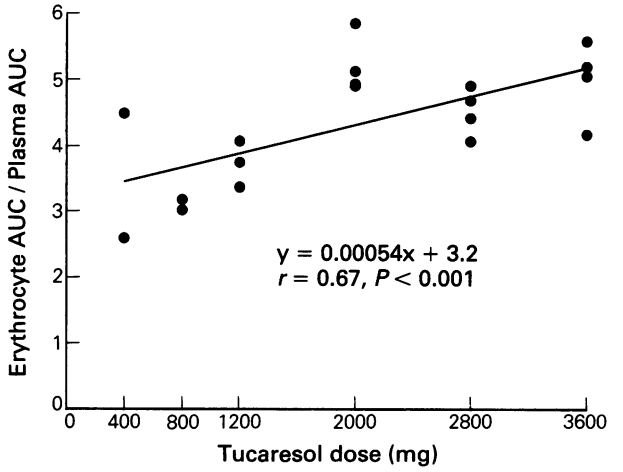

Figure 5 Relationship between the ratio of erythrocyte AUC/plasma AUC and dose of tucaresol.

Oral clearance was not related to dose. AUC was higher in erythrocytes than in plasma and the erythrocyte/plasma ratio increased with dose (r = 0.67, P < 0.001; Figure 5).

Haemoglobin modification—%MOD

Individual values of peak %MOD are given in Table 1. Haemoglobin modification was not detected at doses of

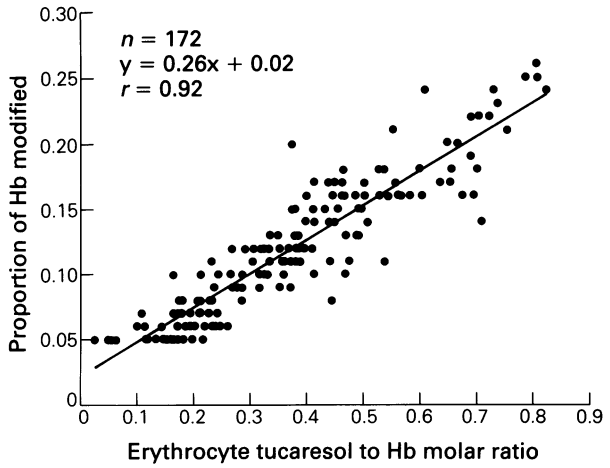

Figure 6 Relationship between erythrocyte tucaresol to haemoglobin molar ratio and proportion of haemoglobin modified to the high affinity form. Data represent all samples with haemoglobin modification >5%.

800 mg and below. Peak %MOD following 3600 mg ranged from 19–26% and occurred at a similar time as maximum drug concentrations in whole blood. The %MOD-time curves were parallel with the erythrocyte tucaresol concentration-time curves (Figure 2). In all subjects %MOD returned to less than 5% by the end of the sampling period. The relationship between %MOD and the molar ratio of erythrocyte tucaresol to haemoglobin ratio (Figure 6) was linear, without evidence of hysteresis.

Tolerability

One subject who received 1200 mg developed a headache and vomited approximately 10 h after drug ingestion and was withdrawn from the study. The first two subjects who took 3600 mg reported slight abdominal discomfort and loose stools. For the remaining two subjects the dose was split into two fractions of 1600 and 2000 mg separated by 7 h and there were no further reports of gastrointestinal symptoms.

Haematology

There were no trends for changes in the erythrocyte, leukocyte or platelet numbers or for erythrocyte haemoglobin F content throughout the study. Three of the four volunteers receiving 2800 mg had an elevation in reticulocyte count above the reference range (< 2%) to peaks of 2.2–3.1% between days 7 and 14. Two of the four subjects receiving 3600 mg had peaks of 2.1 and 2.8% between days 14 and 28. No changes in coagulation or platelet aggregation were observed.

Cardiovascular parameters

There were no trends for changes in resting heart rate or blood pressure and no differences in the exercise-induced heart rates between pre-drug and 24 h post-drug.

Other tests

There were no significant abnormalities of clinical biochemistry or urinalysis.

Discussion

This study has demonstrated that administration of tucaresol sufficient to modify 19 to 26% of haemoglobin to a high affinity form was well tolerated by healthy subjects. This is consistent with the lack of clinical consequences in patients with naturally occurring high affinity haemoglobinopathies, with the exception of polycythaemia. Polycythaemia, an expected response by the bone marrow to a hypoxic stimulus, was observed in animals treated chronically with high doses of tucaresol (unpublished data). The slight increase in reticulocyte count in this study, insufficient to increase haematocrit, may be a threshold effect of tucaresol on erythropoiesis.

The linear relationship between %MOD and the molar concentration ratio of erythrocyte tucaresol to haemoglobin is in agreement with the stoichiometric relationship obtained *in vitro* (Beddell *et al.*, 1984; Keidan *et al.*, 1989). The slope of 0.26 is in agreement with the *in vitro* data indicating that 3–4 mol tucaresol are required per mol of left-shifted haemoglobin tetramer.

The increased oxygen affinity of haemoglobin modified with tucaresol could reduce the tissue oxygen delivery per unit of volume of blood. Oxygen delivery could be maintained by increasing blood flow. Evidence of coronary and systemic vasodilation was observed in anaesthetised dogs following intravenous administration of tucaresol to produce a mean peak 44%MOD (unpublished data). In healthy volunteers receiving intravenous 12C79 (5[2-formyl-3-hydroxyphenoxy]pentanoic acid), an analogue of tucaresol, in doses producing 20, 30 and 40%MOD over 2 h, there was a dose-related increase in the heart rate response to moderate exercise (Nicholls *et al.*, 1989). The lack of a clear effect on the exercise heart rate in this study is probably due to the lower peak %MOD, and perhaps the relatively longer time from baseline to reach peak %MOD.

Tucaresol is an unusual drug in that blood sampling allows measurement of target tissue concentrations (erythrocyte) as well as plasma concentrations. The pharmacokinetics of tucaresol are complex. Plasma concentrations peak earlier than in erythrocytes. Within the first 24 h plasma concentrations fall while erythrocyte concentrations are rising, indicating that there is redistribution of drug from plasma to erythrocyte during this time. At the time of peak whole blood drug concentrations following 3600 mg, approximately 70% of the administered dose is present in blood, assuming a blood volume of approximately 5 l. This indicates that bioavailability is high. Similarly, using peak erythrocyte drug concentrations and assuming an average haematocrit of 0.42, approximately 60% of the administered dose is in the target tissue of the erythrocyte.

The plasma elimination half-life was significantly longer than that from erythrocytes. If drug in plasma and erythrocytes were in dynamic equilibrium during the apparent terminal phase the half-lives should be similar. A possible explanation for this difference is that some drug in plasma may be functionally in a 'deep' compartment. This could occur if some drug is bound to plasma proteins in a very stable conformation with a long half-time of dissociation *in vivo*. The formation of a Schiff's base adduct between the aldehyde group of tucaresol and other proteins could result in a 'reversible' covalent reaction with high stability. This interaction would also need to be reversible by the assay extraction procedure to enable the drug content to be measured. Human plasma protein binding *in vitro* was >98.5% and independent of concentration over a range exceeding the peak concentrations in this study (unpublished data). However, at present the proteins to which tucaresol binds in plasma and the nature of the interaction are unknown.

As dose increased, the ratio of AUC in erythrocytes to that in plasma increased. This indicates that the proportion of drug in erythrocytes compared with plasma increased with dose. This is accompanied by an increasing half-life with dose, while plasma clearance is unaffected. It is unlikely that this change in distribution is due to decreased plasma protein binding from the *in vitro* results mentioned above. The mechanism of this change in distribution is, therefore, not clear.

An elimination half-life of tucaresol from whole blood of approximately 1 week suggests that it is suitable for chronic use in the prophylaxis of the clinical consequences of sickle cell disease. The likelihood of painful crises in a patient with sickle cell disease increases with haematocrit (Baum *et al.*, 1987). As with any agent which inhibits sickling, chronic administration of tucaresol may result in a significant increase in haematocrit due to an inhibition of sickling and stimulation of erythropoiesis. Sudden cessation of therapy could result in a rebound increase in likelihood of a painful crisis. This is much less likely to pose a problem with tucaresol because of its long half-life.

The linear relationship between %MOD and whole blood drug concentration suggests that if facilities to measure %MOD by Hem-O-Scan are unavailable for clinical monitoring, (as the equipment is no longer manufactured), measurement of whole blood drug concentration may suffice. This would need to be validated in a clinical setting in patients with sickle cell disease and with a wider range of haematocrits than in the healthy population used in this study.

In this dose-escalating study of tucaresol in healthy volunteers, no adverse experiences or laboratory abnormalities were reported of sufficient importance to preclude further administration to patients. Tucaresol is well absorbed after oral administration and has a favourable pharmacokinetic profile for use in prophylaxis of the manifestations of sickle cell disease.

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