J. Pharm. Pharmacol. 1985, 37: 878–883
Received April 22, 1985

The sustained release of pyrimethamine base or pyrimethamine pamoate from a biodegradable injectable depot preparation in mice

M. D. COLEMAN*, G. W. MIHALY, S. A. WARD, G. EDWARDS†, R. E. HOWELLS‡ AND A. M. BRECKENRIDGE

Department of Pharmacology and Therapeutics, University of Liverpool, P.O. Box 147, Liverpool L69 3BX and †Departments of Tropical Medicine and ‡Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

The pharmacokinetics and mass fate in mice, of pyrimethamine (425 mg kg^{-1} s.o.) administered subcutaneously either as the base (BASE) or the pamoate salt (PAM) in an injectable oil mixture (benzyl benzoate–peanut oil 50:50 v/v) have been evaluated. Maximum measured plasma pyrimethamine levels after BASE were attained within 24 h, and were twice as high as after PAM. 25% of animals dosed with BASE died; among the survivors plasma drug levels fell rapidly below the minimum inhibitory concentration (MIC) for *Plasmodium berghei* (100–200 ng ml^{-1}) by 5 weeks. In contrast, no mice dosed with PAM died and plasma levels were sustained above the MIC for 13 weeks, drug still being detectable in plasma after four months. Overall, there was no significant difference between areas under the curve from zero time to the time of the final sampling of pyrimethamine following PAM or BASE. The rapid initial elimination of ^{14}C-radioactivity (2·64 ± 0·47% dose day^{-1} over 4 weeks) seen after dosage with [^{14}C]BASE reflected the plasma disposition of pyrimethamine in the mice dosed with BASE. 90% of the excreted ^{14}C was eliminated by one month by which time less than 1% (0·03 ± 0·02%) of the [^{14}C]BASE was recovered from the injection site. Both BASE and [^{14}C]BASE studies suggest that exhaustion of this preparation occurred by 7 weeks. Excretion of ^{14}C-radioactivity after [^{14}C]PAM was gradual and sustained with a low mean daily rate, that was maintained throughout the study i.e. 1·21 ± 0·17% day^{-1} (4 weeks), 0·88 ± 0·28% day^{-1} (8 weeks), 0·5 ± 0·31% day^{-1} (12 weeks), 0·42 ± 0·27% day^{-1} (16 weeks). At the end of the study, 7·11 ± 1·90% of the [^{14}C]PAM remained in the dose site. No visible tissue reactions were observed to either preparation. Overall recovery of radioactivity was 85·00 ± 11·31% for both [^{14}C]PAM and [^{14}C]BASE. These studies indicate that the PAM preparation is worthy of further long term evaluation.

Currently available antimalarials are only effective for short periods. Pyrimethamine, alone or in combination with a sulphonamide or sulphone, is an ideal choice for incorporation into long-acting antimalarial preparations (Coleman et al 1986); pyrimethamine sulphonamide combinations are already extensively used in areas of *Plasmodium falciparum* chloroquine resistance. Recent studies (Werbel et al 1984) have shown that pyrimethamine, when administered in delayed release preparations, protected mice from challenge by *P. berghei* for at least two months. Of these preparations, the poorly soluble pamoate in an aqueous vehicle was superior to the base in sustaining curative pyrimethamine levels over 32 h (Coleman et al 1986).

The present study evaluates the long-term release of pyrimethamine from injectable oil mixtures of both the base and the pamoate salt, administered to mice. This animal is a convenient and widely used experimental model for malarial chemotherapeutic research (Killick-Kendrick & Peters 1978).

* Correspondence.

MATERIALS AND METHODS

Reagents

Pyrimethamine base (BASE) and pyrimethamine 3-*N*-oxide were supplied by Wellcome (Beckenham, Kent, UK). Pyrimethamine pamoate (PAM) was a gift from the Walter Reed Army Institute of Research, Washington DC, USA, through the World Health Organisation. [^{14}C]Pyrimethamine base ([^{14}C]BASE) (specific activity 54 mCi $mmol^{-1}$) labelled in position 2 of the pyrimidine ring, was obtained from Amersham International (Amersham, Bucks, UK) and [^{14}C]pyrimethamine pamoate ([^{14}C]PAM specific activity 27 mCi $mmol^{-1}$) was synthesized for the World Health Organisation in the USA. Proguanil hydrochloride, the internal standard, was supplied by ICI Pharmaceuticals

(Macclesfield, Cheshire, UK). Benzyl benzoate and peanut oil were supplied by Sigma Chemicals (Poole, Dorset, UK). N.C.S. tissue solubilizer, and hydrogen peroxide (30% w/v) were supplied by BDH Chemicals Ltd (Dorset, UK). Emulsifying liquid scintillant (ES 299) was obtained from the Packard Instrument Co. Ltd (Reading, UK). All other solvents were of HPLC grade (Fisons, Loughborough, UK).

Animal studies

Male TFW white mice, mean weight 20 ± 0·5 g, were provided with an Oxoid 41B diet (Oxoid Ltd, London, UK) and free access to drinking water.

Plasma disposition of pyrimethamine

The plasma disposition of pyrimethamine, administered as either BASE or PAM incorporated in a biodegradable oil, was determined in two parallel groups. Mice in group I (n = 20) each received 425 mg kg^{-1} PAM (base equivalent dose), administered subcutaneously in 50 µl of an oil mixture (peanut oil–benzyl benzoate: 50 : 50 v/v). Mice in group II (n = 20) each received 425 mg kg^{-1} BASE, also administered subcutaneously in 50 µl of the same oil mixture. Blood samples (40–90 µl) were taken from the tail vein of each animal at 24, 48, 72, 96 and 168 h, then at weekly intervals for 15 weeks. The samples were collected into microhaematocrit tubes of 20 µl capacity, which were then sealed. Following centrifugation, (1100*g*, 5 min) volumes of plasma, (20–60 µl) accurately measured using a 100 µl capacity Hamilton syringe, were placed in Microcap tubes (1·5 ml, L.I.P. Equipment and Services, Yorks, UK).

Excretion and mass fate of ^{14}C radioactivity

The urinary and faecal excretion and mass fate of radioactivity after the administration of [^{14}C]PAM and [^{14}C]BASE were determined in 2 sets of animals comprising 8 groups, A–H. Mice in groups A, B, C and D (n = 5/group, set 1) received 425 mg kg^{-1} PAM subcutaneously in the oil mixture (peanut oil–benzyl benzoate 50 : 50 v/v). This dose included a tracer of 2·5 µCi, 23 µg) of [^{14}C]PAM. Mice in set 2, i.e. groups E (n = 6), F (n = 7), G (n = 7) and H (n = 6), were each dosed with 425 mg kg^{-1} BASE subcutaneously in the oil mixtures; again the dose contained a tracer of [^{14}C]BASE (1·5 µCi; 6·9 µg).

The mice were housed individually in metabolism cages; urine and faeces were collected at weekly intervals. Urine volumes were recorded and samples were assayed for pyrimethamine and pyrimethamine 3-*N*-oxide by HPLC (Coleman et al 1984). To determine accurately the rate of drug excretion, and the nature of the tissue localization of pyrimethamine at various times during the study, 5 mice from groups A and E, B and F, C and G, and D and H, were killed at intervals of 4, 8, 12 and 16 weeks, respectively. The ^{14}C-concentration at the injection site, major soft organs, and carcasses was determined.

Determination of ^{14}C radioactivity

Sample preparation. After removal of the heart, lung and kidneys, the liver, gut and injection site (i.e. the drug bolus, including immediately surrounding tissue), the tissues were homogenized separately using an Ultra-Turrax blender. The carcasses were dissected and homogenized using a Waring Blender.

Analysis of ^{14}C radioactivity. Samples of urine (20–50 µl) were directly assayed for ^{14}C radioactivity after addition of scintillant fluid (5 ml). Aliquots of the homogenate (50 µl) of liver, gut, faeces, combined heart, lung and kidneys, were incubated overnight with NCS tissue solubilizer (500 µl). Discolouration was achieved by a further 20 min incubation of the digest with hydrogen peroxide (200 µl).

To improve scintillation counting efficiency, the elimination of chemiluminescence was achieved by the addition of glacial acetic acid (60 µl), and after the addition of emulsifying liquid scintillant (20 ml), the samples were vortexed for 30 s, then cooled to 4 °C. Corresponding blank and radioactivity (0·1 µCi) spiked samples were processed identically.

Background radioactivity in samples from mice was determined as described, as was the recovery of ^{14}C-radioactivity from the various tissue homogenates (Coleman et al 1985).

The levels of ^{14}C-radioactivity in samples were determined in duplicate by liquid scintillation counting using a Packard Tri-Carb 4640 liquid scintillation spectrometer.

Determination of pyrimethamine

Pyrimethamine and its 3-*N*-oxide metabolites were determined in plasma and urine by a selective and sensitive microanalytical HPLC method (Coleman et al 1984). This was free from chromatographic interference from the pamoate moiety of pyrimethamine pamoate.

Pharmacokinetic calculations and statistical analysis

The area under plasma concentration/time curve from time = 0 to time = final sample (z) $AUC_{(o-z)}$

was calculated by the trapezoidal rule (Gibaldi & Perrier 1982). Peak plasma concentrations of pyrimethamine and the times at which they were attained were estimated graphically. Statistical analysis of data was by one way analysis of variance, and Student's *t*-test, modified to compensate for the increased likelihood of significant values during multiple *t*-testing (Wallenstein et al 1980). Statistical significance was at the $P < 0.05$ level.

RESULTS

Plasma disposition of unlabelled pyrimethamine

The mean log pyrimethamine plasma concentrations after administration of PAM (group I) and BASE (group II) are shown in Fig. 1, and the resultant pharmacokinetic parameters listed in Table 1. In group II (BASE), plasma concentrations at 24 h were approximately twice as high as those of group I (PAM), and produced severe toxic effects in some animals, 5 of which succumbed to pyrimethamine intoxication within 48 h. At 4 and 7 days following drug administration to group II, (BASE) the pyrimethamine AUCs (AUC_{0-4} days $21{\cdot}11 \pm 7{\cdot}00$ μg day ml^{-1}) and (AUC_{0-7} days $30{\cdot}10 \pm 7{\cdot}81$ μg day ml^{-1}) remained higher ($P < 0{\cdot}001$) than those in group I (AUC_{0-4} days $8{\cdot}31 \pm 4{\cdot}50$ μg day ml^{-1}, AUC_{0-7} days $19{\cdot}41 \pm 6{\cdot}94$ μg day ml^{-1}); however, between 7 and 14 days, pyrimethamine plasma concentrations in the group II (BASE) animals fell steeply from 1·9 to 0·1 μg ml^{-1}. Over the following 3 weeks, plasma levels fluctuated between 0·1 and 0·4 μg ml^{-1}. At five weeks post dose, pyrimethamine concentrations fell below the minimum inhibitory concentration (MIC) for pyrimethamine against *P. berghei*, the rodent malarial parasite (~100–200 ng ml^{-1}, Howells, personal communication).

In contrast, in group I (PAM), no signs of pyrimethamine toxicity were detectable, and peak plasma concentrations were approximately half those of group II. Initially, the AUCs (0–4) and (0–7) in group I were significantly ($P < 0{\cdot}001$) lower than those in group II (Table 1). However, 14 days post-dose, group I pyrimethamine plasma concentrations exceeded those of group II 10-fold (Fig. 1). Only 14 weeks after administration did PAM plasma concentrations fall below the MIC. At 16 weeks, pyrimethamine was still detectable in 9 of the group I mice. Overall, the mean $AUC_{(0-z)}$ of both groups I and II did not significantly differ (Table 1).

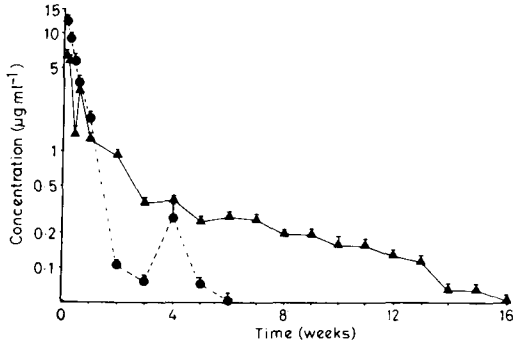

FIG. 1. Semilogarithmic plots of plasma levels (μg ml^{-1} mean ± s.e.m.) of pyrimethamine plotted against time after 425 mg kg^{-1} s.c. pyrimethamine pamoate (▲) or base (●) to 16 weeks.

Excretion and mass fate of ^{14}C-radioactivity

The cumulative excretion of ^{14}C-radioactivity (urinary, faecal and total) and the urinary cumulative excretion of unchanged pyrimethamine over 16 weeks is shown on Fig. 2. Of 26 mice dosed with [^{14}C]BASE, seven died as a result of pyrimethamine toxicity, a similar proportion of fatalities to those in group II (BASE).

Initially ^{14}C-radioactivity and unchanged drug were rapidly eliminated from animals in set 2 ([^{14}C]BASE, Fig. 2), 90% of the eliminated ^{14}C, being excreted four weeks post dose. Low-level elimination of radioactivity and unchanged drug persisted for a further 3 weeks. The mean rate of excretion over the initial 4 weeks (Table 2) of

Table 1. Pharmacokinetic determinates of pyrimethamine at 425 mg kg^{-1} when dosed (s.c.) as the pamoate salt or base in a benzyl benzoate–peanut oil (50 : 50 v/v) mixture. Values are listed as mean ± s.d., for n, peak concentration (Cpk), area under the curve (AUC) from zero to 4 days, zero to 7 days and zero to final sample time (z).

Formulation	n	Cpk (μg ml^{-1})	Tpk (h)	$AUC_{(0-4}$ μg day ml^{-1})	$AUC_{(0-7}$ μg day ml^{-1})	$AUC_{(0-z)}$
Pyrimethamine pamoate (Group I)	20	6·51 ± 3·00	24	8·31 ± 4·50	19·41 ± 6·94	42·51 ± 11·52
Pyrimethamine base (Group II)	15	12·75 ± 3·90	24	21·11 ± 7·00	30·10 ± 7·81	40·73 ± 7·00
P		≤ 0·001		≤ 0·001	≤ 0·001	NS

Table 2. Mean total (urinary and faecal) excretion rate for each group listed as mean ± s.d. after s.c. administration of [^{14}C]pyrimethamine (425 mg kg^{-1}) as the pamoate salt or base in a benzyl benzoate–peanut oil (50 : 50 v/v) mixture.

	Group	A	B	C	D
	n	5	5	5	5
[^{14}C]Pyrimethamine pamoate (set 1)	Time after dosage (days)	28	56	84	112
	Mean total excretion rate (% dose day^{-1})	1·21 ± 0·17 $P \leq 0·05$	0·88 ± 0·28 $P \leq 0·05$ vs A	0·5 ± 0·31	0·42 ± 0·27 $P \leq 0·05$ vs A, B
	Group	E	F	G	H
	n	5	4	5	5
[^{14}C]Pyrimethamine base (set 2)	Time after dosage (days)	28	56	84	112
	Mean total excretion rate (% dose day^{-1})	2·64 ± 0·47 $P \leq 0·05$	0·03 ± 0·02 $P \leq 0·05$	—	—

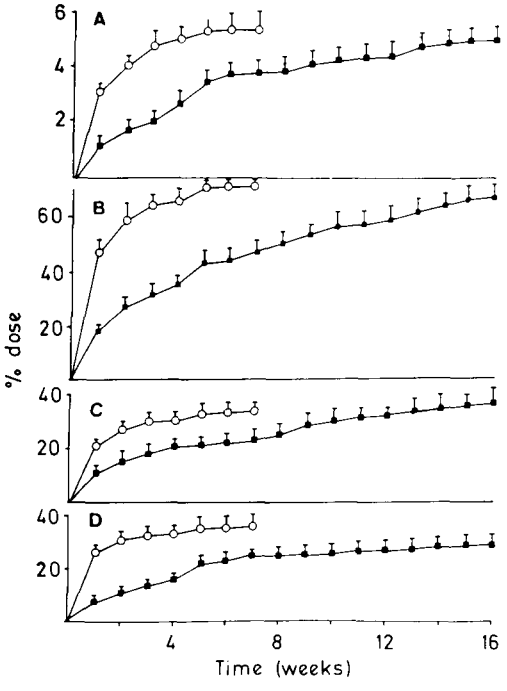

FIG. 2.Cumulative excretion profiles of ^{14}C-radioactivity and pyrimethamine (mean ± s.e.m. % dose) plotted over 16 weeks, after administration of [^{14}C]pyrimethamine pamoate (■) or [^{14}C]pyrimethamine base (○): (A) unchanged drug in urine, (B) total excreted ^{14}C (faeces and urine), (C) faecal ^{14}C excretion, (D) urinary ^{14}C excretion.

^{14}C-radioactivity after administration of BASE was more than twice that of the mice administered PAM (Group E vs Group A; Table 2). Consequently the percentage of [^{14}C]BASE remaining in the dose site was less than 1% (group E; Table 3). By seven weeks the rate of excretion and the residual dose site radioactivity had fallen almost to zero in Set 2 ([^{14}C]BASE, group F). Hence ^{14}C-radioactivity elimination after dosage with base, reflected the plasma disposition of pyrimethamine in the mice dosed with BASE (group II), both studies indicating exhaustion of the pyrimethamine base preparation 7 weeks after dosage. Residual tissue localization in the various organs accounted for 6% of the [^{14}C]BASE dose at 4 weeks, with no residual radioactivity detected thereafter.

In marked contrast, after [^{14}C]PAM, ^{14}C-radioactivity and unchanged drug elimination was gradual and was sustained at a low level for the full 16 weeks of the study (Fig. 2). After [^{14}C]PAM (groups A–D), the mean rate of ^{14}C-radioactivity excretion (Table 2) was significantly lower than that of set 2 ([^{14}C]BASE). In addition, there were no significant differences between groups A, B and C in the rates of ^{14}C radioactivity elimination (Table 2), over the first 12 weeks, after which the excretion rate fell significantly. In contrast to the rapid release of drug from the dose site after [^{14}C]BASE, there was more gradual release of drug after [^{14}C]PAM. After 4 weeks half the dose was recovered from the injection site, and four months after administration, 7·1% of the dose of [^{14}C]PAM still remained in the site of injection (Table 3). Tissue localization in the soft organs was undetectable in groups A–D of set 1.

Over the period of both studies, none of the mice suffered any visible tissue reactions to the drug. After the mice in sets 1 and 2 were killed, examination of the areas around the injection sites revealed

Table 3. Mean ± s.d. residual injection site [^{14}C]pyrimethamine pamoate or [^{14}C]pyrimethamine base listed as % dose, after s.c. administration of [^{14}C]pyrimethamine as the pamoate salt or base at 425 mg kg^{-1} in a benzyl benzoate–peanut oil (50 : 50 v/v mixture).

	Group	A	B	C	D
	n	5	5	5	5
[^{14}C]Pyrimethamine pamoate (set 1)	Days post dosage	28	56	84	112
	[^{14}C]-Pyrimethamine pamoate (% dose)	50·84 ± 16·70	27·71 ± 6·7	11·90 ± 4·41 NS vs D	7·11 ± 1·90 NS vs C
	Group	E	F	G	H
	n	5	4	5	5
[^{14}C]Pyrimethamine base (set 2)	Days post dosage	28	56	84	112
	[^{14}C]-Pyrimethamine base (% dose)	0·88 ± 0·39	0·10 ± 0·10		

no visible internal reactions, however in set 2, of 14 mice, injection sites were only visible in 3 of the group E animals.

In set 1 ([^{14}C]PAM), the injection sites of the mice were clearly visible, and were encapsulated in a thin layer of tissue. During the study less than 6% of the dose of pyrimethamine was excreted unchanged while less than 2% of the dose was accounted for as pyrimethamine 3-*N*-oxide, which is known to be a minor metabolite of pyrimethamine (Hubbell et al 1978; Coleman et al 1985a). The overall recovery of ^{14}C-radioactivity after administration of [^{14}C]PAM and [^{14}C]BASE was 85·00 ± 11·31%.

DISCUSSION

The most promising long acting antimalarial preparation, cycloguanil pamoate (Thompson et al 1963) underwent extensive clinical trials (for review see Elslager 1969) but was poorly tolerated. However, there remains an urgent need for effective long-acting antimalarial preparations, owing to lack of compliance with existing drug regimens (Weber et al 1975), and the logistical problems involved in frequent mass chemotherapy (Howells 1982). Pyrimethamine/sulphonamide combinations continue to be effective in areas of chloroquine resistance (Worth & Werbel 1984), and the present report is a preliminary step in the development of an injectable, sustained release pyrimethamine pamoate/sulphonamide preparation.

From Fig. 1, it is clear that the pyrimethamine base preparation (group II and set 2) had undesirable characteristics. The high maximum measured plasma levels, which were far in excess of those necessary to inhibit *P. berghei*, were associated with severe toxicity leading to the death of some animals. The high initial rate of ^{14}C-radioactivity elimination and unchanged drug was reflected in the rapid fall in pyrimethamine plasma concentrations (Fig. 2) and, at four weeks, 90% of the excreted ^{14}C-radioactivity had been recovered. This high mean rate of excretion (Table 2) was borne out in the low levels of [^{14}C]BASE remaining in the injection site at four weeks (Table 3). Therefore, the BASE preparation was clearly unsuitable.

However, the incorporation of PAM in the oil depot was more encouraging. There was no evidence of toxicity, as maximum measured pyrimethamine plasma concentrations were half those of group II (BASE, Table 1). The AUC data obtained over the first seven days of the PAM gave plasma drug levels lower than BASE-dosed mice (Table 1). Over the initial two weeks, in marked contrast to the [^{14}C]BASE group, the elimination of radioactivity and unchanged drug was gradual and sustained. This was again reflected in the reduced rate of decline in drug plasma levels after PAM administration compared with BASE (Fig. 2). At four weeks, half the dose of [^{14}C]PAM remained at the injection site (Table 3), which correlated well with the low mean rate of excretion (Table 2), sustained throughout the study. Overall, after an initial modest surge over the first 2–4 weeks, the release of pyrimethamine from PAM appeared to approach zero order kinetics (Figs 1, 2).

At the conclusion of these studies, there was no significant difference in the $AUC_{(o-z)}$ of groups I (PAM) or II (BASE) and no difference in the cumulative excretion of either unchanged drug in urine or total ^{14}C-radioactivity in between sets 1 ([^{14}C]PAM) and 2 ([^{14}C]BASE) (Fig. 2).

However, the PAM preparation was superior to the BASE preparation in terms of safety, and maintenance of curative drug plasma concentrations and its increased effectiveness when administered in a low dose aqueous vehicle (Coleman et al 1985b) has been borne out in the present report.

While the ultimate clinical failure of cycloguanil pamoate was principally due to local injection site tissue reactions (Clyde 1969), which were also reported in mice when the preparation was first evaluated (Thompson et al 1963), the lack of external or internal tissue reactions to pyrimethamine in the present study, both as BASE and PAM, suggest that PAM is worthy of further assessment.

Acknowledgements

M. D. C. was supported by the Medical Research Council, G. W. M. by the National Health and Medical Research Council of Australia, S. A. W. by the Mersey Regional Health Authority, and G. E. by the Wolfson Foundation. This work also received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. The authors are grateful to Miss Angela Davies for skilled technical assistance.

REFERENCES

Clyde, D. F. (1969) J. Trop. Med. Hyg. 72: 81–85

Coleman, M. D., Edwards, G. Mihaly, G. W., Howells, R. E., Breckenridge, A. M. (1984) J. Chromatogr. 308: 363–369

Coleman, M. D., Mihaly, G. W., Edwards, G., Ward, S. A., Howells, R. E., Breckenridge, A. M. (1985) J. Pharm. Pharmacol. 37: 170–174

Coleman, M. D., Mihaly, G. W., Edwards, G., Ward, S. A., Howells, R. E., Breckenridge, A. M. (1986) Biopharm. Drug Disp. in press

Elslager, E. (1969) Prog. Drug Res. 13: 170–216

Gibaldi, M., Perrier, D. (1982) Pharmacokinetics (2nd ed.) Marcel Dekker, New York, pp 445–449

Howells, R. E. (1982) Br. Med. Bull. 38: 193–199

Hubbell, J. P., Hemming, M. L., Grace, M. E., Nichol, C. A., Sigel, C. W. (1978) in: Gorrod, J. W. (ed.) Biological Oxidation of Nitrogen. Elsevier/North Holland Biomedical Press, Amsterdam, pp 177–182

Killick-Kendrick, R., Peters, W. (1978) Rodent malaria. Academic Press (London) pp 345–393

Thompson, P. E., Olslewski, B. J., Elslager, E. F., Worth, D. F. (1963) Am. J. Trop. Med. Hyg. 12: 481–493

Wallenstein, S., Zucker, C. L., Fluers, J. L. (1980) Circ. Res. 47: 1–9

Weber, M. C., de V. Clarke, V., Harwin, R. M., Shiff, C. J. (1975) Cent. Afr. J. Med. 21: 187–192

Werbel, L. M., Jacobs, R. L., Steinkampf, R. W., Worth, D. F. (1984) Am. J. Trop. Med. Hyg. 33: 526–533

Worth, D. F., Werbel, L. M. (1984) in: Peters, W., Richards, W. H. G. (eds) Antimalarial Drugs Vol. 2, Current Antimalarials and New Drug Developments. Springer, Berlin, pp. 237–252