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The Parenteral Controlled Release of Liposome Encapsulated Chloroquine in Mice

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Abstract—Free (0·6 mg), and liposome encapsulated chloroquine (0·6, 3 mg), were injected intraperitoneally, intramuscularly and subcutaneously in mice. Intraperitoneal administration of liposome-encapsulated chloroquine resulted in high and long lasting concentrations of chloroquine in the blood compared with intraperitoneal administration of free chloroquine. After administration of the liposome-encapsulated chloroquine the concentrations in the spleen were also higher, indicating that chloroquine liposomes reached the blood compartment intact after intraperitoneal administration. After intramuscular and subcutaneous administration the chloroquine liposomes acted as a local depot, giving a slower release from the subcutaneous fat layer than from the muscle depot. After the 0·6 mg dose a burst effect was found at about 7 h in most of the animals; this was not found after the 3 mg dose. This finding and the slower release after the 3 mg dose than after the 0·6 mg dose could be explained by the formation of aggregates after the injection.

Chloroquine remains an important antimalarial drug (WHO 1984); it is cheap, widely available, well tolerated and orally well absorbed. However, on fast parenteral administration life threatening cardiac toxicity may result because of high concentrations during the distribution phase (White 1985). This could be avoided by a slow parenteral release system. Liposomes have proved to be useful in this context (Näs-sander et al 1990).

Peeters et al (1989a) found that encapsulation of chloroquine by liposomes, for intraperitoneal administration to mice, appeared to result in an increase in the therapeutic index and prophylactic efficacy, possibly owing to an altered distribution of the encapsulated drug. Compared with the maximum safe dose of the free form a tenfold dose of chloroquine in liposome form does not exhibit toxicity.

Chloroquine is partly metabolized in man to the active desethyl metabolite (McChesney & Fitch 1984). The basic and clinical pharmacokinetics in man have recently been reviewed (White 1985, 1988).

Materials and Methods

Materials

Cholesterol, L-α-distearoylphosphatidylcholine (DSPC) and L-α-dipalmitoylphosphatidylglycerol (DPPG) were obtained from Sigma Chemicals (St. Louis, MO, USA).

Desethylchloroquine (des-CQ) and hydroxychloroquine were gifts from Dr F. Schobben (Academic Hospital, Utrecht, The Netherlands).

Chloroquine diphosphate met the requirements of the British Pharmacopoeia. Solvents and other reagents were of analytical grade.

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Preparation of chloroquine-containing liposomes

Negatively charged, gel state, chloroquine-containing, reversed phase evaporation vesicles were prepared as described previously (Szoka & Papahadjopoulos 1978). The molar ratio of the lipids was DSPC:DPPG:cholesterol = 10:1:10. An amount of 2·4 mmol lipid was used. After the extrusion of the resulting liposome dispersion through 600 and 200 nm polycarbonate membranes (Nucleopore Corp., Pleasanton, USA), the non-encapsulated chloroquine was removed by ultracentrifugal sedimentation three times for 45 min at 80 000 *g*. The liposome pellet was restored in isotonic (NaCl) 100 mM acetate buffer pH 7·4 at 4 °C under a nitrogen atmosphere.

The resulting liposome (lipCQ) dispersion was analysed for total chloroquine and lipid phosphate by spectrophotometry, the free chloroquine was measured spectrofluorimetrically. Mean particle size was calculated from dynamic light scattering data.

A justification of the type and chemical composition of the liposomes used in this study and a detailed description of their preparation and characterization has been given by Peeters et al (1989a).

Animal experiments

Outbred Swiss mice, 27·6 ± 4·8 g (mean ± s.d.), were obtained from colonies of the animal facility of the University of Nijmegen. Groups of five mice received chloroquine either free in solution (0·6 mg) or as liposomes (0·6 or 3·0 mg). Injections were given intraperitoneally, intramuscularly in the gluteus muscle or subcutaneously in the pouch of the hind neck region. For different amounts of lipid, the liposome dispersion was diluted to produce equal injection volumes.

At predetermined sampling times, 50 μL heparinized blood was taken from the tail vein and diluted with 200 μL bidistilled water. All samples were stored at −20 °C until analysis. Animals were killed with ether, the vascular system

perfused with phosphate buffer saline and the heart, liver and spleen were removed and stored at −20°C pending analysis.

Assay of chloroquine and desethylchloroquine

Total chloroquine and desethylchloroquine concentrations in whole blood and tissues were measured by a normal phase HPLC method with fluorimetric detection as described by Alvan et al (1982) (column: 5 μm spherical silica (Waters Ass.); mobile phase: acetonitrile:methanol:diethylamine (84·5:15·0:0·5 v/v); flow rate: 0·5 mL min^{-1}).

Heart, liver and spleen tissues were defrosted, weighed and homogenized according to de Groot & Wubs (1987) before further determination.

After addition of 40 μL of 4 mg L^{-1} hydroxychloroquine solution as an internal standard and 1·2 mL freshly prepared 0·1% (v/v) diethylamine and 0·5 mL 5M ammonia, 125 μL of the diluted whole blood samples or tissue samples were extracted with freshly prepared dichloroethane–diethylether (60:40) by end over end rotation at 30 rev min^{-1} for 30 min. After separation of the phases by centrifugation for 15 min at 5000 *g*, the organic layer was evaporated in a clean tube by a stream of nitrogen at 25°C. After reconstitution in 500 μL of eluent, 50 μL was injected. Chloroquine and des-CQ concentrations were calculated by comparing peak height ratios of chloroquine or des-CQ and internal standard of unknown samples to standards. The absolute limit of detection, calculated as 3 times the baseline noise, was 2 ng for chloroquine and 1 ng for des-CQ. The limit of detection expressed as blood concentrations was 400 ng mL^{-1} for chloroquine and 200 ng mL^{-1} for des-CQ.

Pharmacokinetic and statistical evaluation

Areas under the curve were calculated by the linear trapezoidal rule. Results were considered to be normally distributed when the mean was larger than twice the standard distribution of the data set and no indications for deviant distributions existed (Zuidema & Wynne 1989). For difference testing in cases of normally distributed data, Students *t*-test for non-paired results was used; for non-normally distributed data, the Wilcoxon rank sum test for two independent groups was applied. The level of significance was chosen at $P=0·05$. Graphical representation of difference testing was performed, where relevant, by confidence intervals.

Results

Characterization of chloroquine-containing liposomes

Analysis of the liposomal chloroquine dispersion resulted in 22 mg chloroquine-base and 98 μmol phospholipid per mL buffer. Particle size analysis of the vesicles yielded a mean size of 0·26 μm (n=3). Non-liposome associated chloroquine after the clean-up procedure was 2% of the total.

Intraperitoneal administration

Fig. 1 shows the concentration time curves after intraperitoneal administration of 0·6 mg free and 0·6 mg liposome encapsulated chloroquine. The T_{max} value was short when free chloroquine was administered, indicating that uptake in the bloodstream of free chloroquine proceeded immediately.

The profiles after intraperitoneal administration of liposomal encapsulated chloroquine (Fig. 1b) exhibited T_{max} values of 2–8 h.

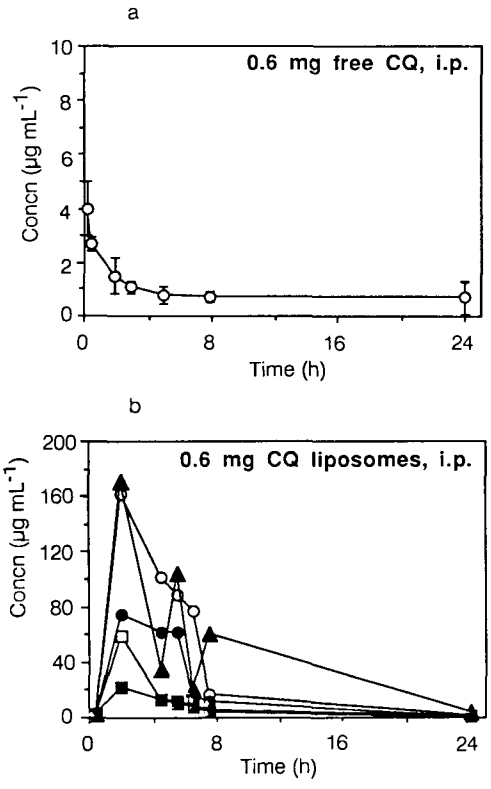

FIG. 1. Chloroquine (CQ) concentration-time curves of intraperitoneally administered 0·6 mg free CQ (a) and 0·6 mg liposome-encapsulated CQ (b). The curve of set a is presented as a mean curve, vertical bars indicate confidence intervals.

After administration of liposome-encapsulated chloroquine, chloroquine blood concentrations were significantly higher over the observation period than after free chloroquine. The differences in $AUC_{0\ 24}$ were also significant ($P=0·002$).

The ratio chloroquine/des-CQ was calculated and values are depicted in Fig. 4. Levels of des-CQ were lower than chloroquine (not separately shown). No differences of any significance in the ratios were found after administration of free 0·6 mg chloroquine. When the drug was given intraperitoneally as the liposome encapsulated form, the ratio was relatively high.

Intramuscular and subcutaneous administration

Table 1 shows the concentrations after intramuscular and subcutaneous administration of free and liposome-associated chloroquine. For both free and liposomal preparations the chloroquine concentrations were of the same order of magnitude when they were given intramuscularly or subcutaneously; however, chloroquine concentrations were higher or similar over the whole observation period when given as liposomes intramuscularly or subcutaneously compared with the free administration form, indicating sustained release from a depot. After the intramuscular dose, the initial concentrations tended to be higher than after the subcutaneous dose, which may indicate a stronger retarding effect of the latter. A burst effect was visible at 7 h in four of the five animals after intramuscular administration.

Chloroquine concentrations in the blood were lower after

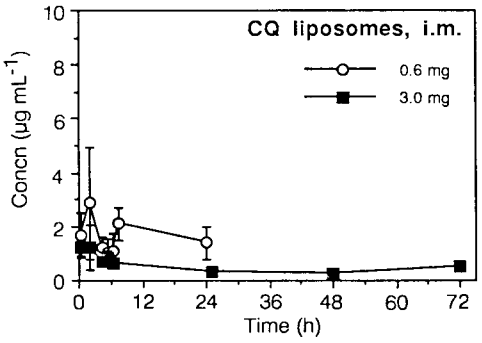

FIG. 2. Chloroquine (CQ) concentration-time curves after intramuscular administration of 0·6 and 3 mg CQ liposomes. Vertical bars indicate confidence intervals.

a 3 mg dose than after a 0·6 mg dose over the first 24 h. (Fig. 2 for the intramuscular administration).

The chloroquine concentrations in the spleen (Fig. 3) were high in the spleen after intraperitoneal administration of liposome-associated chloroquine compared with the other two routes.

The data for des-CQ exhibit a tendency to higher concentrations when 0·6 mg free chloroquine was given compared with the 0·6 mg liposome-encapsulated form. A similar organ distribution was found but with lower concentrations compared with chloroquine itself (results not shown).

Discussion

The effectiveness of liposomes as potential drug carriers in rodent malaria chemotherapy was demonstrated by Peeters et al (1989b). The concentration time profiles after i.p. administration described in the present study correlate well with the results therein described.

After the intraperitoneal injection of liposome encapsulated chloroquine the total chloroquine blood levels were much higher compared with those after the administration of free chloroquine. No toxic effects were observed in the mice treated with the liposome preparation indicating that the major part of chloroquine remained encapsulated in the liposomes. This is supported by the high area under the curve found after liposomal injection (lower apparent distribution volume).

In Fig. 4 the chloroquine/des-CQ ratios are shown for the free and the liposome i.p. and i.m. administrations. The s.c. curve resembles the i.m. curve. The ratio after i.p. administration of liposome-encapsulated chloroquine is significantly higher for most time points than after administration of free chloroquine, whereas the ratios of free and liposome i.m. and s.c. administrations are all comparable. The most likely explanation is that the encapsulated chloroquine is not available for desethylation. This is also strong evidence for transport of intact liposomes from the peritoneal cavity to the bloodstream.

Indication of the intact absorption of liposomes from the peritoneal cavity is also found in the high chloroquine concentrations in the spleen and liver after the intraperitoneal experiments (Fig. 3). Both are organs where liposomes and other colloidal carriers are accumulated by phagocytosis through the action of the macrophages of the MPS-cells (Nässander et al 1990). High concentrations of the desethyl compound were found in the spleen after intraperitoneal administration, suggesting that chloroquine is released from liposomes in the tissue and is subsequently degraded.

The T_{max} values varying from 2–8 h following intraperitoneal administration of liposomes compared with the rapid absorption and elimination of chloroquine after the administration of the free dose, indicate a relatively slow and variable absorption from the peritoneal cavity. The presence of long-lasting relatively high des-CQ concentrations over the observation period indicates a continuing chloroquine release from the liposomes since both chloroquine and des-CQ are rapidly cleared in mice.

From studies with double labelled liposomes which were intraperitoneally injected, it is known that liposomes are

Table 1. Blood concentration of chloroquine following the administration of free chloroquine or liposome-encapsulated chloroquine.

	Mean chloroquine concentrations (μg mL⁻¹).								
	Intraperitoneal			Intramuscular			Subcutaneous		
	Free	Liposomes		Free	Liposomes		Free	Liposomes	
Dose (mg)	0·6	0·6	3·0	0·6	0·6	3·0	0·6	0·6	3·0
Time (h)									
0	0	0	0	0	0	0	0	0	0
0·2	3·99			4·9			5·86		
0·5	2·70	1·65	196	3·49	1·67	1·20	3·84	0·55	0·82
2·0	1·50	97·1	697	4·58	2·86	1·24	2·21	0·51	0·56
2·9	1·07			3·05			1·07		
4·5		44·6	896		1·24	0·96		0·38	0·50
4·9	0·75			4·01			0·77		
5·5		55·4	466		1·03	0·82		0·24	0·51
6·5		26·1	724		1·10	0·64		0·27	0·40
7·5		19·4			2·11			0·42	
7·9	0·71			4·83			0·46		
24·0	0·69	1·48		0·21	1·39		0·22	0·24	
25·0			123			0·35			0·20
48·0			5·07			0·28			0·15
72			0·30			0·53			0·33
$AUC_{24\,h}$ (μg h mL⁻¹)	20·86	539	13003	72·11	41·81	32·24	15·82	8·45	18·95

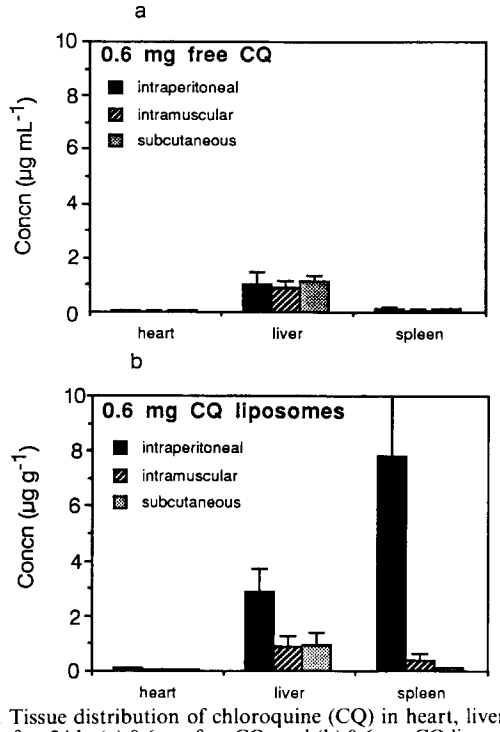

FIG. 3. Tissue distribution of chloroquine (CQ) in heart, liver and spleen after 24 h. (a) 0·6 mg free CQ, and (b) 0·6 mg CQ liposomes. Vertical bars indicate confidence intervals.

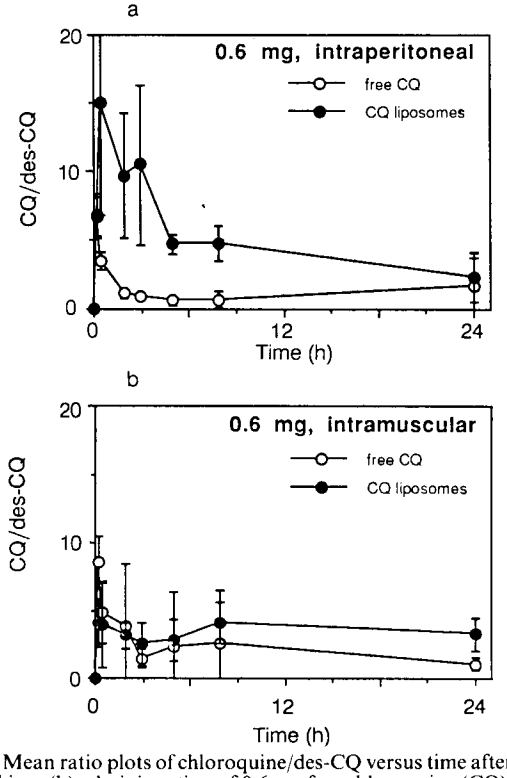

FIG. 4. Mean ratio plots of chloroquine/des-CQ versus time after i.p. (a) and i.m. (b) administration of 0·6 mg free chloroquine (CQ) and CQ liposomes. Vertical bars indicate confidence intervals.

absorbed intact over a few hours with variable speed (Nässander et al 1989). The slow appearance of chloroquine in the circulation can therefore be ascribed to a slow appearance of the liposomes in the circulation.

The data after intraperitoneal injection of chloroquine liposomes differed from the findings after intramuscular and subcutaneous injection. The liposomes were apparently not absorbed as such but acted as sustained release depots in the injection area.

Increasing the dose from 0·6 to 3 mg intramuscularly in this study resulted in lower blood levels, which cannot be explained fully at present. Similar phenomena were observed with intramuscularly administered aqueous suspensions. Factors such as spreading behaviour and the tendency to aggregation have been shown to be critical aspects in dissolution and/or diffusion rate dependent absorption from particles (Hirano & Yamada 1983). In the experiments with the lower dose, the particles may be more vulnerable because of their lower aggregation status. This might also explain the burst effect at about 7 h. This burst was absent in the first 48 h of the higher dose. The increasing concentrations after 48 h observed in all animals, might, however, be ascribed to deaggregation.

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References

Alvan, G., Ekman, L., Linström, B. (1982) Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography. J. Chromat. 229: 241–247

de Groot, G., Wubs, K. L. (1987) A simple enzymatic digestion procedure of intact tissue samples in pharmacokinetic drug analysis. J. Anal. Toxicol. 11: 175–178

Hirano, K., Yamada, H. (1983) Studies on the absorption of practically water-insoluble drugs following injection. VII: Plasma concentrations after different subcutaneous doses of a drug in aqueous suspension in rats. J. Pharm. Sci. 72: 602–607

McChesney, E.W., Fitch, C.D. (1984) 4-Aminoquinolones. In: Peters, W., Richards, W.H.G. (eds) Antimalarial Drugs II. Current Antimalarials and New Drug Developments. Springer Verlag. Berlin, pp 1–59

Nässander, U.K., Moolenaars, E., de Jong, W.H., et al (1989) Immunoliposomes in the treatment of ovarian carcinoma. Pharm. Weekbl. Sci. Ed. 11: D4

Nässander, U.K., Storm, G., Peeters, P.A.M., Crommelin, D.J.A. (1990) Liposomes. In: Chasin, M., Langer, B. (eds), Biodegradable Polymers as Drug Delivery Systems. Marcel Dekker. pp 261–338

Peeters, P. A. M., Huiskamp, C. W. E. M., Eling, W. M. C., Crommelin, D. J. A. (1989a) Chloroquine containing liposomes in the chemotherapy of murine malaria. Parasitology 98: 381–386

Peeters, P. A. M., de Leest, K., Eling, W. M. C., Crommelin, D. J. A. (1989b) Chloroquine (CQ) concentrations in whole blood, plasma and red blood cells after administration of free and liposome encapsulated CQ and their effect on *Plasmodium berghei* infections in mice; effect of lipid composition. Pharm. Res. 6: 787–793

Szoka, F., Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA. 75: 4194–4198

White, N. J. (1985) Clinical pharmacokinetics of anti-malarial drugs. Clinical Pharmacokinetics 10: 187–215

White, N. J. (1988) Drug treatment and prevention of malaria. Eur. J. Clin. Pharmacol. 34: 1–14

World Health Organisation (1984) Advances in malaria chemotherapy, Report of a WHO scientific group. Technical Report Series 711

Zuidema, J., Wynne, H. J. A. (1989) Data reduction problems in biopharmaceutics and pharmacokinetics. Pharm. Weekbl. Sci. Ed. 11: 76–82

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AHR-15010—A Novel Anti-arthritic Agent

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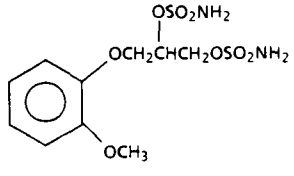
Abstract—AHR-15010 (3-(2-methoxyphenoxy)-1,2-propanediol bissulphamate ester) is a compound of novel structure that displays anti-arthritic activity in adjuvant arthritis in rats. When given orally from days 18 through day 50, (excluding weekends) after adjuvant injection, AHR-15010, at doses of 3·16 to 100 mg kg^{-1}, produced significant anti-inflammatory activity and reduced the severity of the hind paw joint lesions as monitored by X-ray analysis. AHR-15010, however, has no acute anti-inflammatory activity in the Evans Blue-carrageenan pleural effusion assay in rats, has no analgesic activity in mice, and has no activity in a classic, delayed-type, hypersensitivity assay in mice or in a cotton pellet granuloma test in rats. These data, in conjunction with biochemical data showing that AHR-15010 has no prostaglandin synthetase inhibiting activity, suggest that AHR-15010 is an anti-arthritic with a unique mechanism of action. AHR-15010 is a carbonic anhydrase inhibitor. Data are presented that suggest that AHR-15010 and acetazolamide, a prototype carbonic anhydrase inhibitor, may present novel approaches to the treatment of arthritis.

The arthritic diseases are characterized by the loss of the connective tissue structures of the joint. This joint degeneration is mediated by a variety of inflammatory cells and soluble factors released from those cells (Harris 1986). Recent work has also shown that bone resorption is an important clinical feature of the arthritic diseases (Alwan et al 1988; Sambrooke & Reeve 1988). This resorption can be induced by interleukin-1, bradykinin, or PGE_2 (Robinson et al 1975; Lerner et al 1987; Alwan et al 1988). Several drugs, including the NSAIDs and the gold salts, may exert some of their positive effects via inhibition of bone resorption (Katz & Gray 1986; Vargas et al 1987; Klaushofer et al 1988). In further support of this concept, Nolan et al (1989, unpublished data) shown that carbonic anhydrase inhibitors, which have been shown to inhibit bone resorption in-vitro and in-vivo (Minkin & Jennings 1972; Kenny 1985; Raisz et al 1988), display anti-arthritic activity in the rat.



I

I. Structure of AHR-15010, 3-(2-methoxyphenoxy)-1,2-propanediol bissulphamate ester.

In our anti-arthritic programme, evaluation of anti-secretory and anti-arthritic compounds led to the identification of AHR-15010; 3-(2-methoxyphenoxy)-1,2-propanediol bissulphamate ester (I), as a unique agent for the treatment of arthritic diseases. This report describes the anti-arthritic activity of AHR-15010.

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Materials and Methods

Drugs and materials

AHR-15010 was synthesized in the Chemical Research Department at the A. H. Robins Co. (Richmond, VA). Indomethacin, acetazolamide, cyclophosphamide, triamcinolone acetonide, acetylcholine bromide, methylated bovine serum albumin (MBSA), and bovine carbonic anhydrase were from the Sigma Chemical Co. (St. Louis, MO). Cyclosporin (Sandimmune) was from Sandoz, Inc., (East Hanover, NJ), carrageenan (Viscarin) was from Marine colloids, Inc., (Springfield, NJ). *Mycobacterium butyricum* and Complete Freund's Adjuvant were from Difco Laboratories (Detroit, MI). Sheep vesicular glands were from the Wilson Food Corp. (Albert Lea, MN).

In-vivo methods

Rat adjuvant arthritis. Arthritis was induced in female Lewis Wistar rats by a method modified from that of Walz et al (1971). Fifty μL of a suspension of 1·5% *Mycobacterium butyricum* was injected into the subplantar surface of the right hind paw of all rats. Eighteen days later, the volume of both hind limbs was determined by mercury displacement, and the rats with significant uninjected hind paw oedema (paw volume > 2·4 mL) were assigned to treatment groups of 7 by balancing with regard to paw volume size. In the therapeutic dosing regimen, drugs were administered by gavage once daily to the rats beginning on day 18 after adjuvant injection and continuing through day 50 after adjuvant injection (excluding weekends). In the prophylactic experiments drugs were administered daily to groups of 10 rats beginning on the day of adjuvant injection and continuing for 50 days. Oedema was determined on day 29 and day 50 by paw volume difference (from day 18 paw volume in therapeutic experiments and from day 0 paw volume in prophylactic experiments). On day 50 the rats were killed by CO_2 inhalation and the uninjected hind limb cut off above the knee and X-rayed by standard procedures using a dental X-ray unit (Gendex, Model GX-770, Milwaukee, WI). The