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A possible pharmacological explanation for quinacrine failure to treat prion diseases: pharmacokinetic investigations in a ovine model of scrapie

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1 Quinacrine was reported to have a marked *in vitro* antiprion action in mouse neuroblastoma cells. On compassionate grounds, quinacrine was administered to Creutzfeldt–Jakob disease patients, despite the absence of preclinical *in vivo* studies to evaluate efficacy. Quinacrine failed to provide therapeutic benefit. The aim of the study was to investigate possible pharmacokinetic and/or pharmacodynamic explanations for the discrepancy between the proven action of quinacrine *in vitro* and its lack of clinical efficacy.

2 We conducted *in vitro* experiments reproducing the culture conditions in which antiprion effects had been previously observed and recalculated the EC_{50} by determining the actual extracellular (120 nM) and intracellular (6713 nM) quinacrine neuroblastoma concentrations with the reported quinacrine EC_{50} (300 nM).

3 A randomized clinical trial in scrapie-affected ewes confirmed the absence of therapeutic benefit of quinacrine. The *in vivo* quinacrine exposure was evaluated in a pharmacokinetic investigation in healthy ewes. Cerebrospinal fluid concentrations ($<$10.6 and 55 nM after administration of therapeutic and toxic quinacrine doses, respectively) were much lower than the quinacrine extracellular neuroblastoma concentrations corresponding to the reported EC_{50}. The total brain tissue concentrations (3556 nM) obtained after a repeated therapeutic dosage regimen were within the range of the intracellular neuroblastoma quinacrine concentrations.

4 In conclusion, in order to avoid *in vivo* trials for which failure can be predicted, the measurement *in vitro* of the antiprion EC_{50} in both intra- and extracellular biophases should be determined. It can then be established if these *in vitro* antiprion concentrations are achievable *in vivo*.
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Abbreviations: AUC, area under the curve; CJD, Creutzfeldt–Jakob disease; Cl/F, apparent plasma clearance; C_{max}, maximum concentration; MRT, mean residence time; PrP, prion protein; PrP^C, normal cellular prion protein; PrP^{Sc}, abnormal form of the PrP^C protein; ScN2a, scrapie-infected neuroblastoma; T_{max}, time to maximum concentration; V_{ss}/F, apparent steady-state volume of distribution

Introduction

Prion accumulation is considered to be a key event in the pathophysiology of Creutzfeldt–Jakob disease (CJD) (Bruce *et al*., 1989; Jendroska *et al*., 1991), and cellular models for prion disorders have been used to screen the efficacy of many inhibitors of abnormal form of the PrP^C protein (PrP^{Sc}) formation. Thus, a 6-day treatment with the anti-malarial drug quinacrine or with a phenothiazide derivative chlorpromazine cured a mouse neuroblastoma cell line (ScN2a) that was chronically infected with prions (Doh-Ura *et al*., 2000; Korth *et al*., 2001). The median effective concentration (‘EC_{50}’) for quinacrine and chlorpromazine (i.e. the nominal concentration of the test solution producing half-maximal inhibition of PrP^{Sc} formation) reported in this study were 300 nM (142 ng ml^{-1}) and 3000 nM, respectively. Based on a review of the literature, it was anticipated that this effective *in vitro* concentration of quinacrine would be attainable in the *in vivo* biophase (not precisely identified at present). However, to date all patients with CJD treated with quinacrine at the dosage usually recommended in man for other conditions (approximately 300 mg per day) (Shannon *et al*., 1944; Goodman & Gilman, 1960) died without evidence of any retardation in disease progression (Furukawa *et al*., 2002; Follette, 2003).

The initial hypothesis was that the lack of quinacrine efficacy resulted from an inability to treat patients with a dosage regimen that would produce the same biophase concentration *in vivo* as *in vitro*. Scrapie is a naturally occurring disease of sheep, which provides a relevant model for testing this hypothesis, as scrapie is a prion disease for which the pathophysiological mechanisms of infection are likely to be similar to those occurring spontaneously in CJD.

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A second hypothesis was that the reported *in vitro* EC_{50} (300 nM) was not the actual *in vitro* biophase EC_{50}. Indeed, the *in vitro* EC_{50} for quinacrine reported by Korth *et al*. (2001) was only the nominal (calculated) concentration of the quinacrine solution added to the culture medium, which enabled half the maximum effect on prion replication in neuroblastoma cell lines, to be obtained with repeated administration in a volume of 4 ml for 2–7 days. It is not established that this culture medium 'EC_{50}' corresponds to the true EC_{50} in the *in vitro* biophase. Indeed, under both *in vitro* and *in vivo* conditions, the drug has to diffuse to the biophase and it can be removed and/or trapped by the neuroblastoma cellular organelles. Thus, it cannot be assumed that the concentration in the *in vitro* biophase is equal to the nominal concentration of the test solution added to the culture to obtain half quinacrine efficacy. Therefore, the *in vitro* EC_{50} should be determined experimentally from measured *in vitro* drug concentrations. This is particularly important when the *in vitro* effect of interest develops over several days.

The objectives of the present study were: (i) to determine if quinacrine together with chlorpromazine is effective in a controlled clinical trial in a naturally occurring prion disease of sheep; (ii) to recalculate the *in vitro* EC_{50} of quinacrine by establishing its disposition when added to the culture medium of N2a cells *in vitro* at the concentration equivalent to the reported 'EC_{50}'; (iii) to establish the *in vivo* exposure to quinacrine after the administration of therapeutic and toxic dosage regimens; and (iv) to compare the quinacrine concentrations in the N2a cell model with those obtained experimentally in the central nervous system (CNS) of ewes treated with quinacrine.

Methods

Animal procedures

All procedures involving animals were performed in accordance with French legal requirements regarding the protection of laboratory animals and with the authorization for animal experimentation no. 001889 of the French Ministry of Agriculture. The ewes used in experiments 1 and 3 were maintained under natural photoperiod conditions and they received daily rations of concentrate. Hay and water were given *ad libitum*.

Clinical trial

Experiment 1 was designed to evaluate the clinical efficacy of quinacrine in a randomized clinical trial in naturally infected scrapie ewes. The trial was carried out in 23 Manech red-faced ewes with naturally occurring scrapie. The scrapie diagnosis was established from clinical signs of pruritus, behavioural changes, tremor and locomotion incoordination, and confirmed by histopathology on brain samples after necropsy as described previously (Schelcher *et al*., 1999). The ewes were ranked in order of severity of clinical signs, pairs of ewes with similar clinical signs were formed and, within each pair, individual ewes were randomly allocated to treated or untreated (placebo) groups. At the time of inclusion in the clinical trial, the mean body weights ($\pm$s.d.) of 11 treated and 12 untreated scrapie ewes were 41.6 ± 6.4 and 41.7 ± 6.6 kg, respectively. The 11 ewes in the treated group received 150 mg (317 μmol) *in toto* of quinacrine dihydrochloride and 100 mg (281 μmol) chlorpromazine daily by injection into the gluteal muscle, for 7 days in the case of six ewes and for a nominal period of 30 days (except 1 day per week) for five ewes. The volumes of quinacrine and chlorpromazine solutions intramuscularly (i.m.) administered were 5 and 4 ml, respectively. Chlorpromazine was administered with quinacrine as recommended for human therapy of CJD (Korth *et al*., 2001). The 12 ewes in the untreated group received the equivalent volume of vehicle. The dosage regimen of quinacrine administered to the ewes was consistent with the dose used in humans (300 mg daily; Shannon *et al*., 1944). Blood samples were collected by venipuncture (jugular vein) from scrapie-infected ewes once each week during the time course of the disease. These ewes were killed as soon as they manifested clinical signs of irreversible recumbency.

Quinacrine disposition in N2a cells

Experiment 2 was designed: (i) to recalculate *a posteriori* the EC_{50} of quinacrine by determining both intra- and extracellular concentrations obtained when quinacrine was added to the culture medium of N2a cells at a nominal concentration equivalent to the *in vitro* EC_{50} reported by Korth *et al*. (2001); and (ii) to determine the relationship between intra- and extracellular quinacrine concentrations when increasing quinacrine amounts were added to the culture medium of N2a cells.

The mouse neuroblastoma cell line (N2a) was stably transfected as previously described (Lehmann & Harris, 1995). Transfected cells were plated at a density of 40,000 cm^{-2} into 25 cm^2 flasks of 5 ml of OptiMEM (GIBCO, BRL, Cergy Pontoise, France) containing 10% foetal calf serum, and penicillin–streptomycin. These culture conditions were almost the same as those previously used (Korth *et al*., 2001), except that the cells were split at day 4 using 0.05 (w v^{-1}) trypsin-EDTA (GIBCO, BRL) and were not infected with scrapie. The medium was changed every 48 h together with quinacrine, except on the 7th day of culture when the medium was collected after 24 h. The viability of neuroblastoma cells was assessed by counting in satellite flasks. The media from the final 24 or 48 h of culture were stored at -20°C until assayed for quinacrine content. At the end of the treatment period, cells were washed four times with isotonic saline solution, scraped and resuspended in 1 ml of deionized water and stored at -20°C until assayed. Intracellular concentrations of quinacrine were assayed after sonication of cells.

In a first series of *in vitro* experiments reproducing the culture conditions in which antiprion effects were observed (Korth *et al*., 2001), the time courses of intra- and extracellular quinacrine concentrations were measured over a period of 2–7 days in the presence of a nominal quinacrine concentration of 300 nM.

In a second set of experiments, the N2a cells were cultured in the presence of different concentrations of quinacrine (from 0 to 850 nM) for 2 days. At the end of the culture period, the quinacrine concentrations in the media and in the cells were determined.

In vivo *quinacrine disposition*

The objectives in Experiment 3 were: (i) to determine the overall *in vivo* quinacrine exposure in the animal model to enable comparison to exposure in man, and (ii) to compare the concentrations of quinacrine in the CNS of healthy ewes, treated with either a therapeutic dose or a toxic dose of quinacrine, with quinacrine concentrations that were effective *in vitro*.

The *in vivo* disposition of quinacrine was investigated in seven healthy Lacaune ewes, under conditions reproducing the therapeutic dosage regimen of quinacrine in the clinical trial. The ewes received 8 daily (day-0 to -7) i.m. injections of a therapeutic dose of $3\,\mathrm{mg\,kg^{-1}\,day^{-1}}$ of quinacrine. The daily injections were administered in turn in the gluteal, vastus lateris, brachiocephalicus or longissimus dorsi muscles, and in the right or left sides in an 8×8 Latin square design. One ewe has to be excluded from the experiment for an unrelated reason.

On day-0 and -7, blood samples were serially collected by direct venipuncture (jugular vein) within the hour preceding quinacrine i.m. administration and 10, 30, 60, 90, and 120 min after quinacrine administration, then at 2-h intervals until 10 h post-administration and finally at 23–24 h post-administration. From day-2 to -6, a blood sample was collected daily within the hour preceding quinacrine i.m. administration to determine trough plasma concentration. At 24 h after the final injection of quinacrine (day-8), cerebrospinal fluid (CSF) was sampled from the cisterna magna of four ewes. These ewes were then euthanized with intravenous (i.v.) pentobarbitone and exsanguinated. The brains were immediately removed and homogenized at 4°C in 15% ($\mathrm{w\,v^{-1}}$) deionized water and stored at −20°C until assayed.

Jugular venous blood samples were collected from the three remaining ewes daily until day-15, then every 2 or 3 days until day-21 and on day-28, when plasma quinacrine concentrations were no longer detectable. On day-28, CSF was sampled from the cisterna magna (two ewes) or from the lombosacral space (one ewe). The ewes were immediately euthanized and the brains homogenized at 4°C in 30% ($\mathrm{w\,v^{-1}}$) deionized water and stored at −20°C until assayed.

A second series of experiments was performed using three healthy ewes to determine CNS exposure to quinacrine over a wide range of quinacrine doses. Two ewes received an i.m. injection of a single therapeutic dose of 150 mg *in toto* of quinacrine and one ewe received a slow 5.4 h i.v. quinacrine infusion of a toxic dose of 2600 mg *in toto*. This i.v. dose was injected into the right jugular vein. At 24 h after the administration of quinacrine, a blood sample was taken from the left jugular vein *via* an indwelling catheter and CSF was sampled from the cisterna magna of all three ewes. The ewes were immediately euthanized and the brains homogenized at 4°C in 15% ($\mathrm{w\,v^{-1}}$) deionized water and stored at −20°C until assayed.

Sampling

Blood samples (5 ml) were collected in heparinized tubes and centrifuged for 10 min at $3000 \times g$. The plasma was removed and stored at −20°C until assayed. CSF was collected from anaesthetized animals (sodium pentobarbitone, Nesdonal®, Merial, Lyon, France) by puncture of the cisterna magna or the lombosacral space with a 20-gauge needle. A volume of 1–9 ml of CSF was gently withdrawn and centrifuged for 10 min at $1500 \times g$ to remove cells and stored at −20°C until assayed.

Quinacrine assay

Quinacrine concentrations were determined using a validated high-performance liquid chromatography (HPLC) method in which the internal standard and all biological samples were extracted by liquid/liquid extraction. The HPLC apparatus consisted of a pump system equipped with an automatic injector and a variable-wavelength fluorescence monitor. The separation was achieved by reverse phase column (Inertsil ODS3 column, 3 μm, $150 \times 4.0\,\mathrm{mm^2}$). The column was equilibrated at a flow rate of $0.3\,\mathrm{ml\,min^{-1}}$, with a mobile phase consisting of methanol : 25 mM citrate buffer (pH = 4.0) (60 : 40, $\mathrm{v\,v^{-1}}$) containing 0.1 mM benzamidine. As far as possible, polypropylene was used for collecting, storing and assaying samples. The adsorption of drug on to materials during the assay was minimized by including a competing hydrophobic molecule, benzamidine in the mobile phase. The fluorescence detector was set at 300 nm (excitation) and 495 nm (emission). The sample volumes used in the assay were 200 μl for the plasma and 100 μl for the other biological samples. Quinacrine was extracted from biological samples with 1 ml (3 ml for the plasma samples) of 1,2 dichloroethane and 100 μl of 0.2 M sodium hydroxide and 100 μl of 277 nM ethodin as internal standard. Dichloroethane extracts of the matrices were evaporated under nitrogen at 40°C and resuspended in 100 μl DL-lactic acid (0.85%) before injection of a volume of 50 μl on to the column. The mean recoveries of quinacrine from the culture media and from the plasma were 85 and 50%, respectively. The quinacrine assay was performed accurately and reproducibly in the range of 21–254 nM. Within- and between-day precision was less than 15%. The validated quantification limit of the assay was 5.3 nM for the plasma and 10.6 nM for the CSF and culture media.

Pharmacokinetic analysis

Both compartmental and statistical moment approaches were used for the pharmacokinetic analysis of quinacrine concentrations, using WinNonlin 4.0 (Pharsight Corporation, Mountain View, CA, U.S.A.). The maximum concentration ($C_{\max}$) and time to maximum concentration ($T_{\max}$) were determined directly from plasma concentrations obtained after the first quinacrine i.m. administration, for each animal. The area under the curve ($\mathrm{AUC_{0-24\,h}}$) for plasma quinacrine concentrations was calculated from $t = 0$ to 24 h, after the first and eighth quinacrine administrations using the arithmetic trapezoidal rule. The $\mathrm{AUC_{0-inf}}$ and the area under the first moment curve (AUMC) for quinacrine plasma concentrations after the first administration were calculated using the linear trapezoidal rule with extrapolation to infinity. The mean residence time (MRT, h) of quinacrine for a single administration was calculated using the equation

$$\mathrm{MRT} = \frac{\mathrm{AUMC}}{\mathrm{AUC_{0-inf}}} \tag{1}$$

where AUMC is the area under the moment curve observed after i.m. administration of quinacrine.

The plasma concentration *versus* time curves after the eight i.m. quinacrine injections at the dose rate of $3\,\mathrm{mg\,kg^{-1}\,day^{-1}}$ were fitted with a polyexponential equation to assess possible dose and time dependencies of quinacrine disposition. The parameters were estimated by nonlinear regression. The number of exponents was determined by application of the Akaike's Information Criterion (Yamaoka *et al*., 1978). The data points were weighted with the inverse of the squared fitted value. The goodness of fit of the described model was assessed using least-squares criteria. A triexponential equation describing a bicompartmental open model with first-order absorption was selected

$$C_{(t)} = Y_1 \exp(-\lambda_1 t) + Y_2 \exp(-\lambda_2 t) - (Y_1 + Y_2)\exp(-K_{01} t) \quad (2)$$

where $C_{(t)}$ represents the plasma quinacrine concentration at time t; Y_i (nM) the coefficient of the ith exponential term; λ_1 and λ_2 the first-order rate constants of the initial and terminal phases; and K_{01}(h^{-1}) the first-order absorption rate constant.

The plasma half-life for the terminal phase was calculated using the equation

$$t_{1/2} = \frac{0.693}{\lambda_2} \quad (3)$$

with λ_2 as defined above.

The apparent steady-state volume of distribution (V_{ss}/F) was obtained with equation (4)

$$\frac{V_{ss}}{F} = \frac{V_c}{F}\left(1 + \frac{K_{12}}{K_{21}}\right) \quad (4)$$

where F is the relative bioavailability and V_c/F is the apparent volume of the central compartment, K_{12} is the first-order rate constant between central and peripheral compartments and K_{21} is the first-order rate constant between peripheral and central compartments.

The apparent plasma clearance (Cl/F) was obtained with equation (5)

$$\mathrm{Cl}/F = K_{10} V_c/F \quad (5)$$

with V_c/F as defined above and K_{10} the rate constant of quinacrine elimination.

Materials

Drug solutions were freshly prepared for *in vitro* and *in vivo* use. Quinacrine dihydrochloride (molecular weight: 472.9 Da), chlorpromazine–HCl, ethodin, benzamidine and 1,2 dichlororethane were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Quinacrine was dissolved in saline to produce a concentration of 63.3 mM, except for the seven ewes in Experiment 3 for which quinacrine was dissolved in saline and dimethyl sulphoxide (50 : 50, v v^{-1}). Chlorpromazine-HCl was dissolved at the concentration of 70.4 mM in vehicle containing 11.4 mM ascorbic acid, 5.3 mM sodium bisulphite, 7.9 mM sodium sulphite, 17.1 mM sodium chloride and 2% benzyl alcohol. Stock solutions of quinacrine were filtered throughout a 0.2 μm syringe filter for *in vitro* use.

Statistical analysis

Results are reported as mean $\pm$ s.d. (or median). Statistical analyses were performed using SYSTAT 8.0 (SPSS Inc., Chicago, IL, U.S.A.). In Experiment 1, the median delay between inclusion in the clinical trial and death was determined for both treated and control groups. The percentage of ewes that died from the beginning of treatment was compared for the two treated and untreated groups with the log-rank test for equality of survival (Kaplan–Meier test).

Results

Experiment 1

The median survival time of untreated scrapie-affected control ewes (36 days, range 13–72 days, $n = 12$) did not differ from that of ewes treated for 7 days (45 days, range 9–90 days, $n = 6$) or for a nominal period of 30 days (22 days, range 6–91 days, $n = 5$, Figure 1). Plasma quinacrine concentrations of the five treated diseased ewes varied from 47 to 721 nM (22–341 ng ml^{-1}) 7 or 9 days after the beginning of the 7-day quinacrine treatment.

Experiment 2

The time course of intra- and extracellular quinacrine concentrations was determined during a 2- to 7-day period of N2a cell culture in the absence and presence of a nominal quinacrine concentration of 300 nM (Figure 2). A 34 $\pm$ 8% loss of quinacrine occurred after the dilution and filtration of a standard quinacrine solution of 21 μM through a 0.2 μm filter and 16 $\pm$ 3% loss was due to adsorption to the culture disk. The quinacrine concentrations measured in the culture media remained relatively constant during the period of culture (mean: 120 nM; range: 100–140 nM) and were approximately 60% lower than the nominal concentration of the added solution.

The mean intracellular quinacrine concentrations were calculated from an estimated 50 μl volume of subconfluent cells (i.e. a layer of 25 cm^2 and 20 μm depth) to range from 2057 to 6713 nM (973–3175 ng ml^{-1}) during the 7 days of culture. The ratio between the intra- and extracellular concentrations of quinacrine varied between 18 and 58 and tended to remain constant between day-2 and -6 of culture.

In the second *in vitro* experiment, the measured extracellular and intracellular quinacrine concentrations increased linearly with the added quinacrine concentrations (slope 0.43 and 13.8,

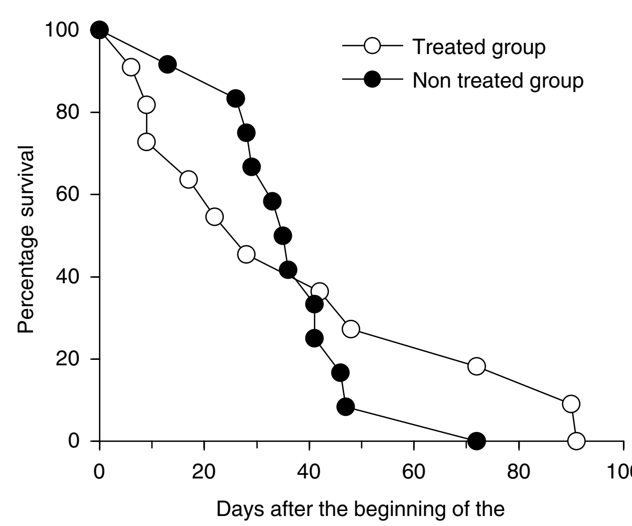

Figure 1 Survival curves for scrapie-infected ewes (days after commencing a combined quinacrine and chlorpromazine treatment). There was no significant difference in survival between the two groups (11 treated ewes *versus* 12 untreated ewes).

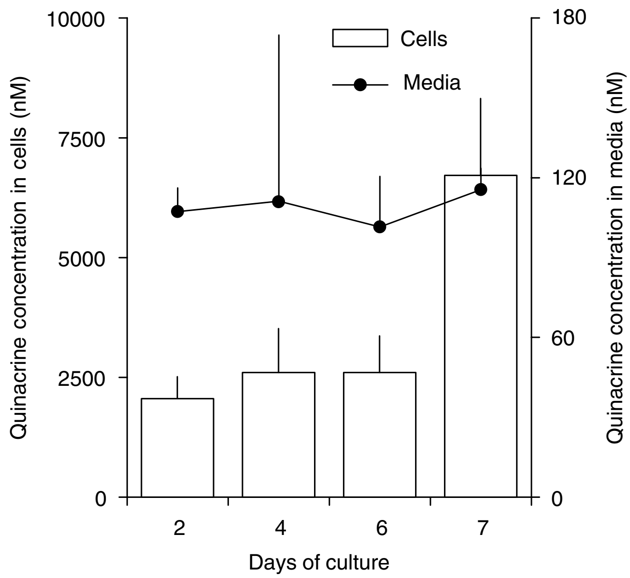

Figure 2 Distribution of quinacrine between extracellular and intracellular compartments in N2a neuroblastoma cells during the 7-day culture. Cells were cultured in the absence or presence of a nominal quinacrine concentration of 300 nM. At every change of medium, the concentration of quinacrine remaining in the medium and present in cells after 24–48 h of culture was determined. Values represent the mean ± s.d. of an experiment performed in duplicate. Note that the scales of quinacrine concentration differ for the cells (left ordinate) and the culture media (right ordinate).

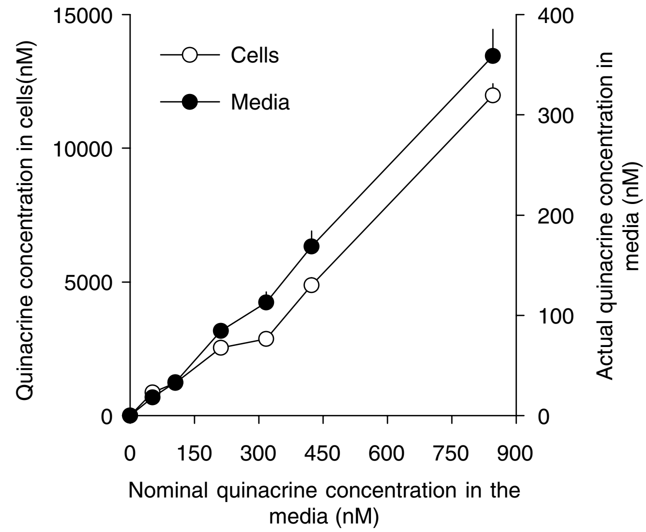

Figure 3 Distribution of quinacrine between extracellular and intracellular compartments in N2a neuroblastoma cells as a function of the quinacrine concentration, added to the culture medium. The N2a cells were cultured in the presence of seven concentrations of quinacrine (from 0 to 850 nM) for 2 days. At the end of the culture period, quinacrine concentration in the media and cells was determined. Values represent the mean ± s.d. of an experiment performed in triplicate. Note that the scales of quinacrine concentration differ for the cells (left ordinate) and the culture media (right ordinate).

for intra- and extracellular concentrations, respectively; $R^2 = 0.99$, coefficient of determination, Figure 3). For a nominal medium concentration of 300 nM (i.e. the reported EC_{50}), the intra- and extracellular quinacrine concentrations calculated from the regression line were 3761 nM (1779 ng ml^{-1}) and 120 nM (57 ng ml^{-1}), respectively. The ratio between the intra- and extracellular concentrations of quinacrine varied between 25 and 47.

Experiment 3

The mean plasma quinacrine concentration *versus* time profile after the first i.m. quinacrine injection at the dose rate of 3 mg kg^{-1} is presented in Figure 4. The mean (± s.d.) quinacrine $AUC_{0-24\,h}$ after the first i.m. injection was 898 ± 593 nM h (425 ± 280 ng ml^{-1} h), giving an average plasma quinacrine concentration of 37.4 ± 24.7 nM (17.7 ± 11.7 ng ml^{-1}) over the first 24 h. The quinacrine MRT for a single dose was 13.43 ± 5.90 h. The mean plasma maximum quinacrine concentration was 189 ± 152 nM (89 ± 72 ng ml^{-1}) and mean time to maximal plasma concentration was 0.79 ± 0.39 h.

After the eighth quinacrine administration, the mean (± s.d.) $AUC_{0-24\,h}$ was 2958 ± 1918 nM h (1399 ± 907 ng ml^{-1} h), giving an average plasma quinacrine concentration of 123 ± 80 nM (58 ± 38 ng ml^{-1}) and indicating an accumulation ratio of approximately 3 between the first and the eighth injections.

Figure 5 illustrates the time courses of observed and fitted plasma quinacrine concentrations for a representative healthy ewe for an 8-day i.m. quinacrine treatment of 3 mg kg^{-1} day^{-1}. Visual inspection of Figure 5 indicates that plasma quinacrine

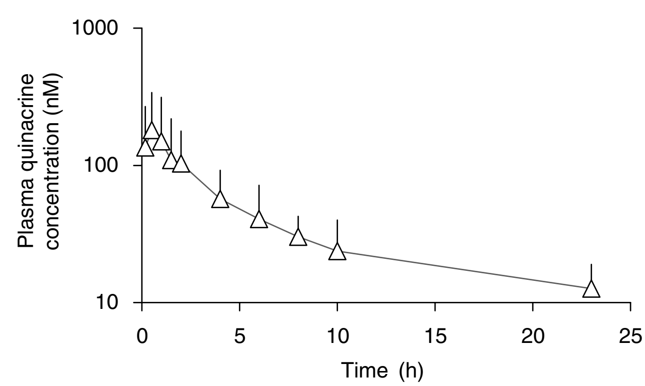

Figure 4 Semilogarithmic plot of mean plasma quinacrine concentration (nM) *versus* time (h) after a single i.m. quinacrine injection at the dose rate of 3 mg kg^{-1} in seven ewes.

concentrations increased almost linearly from day-0 to -6 and tended to remain constant from day-6 to -8. Table 1 presents the mean values of the quinacrine pharmacokinetic parameters as obtained by fitting the plasma quinacrine concentrations after an 8-day i.m. quinacrine treatment. After the final injection, the plasma concentration decreased, with a terminal half-life (mean ± s.d.) of 52 ± 11 h, to a value similar to the quantification limit of the assay at day-16. The apparent quinacrine clearance (Cl/*F*) was 3.04 ± 0.70 l h^{-1} kg^{-1}. The apparent steady-state volume of distribution (V_{ss}/F) was 185 ± 42 l kg^{-1}.

At 20 days after the 8-day i.m. quinacrine treatment, quinacrine concentrations in the plasma, brain tissue and CSF were below the limit of quantification of the assay, except in the nervous tissue of one ewe, for which the value obtained was 63 nM.

Quinacrine concentrations were much higher in the nervous system than in plasma and CSF (Table 2). The ratios of the mean brain tissue concentration to mean plasma quinacrine concentration were 76, 53 and 24, 24 h after a single therapeutic quinacrine dose, repeated therapeutic doses and a toxic dose, respectively. The brain tissue/CSF ratio was 979 after the toxic dose of quinacrine and more than 335 after the chronic therapeutic dosage regimen.

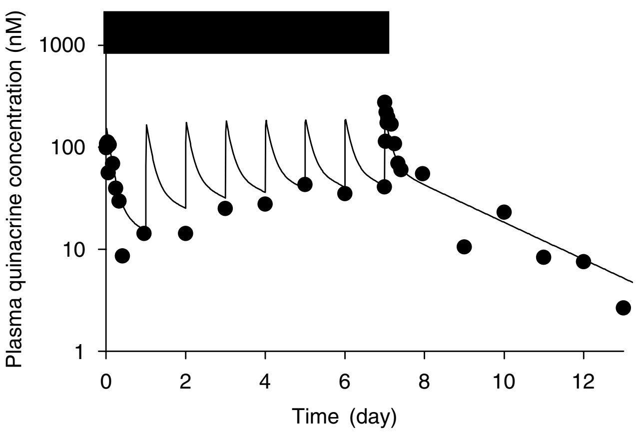

Figure 5 Observed (point) and fitted (line) plasma quinacrine concentrations (nM) in a representative ewe during 8 days of i.m. quinacrine administration at the dose rate of 3 mg kg^{-1} day^{-1} and for 5 further days after termination of dosing. Horizontal bar indicates the period of the quinacrine treatment. The good fitting supports the linearity (dose and time) of quinacrine disposition in our experimental conditions.

Table 1 Pharmacokinetic parameters (mean ± s.d.) describing the plasma disposition of quinacrine after an 8-day i.m. quinacrine treatment at the dose of 3 mg kg^{-1} day^{-1} in three ewes

Parameters (units)	*Mean ± s.d.*
K_{10} (h^{-1})	0.069 ± 0.021
K_{12} (h^{-1})	0.216 ± 0.079
K_{21} (h^{-1})	0.069 ± 0.012
K_{01} (h^{-1})	10.9 ± 6.9
$t_{1/2}$ (h)	52 ± 11
V_{ss}/F (l kg^{-1})	185 ± 42
Cl/F (l kg^{-1} h^{-1})	3.04 ± 0.70

K_{01} is the apparent absorption rate constant; K_{10} is the rate constant of quinacrine elimination; K_{12} is the first-order rate constant between central and peripheral compartments; and K_{21} is the first-order rate constant between peripheral and central compartments. $t_{1/2}$ is the plasma half-life. Cl/F is the apparent clearance and V_{ss}/F is the apparent steady-state volume of distribution.

Discussion

The results of this investigation failed to show any therapeutic benefit of a combined quinacrine and chlorpromazine treatment regimen in a controlled clinical trial in naturally infected scrapie ewes. Using 12 animals per arm, the power of this trial was theoretically sufficient to demonstrate a significant decrease in mortality rate of 24% for a unilateral risk of 5% and a power of 80%. This is satisfactory for this type of exploratory trial designed to demonstrate the existence or not of drug efficacy. This negative result is consistent with observations made in a model of mouse-adapted CJD (Collins *et al*., 2002) and in humans (Furukawa *et al*., 2002; Follette, 2003). However, the two mortality curves in our investigation were not parallel. Therefore, it cannot be totally excluded that treatment accelerated the death in the most affected ewes, while slowing disease progression in the less affected ewes.

In order to avoid the generation of debatable data with the *in vitro* test system, a second aim of our study was to determine why an antiprion drug (quinacrine) that was active *in vitro* was not clinically effective when administered to human patients and sheep. It was hypothesized that one explanation for the lack of clinical efficacy is the impossibility of treating patients with a quinacrine dosage regimen that enables similar quinacrine biophase concentrations to be achieved *in vivo* as *in vitro*. A second possible explanation is that while the reported quinacrine *in vitro* EC_{50} (300 nM) is the medium concentration required to obtain an effect on a neuroblastoma test system, this may not be the concentration required in the target biophase *in vivo* for clinical efficacy. The *in vitro* quinacrine disposition study showed first that the nonspecific quinacrine loss was approximately 50%, in agreement with the potential of quinacrine to be adsorbed on to surfaces (Björkman & Elisson, 1987). Secondly, the study demonstrated that quinacrine was relatively stable over 7 days of N2a cell culture. In addition, it is estimated that approximately 25% of the quinacrine loss is attributable to uptake of drug by the cells.

In the presence of a nominal concentration of quinacrine equivalent to the reported *in vitro* quinacrine EC_{50} (300 nM), the intracellular quinacrine concentrations (2057–6713 nM) attained values that were approximately 30 to 50 times higher than the extracellular levels (100–140 nM). This ratio is not very different to that which could be predicted from the pH partitioning hypothesis. Indeed, considering that quinacrine is a weak base with $pK_a = 10.4$ and assuming that the pH values of the culture medium, the neuroblastoma cytosol and the intralysosomal space are 7.4, 7.2 and 5, respectively, and that

Table 2 Quinacrine concentrations (nM, mean ± s.d.) in plasma, CSF and brain tissue in ewes for different conditions

		Quinacrine concentrations (nM)		
Dosage regimen	*Delay after the (last) dose*	*Plasma*	*CSF*	*Brain*
150 mg i.m. *in toto* ($n = 2$)	24 h	13.7 ± 4.5	< 10.6[a]	1043 ± 199
2600 mg i.v. infusion ($n = 1$)	24 h	2233	55	53 810
3 mg kg^{-1} i.m. daily for 8 days ($n = 4$)	24 h	66.6 ± 46.7	< 10.6[a]	3556 ± 965
3 mg kg^{-1} i.m. daily for 8 days ($n = 3$)	28 days	< 5.3[a]	< 10.6[a]	< 35[a] ($n = 2$) and 63 nM for one ewe

[a]Below the level of quantification of the assay.

the lysosomial space represents about 5% of the cell volume, the predicted theoretical ratio of quinacrine concentrations between the intra- and extracellular spaces was about 13. Thus, the present experiment suggests that the antiprion *in vitro* EC_{50} may lie between 2000 and 7000 nM if the biophase is intracellular, but be only 120 nM if the biophase is extracellular. This extracellular concentration is almost identical to the free quinacrine concentration, as the binding to protein is very limited in the culture medium, whereas the intracellular concentration like the total tissue concentration comprises both free and bound quinacrine.

The effective quinacrine concentrations in the neuroblastoma cell model were compared to those obtained in the two putative biophases of the CNS, that is the brain tissue (representative of an intracellular biophase) and CSF (representative of an extracellular biophase) (De Lange & Danhof, 2002). The comparison was made under similar conditions to those prevailing in the sheep clinical trial. When quinacrine was administered at the recommended therapeutic dosage regimen ($3\,mg\,kg^{-1}\,day^{-1}$), plasma quinacrine concentrations increased progressively and attained an apparent steady-state level of approximately 120 nM after the eighth quinacrine administration. This is consistent with a quinacrine terminal half-life of 52 h. These results indicated that the ewes had been appropriately exposed during the clinical trial, despite considerable interindividual variability. Moreover, our data are consistent with findings in man. In humans, the recommended dosage regimen (800 mg *per os* the first day, then 100 mg three times daily) produces a similar quinacrine exposure, with plasma quinacrine concentrations in the range of 100–200 nM (Shannon *et al*., 1944; Nakajima *et al*., 2004). In addition, in sheep, the data indicate that the blood/plasma ratio of quinacrine concentrations was approximately 1.15 (unpublished observations) demonstrating that quinacrine is poorly accumulated in red blood cells as previously reported by Shannon *et al*. (1944).

Despite the variability in exposure to quinacrine, after both a single therapeutic and a toxic quinacrine dose, the relationship between the plasma and brain tissue quinacrine concentrations remained similar (range of brain tissue to plasma quinacrine concentrations ratio of 24–76). This confirms that plasma concentration is the driving concentration influencing drug distribution to and accumulation in the tissue compartment (Gibaldi & Perrier, 1982). Another fundamental tenet in pharmacokinetics is that it is the free drug concentration (and not the total drug concentration) which is the driving concentration for distribution. Therefore, it is the free drug plasma concentration which should be taken into account when considering drug efficacy and also when comparing *in vitro* and *in vivo* conditions.

The transport of (free) quinacrine across the blood–brain barrier was shown to involve both an influx system (organic cation transporter-like machinery) and an efflux system *via* the P-gp, which might restrict the entry of quinacrine into the brain (Dohgu *et al*., 2004). In the present experiment, the free drug concentration *in vivo* was not directly measured but can be estimated from the plasma/CSF concentrations ratio. Indeed, the CSF is an ultrafiltrate of plasma, virtually devoid of plasma protein and the drug concentration in the CSF represents the maximal value of the plasma free drug concentrations. The CSF quinacrine concentration was lower than the level of quantification of the analytical technique (10.6 nM) 24 h after repeated i.m. administration of $3\,mg\,kg^{-1}\,day^{-1}$ of quinacrine, suggesting that the free quinacrine concentration was also less than 10.6 nM. After administration of a toxic quinacrine dose, quinacrine in CSF was measurable (55 nM) and the estimated plasma (total) to CSF (free) concentration ratio was 40, suggesting that the free quinacrine fraction in the plasma was greater than or equal to 2.5%. This latter value is of the same order as that reported for the free fraction in human plasma (Shannon *et al*., 1944; Goodman & Gilman, 1960), suggesting that the quinacrine concentrations in the CSF are similar to or slightly lower than the plasma free quinacrine concentrations. Considering this free fraction (2.5%), the estimated free plasma quinacrine concentration after a therapeutic dose of quinacrine ($3\,mg\,kg^{-1}\,day^{-1}$) for 8 days ranges from 0.5 to 3 nM, that is, much less than the reported *in vitro* EC_{50} (120 nM). Hence, if the biophase for antiprion activity is extracellular, that is, if the CSF quinacrine concentration is the relevant active quinacrine concentration, the current quinacrine dosage regimen will be wholly unable to achieve an *in vivo* therapeutic antiprion concentration.

On the other hand, it has been suggested that quinacrine interacts with the prion protein, PrP^C within the endolysosomes (Doh-Ura *et al*., 2000), and that this interaction could prevent its conversion into the pathogenic form, PrP^{Sc}, in the endocytic pathway (Vogtherr *et al*., 2003). In this investigation, the total quinacrine concentration in the brain tissue was much higher (about 1000-fold) than the estimated CSF quinacrine concentrations (0.5–3 nM), attaining a concentration of 3556 nM after an 8-day treatment, that is, a tissue/CSF ratio much greater than the ratio of 13 predicted solely from equilibrium pH–pK_a partition considerations, and assuming that the pH of CSF is equivalent to that of the culture medium (7.4). This finding is consistent with tissue trapping of the drug (Dubin *et al*., 1982) and with previous reports that quinacrine is concentrated in tissues, with only low concentrations in the CSF (Shannon *et al*., 1944). Lysosomal trapping of quinacrine (O'Neill *et al*., 1998) might account for most of the concentration of this drug in nervous tissue, but the extensive *in vivo* uptake of quinacrine by cells may also involve binding to other cell organelles or macromolecules. The lysosomes might be a privileged site of action for quinacrine, but this possibility does not exclude a plasma membrane biophase (Shyng *et al*., 1993).

Assuming that there is a lysosomal biophase, the total neuroblastoma and total brain tissue concentrations may be considered as relevant in relation to efficacy. As the total brain quinacrine concentration was of the same order as the *in vitro* measured EC_{50} (2000–7000 nM), attaining 3556 nM after a therapeutic treatment of quinacrine ($3\,mg\,kg^{-1}\,day^{-1}$), it can be reasonably assumed that the present dosage regimen sufficed to achieve an appropriate *in vivo* lysosomal quinacrine concentration. Consequently, it is likely that the lack of *in vivo* quinacrine efficacy in the clinical trial was of pharmacodynamic rather than pharmacokinetic origin. In agreement with this hypothesis, Barret *et al*. (2003) demonstrated that quinacrine could interact with PrP^C to inhibit PrP^{Sc} formation in ScN2a cells, but was unable to affect the protease resistance of pre-existing PrP^{Sc} from brain homogenates of BSE-infected mice.

In conclusion, if the quinacrine biophase is located in the extracellular compartment (or intracellularly in the cytosol), the range of measured quinacrine concentrations required to obtain a 50% efficacy level in the neuroblastoma will clearly never be achieved *in vivo*, even with a toxic dose of quinacrine. In contrast, if the *in vivo* quinacrine biophase is lysosomal, appropriate quinacrine exposure can be achieved with a currently recommended therapeutic dosage regimen. In this circumstance, the lack of quinacrine clinical efficacy suggests that the *in vitro* quinacrine action of recovery of the neuroblastoma is not a relevant *in vivo* mechanism of action to obtain clinical improvement in treated patients.

Finally, from these experiments it can be recommended that in future investigations of putative antiprion drugs, the *in vitro* drug potency should be determined by measuring the actual *in vitro* drug concentration in the potential biophases. It should not be assumed that the *in vitro* biophase concentration is equal to the nominal concentration in the culture system. *In vivo* pharmacokinetic investigations are also required to predict whether a dosage regimen that has both antiprion effect and is safe will achieve an appropriate *in vivo* concentration in the possible biophases.

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