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[^{3}H]-Trimetazidine mitochondrial binding sites: regulation by cations, effect of trimetazidine derivatives and other agents and interaction with an endogenous substance

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1 Trimetazidine, an antiischaemic drug, has been shown to restore impaired mitochondrial functions. Specific binding sites for [^{3}H]-trimetazidine have been previously detected in liver mitochondria. In the present study we confirm this observation and provide additional evidence for the involvement of these sites in the pharmacological effects of the drug.

2 Inhibition experiments using a series of trimetazidine derivatives revealed the presence of three classes of binding sites. An N-benzyl substituted analogue of trimetazidine exhibited a very high affinity ($K_i = 7$ nM) for one of these classes of sites.

3 Compounds from different pharmacological classes were evaluated for their ability to inhibit [^{3}H]-trimetazidine binding. Among the drugs tested pentazocine, ifenprodil, opipramol, perphenazine, haloperidol, and to a lower extent prenylamine, carbetapentane and dextromethorphan competed with high affinity, suggesting a similarity of high affinity [^{3}H]-trimetazidine sites with sigma receptors.

4 [^{3}H]-Trimetazidine binding was modulated by pH. Neutral trimetazidine had about 10 fold higher affinity than protonated trimetazidine for its mitochondrial binding sites. Various cations also affected [^{3}H]-trimetazidine binding. Ca^{2+} was the most potent inhibitor and totally suppressed the binding of [^{3}H]-trimetazidine to the sites of medium affinity.

5 An endogenous cytosolic ligand was able to displace [^{3}H]-trimetazidine from its binding sites. Its activity was not affected by boiling for 15 min, suggesting a non-protein compound.

6 These data suggest that mitochondrial [^{3}H]-trimetazidine binding sites could have a physiological relevance and be involved in the antiischaemic effects of the drug.
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Abbreviations: DTG, 1,3-di(2-tolyl)guanidine; EDTA, ethylenediamine,tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid; MTP, mitochondrial transition pore; 3-PPP, 3-(3-hydroxyphenyl)-N-(1-propyl)piperidine; Tris, 2-amino-2-hydroxymethyl-propan-1,3-diol

Introduction

Since the demonstration of the antiischaemic effect of trimetazidine both by clinical (Detry, 1993) and experimental studies (for a review see Albengres *et al*., 1998), many efforts have been made to identify a cellular target responsible for these effects. During the last years pharmacological studies have focused on mitochondria. Indeed, at least four experimental findings indicate that trimetazidine could act selectively in protecting mitochondria: (1) it improves mitochondrial function during ischaemic damage (Guarnieri & Muscari, 1993); (2) it reverses impairment of oxidative phosphorylation induced by cyclosporine A (Salducci *et al*., 1996); (3) it inhibits mitochondrial swelling generated by high Ca^{2+} concentrations in the presence of a prooxidant (Elimadi *et al*., 1997); and (4) it counteracts the hepatic injury associated with ischaemia-reperfusion by preserving mitochondrial functions (Elimadi *et al*., 1998). Taken together, these results suggest that the mitochondrion is the major target responsible for the antiischaemic effects of trimetazidine and imply the probable existence of specific mitochondrial target sites. We recently identified such binding sites in mitochondria purified from rat liver using [^{3}H]-trimetazidine as a ligand (Morin *et al*., 1998), and showed that [^{3}H]-trimetazidine labels two classes of binding sites as revealed by equilibrium and displacement experiments yielding low Hill-coefficients. The nature of these sites was not established but we have shown that they are not related to other previously described mitochondrial sites. In addition we observed a relationship between the ability of several amphiphilic cations to displace [^{3}H]-trimetazidine and their ability to inhibit mitochondrial swelling.

In the present study we have progressed in the characterization of these sites. First, we have demonstrated that they are not restricted to liver mitochondria but are also present in purified brain mitochondria. Furthermore, we have studied the effect of pH and of monovalent and divalent cations on trimetazidine binding as well as the interaction with trimetazidine derivatives and drugs of different pharmacological classes. Finally, we provide evidence for the existence of a

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possible endogenous ligand able to modulate trimetazidine binding.

Methods

Isolation of liver and brain mitochondria

Male Wistar rats weighing approximately 250–300 g were decapitated. Their livers were excised rapidly and placed in a medium containing 250 mM sucrose, 50 mM 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) and 5 mM ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), pH 7.8 at 4°C. Tissue samples (28 g) were minced and homogenized (10 g tissue in 60 ml medium) on ice using a Teflon Potter homogenizer. The homogenates were centrifuged at $600 \times g$ for 10 min (Sorvall® RC 28 S) and the supernatants were centrifuged for 10 min at $3300 \times g$ to obtain mitochondrial pellets. The resulting supernatants were kept on ice to prepare the cytosolic fraction. Mitochondrial pellets were washed twice with the same medium and centrifuged at $3300 \times g$ for 10 min.

The resulting pellets were washed with medium from which EGTA was omitted and centrifuged for 10 min at $3300 \times g$. The final pellets were suspended in 36 ml of Tris-sucrose buffer (without EGTA) and mitochondria were purified on a discontinuous sucrose gradient according to Morin *et al.* (1998). The purified mitochondrial suspensions were stored on ice before use. Protein content was determined by the method of Lowry *et al.* (1951). The supernatants kept on ice were centrifuged at $125{,}000 \times g$ for 90 min. The pellets were discarded and the supernatants centrifuged at $226{,}000 \times g$ for 90 min. The final supernatants were considered as the purified cytosolic fraction.

Rat brain mitochondria were isolated by differential centrifugation and purified on a percoll gradient according to Sims (1990). Briefly, brains were removed, sliced and washed in a buffer containing 320 mM sucrose, 10 mM Tris and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4 at 4°C. The brains were then homogenized in 10 ml per g of tissue of the same buffer and centrifuged 3 min at $1330 \times g$ (Sorvall® RC 28 S). The supernatants (S_1) were kept on ice and the pellets were suspended in 5 ml per g of tissue of the same buffer and centrifuged at $1330 \times g$ for 3 min. The pellets were discarded, the supernatants S_2 pooled to S_1 and centrifuged at $21{,}200 \times g$ for 10 min. The final pellets were suspended in 15% (v/v) percoll (10 ml g^{-1} of tissue originally homogenized) and layered on a discontinuous density gradient of 3.5 ml each of 23 and 40% percoll. The tubes were centrifuged at $30{,}700 \times g$ for 5 min. The purified mitochondrial rings were recovered at the interface of the 23–40% fractions using a Pasteur pipette and rinsed in the isolation buffer.

All animal procedures used in this study are in strict accordance with the French Agency's policy on animal experimentation (Ministère de l'Agriculture et de la Forêt, authorization No. 00768). Agreement for publication of our results has been given by all companies concerned.

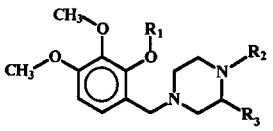
Binding experiments

For inhibition experiments, purified mitochondria were suspended in a Tris buffer (50 mM Tris, 250 mM sucrose, pH 7.4) (2 mg ml^{-1} protein), and incubated with 7–10 nM [^{3}H]-trimetazidine (83 Ci $mmole^{-1}$) and 24 to 28 different concentrations of competing drug or Tris buffer for 45 min at 25°C in a total volume of 400 μl. Specific binding was defined as the difference between total binding and binding in the presence of 1 mM of the trimetazidine derivative S-00226 (Figure 1). For saturation analysis [^{14}C]-trimetazidine (0.5–550 μM; specific activity 54.9 mCi $mmol^{-1}$) was used and the non-specific binding was defined in the presence of 50 mM S-00226. The protein concentration was 2 mg ml^{-1} protein (liver) or 0.7 mg ml^{-1} protein (brain).

At the end of each incubation, bound and free ligands were separated by rapid filtration through Whatman GF/B glass fibre filters (presoaked in 0.1% polyethylenimine). Each filter was washed twice with an additional 5 ml of ice-cold phosphate buffer (25 mM) and counted in a liquid scintillation counter Packard 1600 TR with an efficiency of 45%. The filtration process was rapid enough to avoid the dissociation of radiolabelled trimetazidine from its binding sites. In addition, in all cases radiolabelled trimetazidine binding to glass fibre filters was very low, less than 0.05% of total ligand concentration.

Drugs

Labelled (^{3}H and ^{14}C) and unlabelled trimetazidine (Figure 1) were kindly provided by Servier Laboratories (Neuilly-sur-



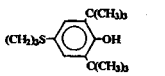
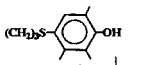
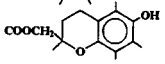
Drugs	R_1	R_2	R_3
Trimetazidine	CH_3	H	H
S-00218	CH_3	CHO	H
S-00224	CH_3	CH_3	H
S-00226	CH_3	CH_2-CH_3	H
S-00240	CH_3	$CH(C_6H_5)_2$	H
S-00526	CH_3		H
S-00700	CH_3	-$(CH_2)_4$-	
S-15176	CH_3		H
S-15739	CH_3		H
S-15742	CH_3		H
S-20596	CH_3	CO-CH_2-NH_2	H
S-64329	CH_3	CH_2-C_6H_5	H
S-16842	H	CH_2-CH_3	H
S-16858	H	H	H
S-16893	H	CH_2-$CH(CH_3)_2$	H
S-16894	H	$(CH_2)_3$-CH_3	H
S-16895	H	$(CH_2)_6$-CH_3	H
S-16949	H	$(CH_2)_3$-CH_2OH	H
S-16950	H	CH_2-C_6H_5	H
S-17060	H	$(CH_2)_{17}$-CH_3	H
S-17080	H	$(CH_2)_{15}$-CH_3	H
S-17192	H	$(CH_2)_{11}$-CH_3	H
S-17316	H	CH_3	H

Figure 1 Chemical structures of trimetazidine (1-(2,3,4-trimethoxybenzyl)piperazine) and trimetazidine derivatives.

Seine, France). Trimetazidine derivatives (Figure 1) either were provided by Servier Laboratories or synthesized by Synthe-Pharma (Seysses, France) at the request of Servier Laboratories. Other drugs and chemicals were obtained from Sigma (St Quentin Fallavier, France) or Merck (Nogent-sur-Marne, France) and were of the highest purity available.

Data analysis

The binding data for equilibrium saturation experiments were fitted to equation (1) describing the interaction of a selective ligand with two classes of high and low affinity binding sites. B_{max}, F and Kd denote the site density, the free ligand and the dissociation constant of each class, respectively.

$$B = \frac{B_{max_1}.F}{F + Kd_1} + \frac{B_{max_2}.F}{F + Kd_2} \quad (1)$$

In inhibition experiments, the displacement curves were fitted to the general binding isotherm (Hill model):

$$B = \frac{Beq.IC_{50}{}^{nH}}{IC_{50}{}^{nH} + C^{nH}} \quad (2)$$

where B is the number of binding sites observed in the presence of a particular inhibitor concentration (C), Beq the bound ligand at equilibrium, IC_{50} the concentration that inhibits 50% of the maximal binding and nH the pseudo-Hill coefficient (Weiland & Molinoff, 1981).

A nH value of 1 corresponds to a competitive interaction and thus indicates the presence of one class of binding sites. When nH value was statistically lower than 1 (according to the Aikake criterion), a competitive model considering a competitive interaction including two classes or more of binding sites was applied. The following equation was used:

$$B = \frac{Beq_1.IC_{501}}{IC_{501} + C} + \frac{Beq_2 IC_{502}}{IC_{502} + C} + \cdots\cdots + \frac{Beq_n.IC_{50n}}{IC_{50n} + C} \quad (3)$$

Data from all binding experiments were analysed by means of non-linear regression using commercially available software (Micropharm, INSERM 1990; Urien, 1995). All data are presented as the mean ± s.e.mean of three or more individual experiments.

Results

Evidence for the existence of trimetazidine binding sites on liver and brain mitochondria

Binding studies of [^{14}C]-trimetazidine were performed with purified rat liver and brain mitochondria. As described previously, the binding of [^{14}C]-trimetazidine to liver mitochondria was saturable. Scatchard analysis of the data confirmed the presence of two populations of binding sites, the high affinity sites representing only 5% of the total (Table 1). An accumulation of the drug inside mitochondria without binding was also considered. Indeed, we recently showed that timetazidine can cross cellular membranes transferring protons from an acidified cellular compartment to the extracellular space (Reymond *et al*., 1999).

The same binding parameters were found when mitochondria were broken, subjected to hypo-osmotic shock and sonication, or frozen. These procedures eliminate the possibility of a trimetazidine uptake without binding. [^{14}C]-Trimetazidine binding sites were also present in purified rat brain mitochondria. The Scatchard plot was linear and the Hill coefficient was close to unity indicating the presence of only one class of binding sites (Figure 2). The total binding capacity was 507 pmol mg^{-1} protein but no high affinity sites could be detected. However, displacement experiments allowed us to detect them (results not shown). This discrepancy between displacement and equilibrium experiments is due to the low selectivity of [^{14}C]-trimetazidine between the two sites, and especially to the low proportion of high affinity binding sites in rat brain yielding their detection impossible by equilibrium experiments. Clearly, the study of these sites requires a better ligand.

Effect of trimetazidine derivatives on [^{3}H]-trimetazidine binding

The need for a better ligand to label trimetazidine sites prompted us to investigate the effect of different trimetazidine derivatives on [^{3}H]-trimetazidine binding. Thus, we examined a

Table 1 Equilibrium binding parameters of [^{14}C]-trimetazidine for mitochondrial binding sites in rat brain and liver

	Liver		
	site 1	*site 2*	*Brain*
Kd (μM)	0.96 ± 0.11	104 ± 31	223 ± 44
B_{max} (pmol [mg protein]$^{-1}$)	5 ± 1	108 ± 19	507 ± 52

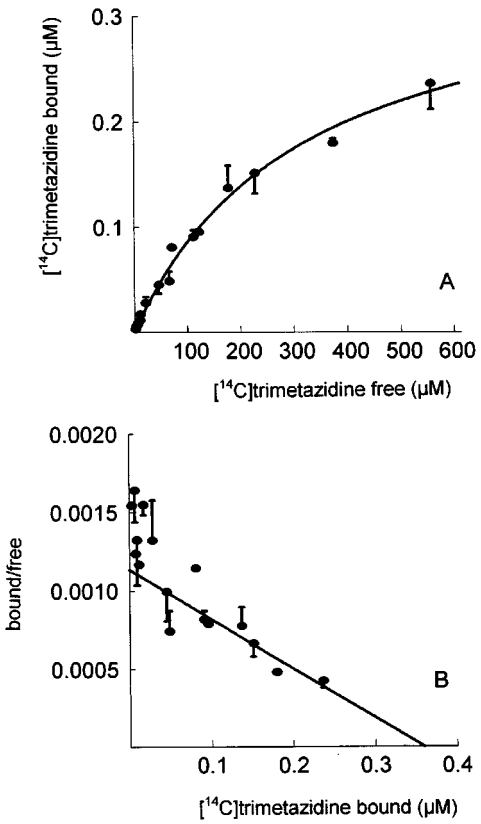


Figure 2 Equilibrium binding of [^{14}C]-trimetazidine to rat brain mitochondria. [^{14}C]-Trimetazidine (0.5–550 μM) was incubated with mitochondria (0.7 mg ml^{-1}) for 45 min at 25°C. Non-specific binding was determined in presence of 50 mM S-00226. B_{max} and K_d values were 0.355 and 223 μM, respectively. Values are means for three independent experiments performed in duplicate. Equilibrium binding parameters were estimated by a non-linear regression analysis. (A) Direct plot. (B) Scatchard plot.

series of compounds substituted either on the ortho-oxygen, the piperazine ring, or both (Table 2). We generated inhibition binding curves by displacement of a low concentration of [^{3}H]-trimetazidine (8 nM). This protocol permitted detection of the presence of several binding sites, and under these conditions IC_{50} values of the competing drugs were close to their K_i values (Swillens *et al*., 1995). The displacement of [^{3}H]-trimetazidine by trimetazidine indicated that 7–10 nM [^{3}H]-trimetazidine labelled both families of sites to a comparable extent (Table 2), confirming our previous observation (Morin *et al*., 1998). The same results were obtained with some (S-16858, S-00226, S-00700) but not all derivatives. Indeed, according to the displacing agent used, the ratio of high to low affinity sites varied from 25/75 to 65/35, and four drugs displaced all [^{3}H]-trimetazidine binding sites with the same affinity. Some of these drugs showed a high affinity for site1, especially S-64329 ($IC_{50}=0.02$ μM) which was also highly selective for this site with an IC_{50} ratio of 1710 (Table 2; Figure 3).

These puzzling results are difficult to interpret. The only compatible assumption is the presence of more than two families of binding sites. Another trimetazidine derivative, S-16950, suggested a solution to the problem. Indeed, S-16950 inhibition of [^{3}H]-trimetazidine binding resulted in very shallow competition curves with a nH value of 0.34 ± 0.02 and a mean IC_{50} value of 0.32 ± 0.14 μM. When these data were subjected to computer modelling, it was found that the inhibition curve was better described by a three-site rather than a two-site model ($P<0.01$; Figure 3). The one-site model was unacceptable. At this particular [^{3}H]-trimetazidine concentration (7–10 nM), S-16950 IC_{50} values were 7,320 and 45,000 nM corresponding to binding site proportions of 41, 25 and 34% of the total, respectively (Figure 3). Interestingly, the combination of these percentages accounts approximately for all binding ratios (expressed as the percentage of the total number of binding sites) obtained in Table 2. For example, S-15739 showed a ratio of binding sites (site1/site2) of 65/35 (65% corresponding to the sum 41+25%), whereas S-00218 showed the reverse (35/65). For most drugs, the ratio was within the range 40/60–60/40 indicating that these drugs discriminate between the high (41%) and the two lower (25+34%) families of sites.

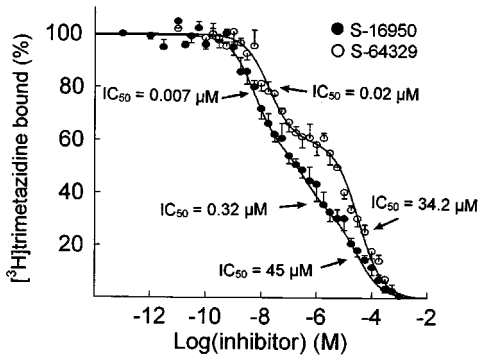


Figure 3 Inhibition of specific [^{3}H]-trimetazidine binding to rat liver mitochondria (2 mg ml^{-1}) by increasing concentrations of S-16950 and S-64329. Data are plotted as percentages of the control [^{3}H]-trimetazidine binding value (in the absence of inhibitor : [^{3}H]-trimetazidine bound =100%). [^{3}H]-Trimetazidine concentration was 8 nM. Control binding values varied between 20 to 30 fmol mg^{-1} of protein and non-specific binding between 27 to 40%. The pseudo-Hill coefficient nH being lower than 1 (S-16950, nH=0.34±0.02; S-64329, nH=0.34±0.04), the curves were fitted to a two-site model. This model was not satisfactory for S-16950, but fitting to a three-site model significantly improved the goodness of fit ($P<0.01$).

Table 2 Inhibition concentrations and proportions of high and low affinity binding sites of trimetazidine derivatives for [^{3}H]-trimetazidine binding sites in rat liver mitochondria

Drugs	*IC_{50} (μM) site 1*	*site 2*	*site 2/site 1*	*Binding site ratio (%) site 1/site 2* (±s.e.mean)
Trimetazidine	2.40±0.31	95.2±20.1	39.6	51/49 (±4)
S-64329	0.02±0.003	34.2±5.54	1710	40/60 (±2)
S-16842	0.43±0.10	299±78	695	53/47 (±2)
S-16858	6.80±2.60	189±52	27.8	50/50 (±3)
S-16893	0.08±0.002	128±43	1600	44/56 (±4)
S-16894	0.07±0.04	96.2±19.5	1374	47/53 (±0.5)
S-16895	0.08±0.02	90.9±42.1	1136	57/43 (±4)
S-16949	0.61±0.49	184±61	302	29/71 (±3)
S-15739	0.54±0.10	17.1±4.63	31.6	65/35 (±4)
S-15742	0.83±0.53	16.7±1.60	20.1	21/79 (±1)
S-15176	0.60±0.14	67.8±26.1	113	60/40 (±7)
S-17060	3.10±0.70			100
S-17080	0.82±0.17	67.7±0.20	82.5	56/44 (±7)
S-17192	1.35±0.25	48.2±0.45	35.7	70/30 (±4)
S-17316	1.65±0.45	486±93	294	57/43 (±12)
S-00218	2.70±0.30	105±21	38.9	34/66 (±2)
S-00224	1.50±0.31	141±23	94.0	46/54 (±5)
S-00226	0.56±0.12	67.9±13.5	121	50/50 (±2)
S-00240	12.4±0.15			100
S-00526	16.4±2.74			100
S-00700	0.42±0.07	218±1	519	52/48 (±7)
S-20596	45.7±7.95			100

Data shown are the mean±s.e.mean of 3–4 experiments done in duplicate. [^{3}H]-trimetazidine concentration was 7–10 nM. IC_{50} values were estimated by a non-linear regression analysis either by a Hill or a competitive model considering two classes of binding sites when the pseudo-Hill coefficient nH was lower than 1. When nH=1 (one-site model) only one set of parameters is shown. All drugs displaced 100% of specific [^{3}H]-trimetazidine binding.

Dependence of [3H]-trimetazidine binding on pH and ions

Since trimetazidine is a dibasic compound with two p*K*a values, we investigated the effect of pH and cations on [3H]-trimetazidine binding. The pH-dependent binding curve of trimetazidine is shown in Figure 4A with specific binding increasing up to pH = 8.5 and a mid-point being reached close to pH = 7.5. In fact, this binding curve resembles an ionization curve, and indeed such a protonation curve can be shown to mimic approximately the binding curve. In Figure 4A, a protonation curve for a base of p*K*a = 7.6 is compared with the binding curve, revealing a partial fit.

This pH-dependent binding profile prompted us to determine which binding sites were affected by pH. We carried out competition studies in the presence of S-16950 at three pH values: 6, 7.4 and 9 (Figure 4B). All [3H]-trimetazidine binding sites decreased with pH. The high affinity sites were more greatly affected, decreasing from 40 to 2.2 fmol mg^{-1} protein (−94.5%) when pH varied from 9 to 6.

The ability of [3H]-trimetazidine to bind to its sites was also found to be dependent on some ions present in the medium. As shown in Figure 5A, [3H]-trimetazidine specific binding was inhibited in a concentration-dependent manner by NaCl, KCl, $MgCl_2$ and $CaCl_2$, the latter being the most potent. The weak inhibition effect of NaCl was assigned to the Cl^- ion because Na^+-acetate did not significantly modify binding at concentrations up to 150 mM. Hence, Na^+ was without effect. In contrast to Na^+-acetate, K^+-, Mg^+- and Ca^{2+}-acetate decreased binding activity (data not shown) but less than their chloride salts. These results confirm the inhibition of [3H]-trimetazidine binding by Cl^-, K^+, Mg^{2+} and Ca^{2+}.

In order to determine which family of [3H]-trimetazidine binding sites was affected by these ions, we performed competition experiments with S-16950 in the presence of 60 mM $CaCl_2$. As expected, Ca^{2+} reduced the binding of [3H]-trimetazidine (Figure 5B). The higher and lower affinity sites were not affected, whereas [3H]-trimetazidine binding to the medium affinity sites was almost completely suppressed (Figure 5B).

Pharmacological profile of [3H]-trimetazidine binding sites

In a previous study (Morin *et al*., 1998), we observed that [3H]-trimetazidine binding sites differed from known mitochondrial sites and interacted with a number of drugs. Here we continue the pharmacological characterization of these sites, investigating the effects of several classes of drugs.

No antiischaemic agent (Table 3) competed with very high affinity, prenylamine and flunarizine being the most effective. Four drugs displaced total specific [3H]-trimetazidine binding in a monophasic manner, demonstrating a relatively high affinity for all [3H]-trimetazidine binding sites. Among the other classes of drugs, the antidepressant opipramol, the antipsychotics perphenazine and haloperidol, and the local anaesthetic butacaine were the most effective with IC_{50} values

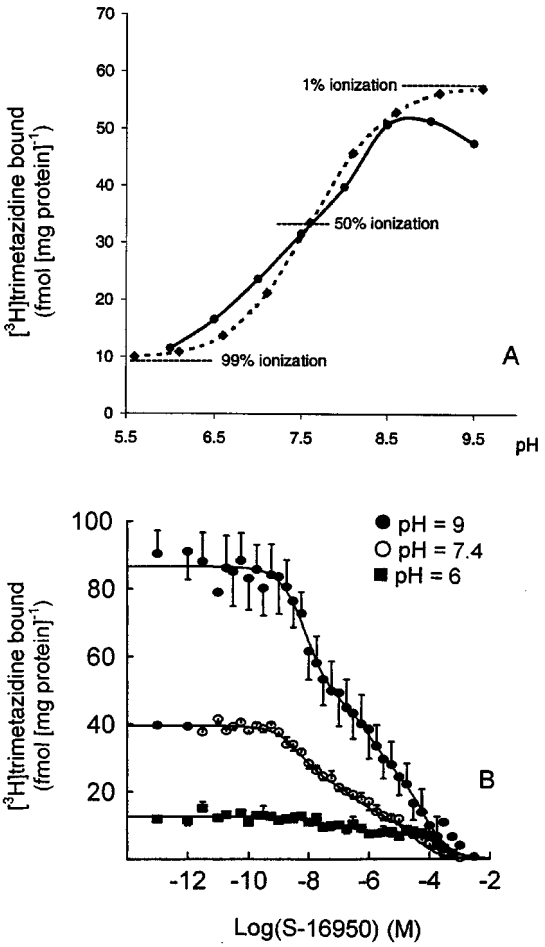


Figure 4 Effect of pH on [3H]-trimetazidine binding to rat liver mitochondria. (A) [3H]-Trimetazidine binding to mitochondria (full curve), compared with the protonation curve (broken line running from 99 to 1% ionization) of a base with p*K*a = 7.6. (B) Inhibition of [3H]-trimetazidine binding by increasing concentrations of S-16950 at different pH. [3H]-Trimetazidine concentration was 8 nM. The curves were fitted to a three-site model. IC_{50} values were 0.008, 1.1 and 64.4 μM at pH = 9, 0.005, 0.34 and 28.9 μM at the pH = 7.4 and 0.011, 0.19 and 216 μM at pH = 6.

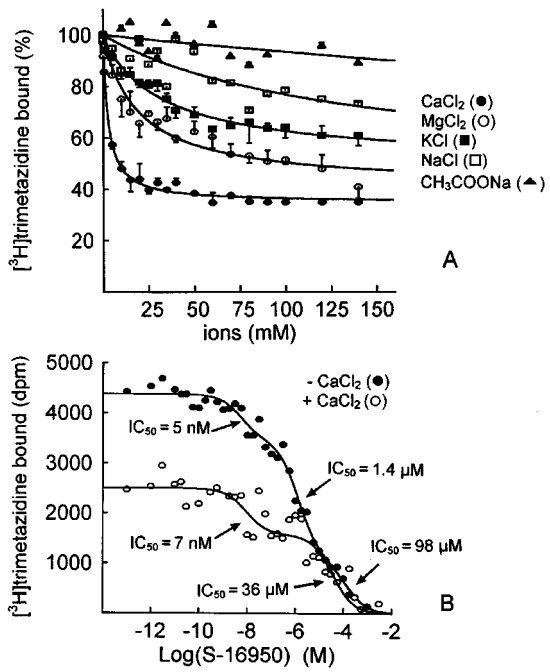


Figure 5 Effect of cations on specific [3H]-trimetazidine binding to rat liver mitochondria. (A) Inhibition of [3H]-trimetazidine binding (8 nM) by increasing concentrations of various cations used as acetate and chloride salts. Data are plotted as percentages of the control [3H]-trimetazidine binding value (in the absence of cation : [3H]-trimetazidine bound = 100%). (B) Inhibition of [3H]-trimetazidine binding by increasing concentrations of S-16950 in the absence or in

Table 3 Inhibition concentrations and proportions of high and low affinity binding sites of antiischaemic drugs for [3H]-trimetazidine binding sites in rat liver mitochondria

	IC_{50} (μM)		*Binding site ratio* (%)
Drugs	*site 1*	*site 2*	*site 1/site 2* (±s.e.mean)
Antianginal and vasodilator agents			
prenylamine	0.21±0.05	19.3±3.60	49/51 (±2)
flunarizine	0.35±0.16	38.7±11.9	36/64 (±1)
amiodarone	0.42±0.005		100
(+)propranolol	0.52±0.15	16.3±5.67	54/46 (±4)
alprenolol	0.54±0.01	33.8±2.65	59/41 (±5)
bepridil	0.74±0.18	29.3±9.00	55/45 (±0.5)
(−)propranolol	1.11±0.29	48.2±3.45	69/31 (±4)
tolazoline	1.35±0.15	141±16.4	33/67 (±1)
verapamil	1.55±0.05	95.4±25.9	61/39 (±0.5)
cinnarizine	2.40±0.68		100
nicardipine	3.90±0.30		100
diltiazem	4.57±1.05	232±44.8	39/61 (±3)
lidoflazine	6.73±0.39		100

The inhibition of the specific binding of 7–10 nM [3H]-trimetazidine was determined and calculated as described for Table 2. Data shown are the mean±s.e.mean of 3–4 experiments done in duplicate and all drugs displaced 100% of specific [3H]-trimetazidine binding.

Table 4 Inhibition concentrations and proportions of high and low affinity binding sites of several classes of antiischaemic drugs for [3H]-trimetazidine binding sites in rat liver mitochondria

	IC_{50} (μM)		*Binding site ratio* (%)
Drugs	*site 1*	*site 2*	*site 1/site 2* (±s.e.mean)
Antipsychotic agents			
perphenazine	0.05±0.01	11.6±1.80	47/53 (±1)
haloperidol	0.07±0.02	6.47±1.32	26/74 (±5)
trifluoperazine	0.32±0.08	47.7±8.85	53/47 (±1)
chlorpromazine	0.53±0.11	19.5±3.25	50/50 (±5)
flupentixol	0.63±0.19	56.1±12.3	51/49 (±5)
thioridazine	10.0±0.40		100
Antidepressant and antihistaminic agents			
opipramol	0.02±0.002	98.9±5.75	53/47 (±4)
mepyramine	0.27±0.04	30.3±0.65	52/48 (±2)
chlorphenamine	0.39±0.09	42.5±4.90	39/61 (±5)
imipramine	0.42±0.13	43.2±13.1	49/51 (±5)
amitriptyline	0.44±0.16	40.8±7.48	50/50 (±1)
promethazine	0.64±0.05	22.6±2.20	38/62 (±6)
desipramine	1.37±0.15	52.2±9.58	66/34 (±4)
Local anaesthetics			
butacaine	0.04±0.02	5.80±1.81	31/69 (±4)
proparacaine	0.10±0.03	15.4±5.64	53/47 (±3)
meprylcaine	0.12±0.07	51.7±5.55	25/75 (±0.5)
dibucaine	1.22±0.39	86.7±15.0	50/50 (±3)
lidocaine	4.65±0.75	506±208	33/67 (±5)
tetracaine	5.80±0.10		100
Miscellaneous compounds			
lobeline	0.06±0.02	15.7±3.73	27/73 (±1)
proadifen	0.06±0.01	27.1±3.00	38/62 (±3)
hydroxyzine	0.08±0.02	108±38.5	57/43 (±3)
dextromethorphan	0.50±0.30	345±53.9	56/44 (±0.5)
quinacrine	2.50±1.40	106±49.5	63/37 (±2)
chloroquine	2.83±0.47		100
idebenone	5.75±0.25		100
glibenclamide	5.75±0.95	1096±156	25/75 (±5)
quinidine	36.0±4.70		100
quinine	180±50.8		100
Sigma ligands			
pentazocine	0.01±0.002	32.2±2.85	25/75 (±0.5)
ifenprodil	0.02±0.001	35.2±2.80	25/75 (±1)
DTG	0.08±0.03	18.0±0.60	58/42 (±4)
carbetapentane	0.11±0.01	45.7±20.1	63/37 (±5)
3-PPP	1.80±0.35	813±454	53/47 (±3)

The inhibition of the specific binding of 7–10 mM [3H]-trimetazidine was determined and calculated as described in Table 2. Data shown are the mean±s.e.mean of 3–4 experiments done in duplicate and all drugs displaced 100% of specific [3H]-trimetazidine binding.

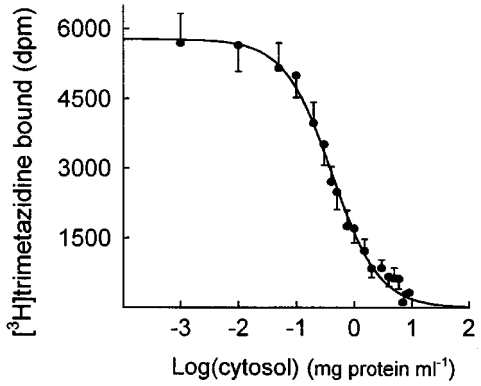


Figure 6 Effect of increasing concentration of cytosol on specific [^{3}H]-trimetazidine binding to rat liver mitochondria. The plot is representative of nine experiments performed with five different preparations of cytosol.

for the high affinity sites of 0.02, 0.04, 0.07 and 0.04 μM, respectively (Table 4); (−)-lobeline, proadifen and to a lesser extent dextromethorphan were also effective. Given that the most effective drugs belong to different pharmacological classes, we looked for another common property and found that they are all known to interact with sigma receptors. This prompted us to study the effect of more selective sigma receptor ligands, i.e. pentazocine, ifenprodil, 1,3-di(2-tolyl)-guanidine (DTG), carbetapentane and 3-(3-hydroxyphenyl)-N-(1-propyl)piperidine (3-PPP) (Quirion *et al*., 1992). Indeed, these compounds inhibited [^{3}H]-trimetazidine binding with IC_{50} values close to their sigma receptor affinity (Dehaven-Hudkins *et al*., 1994; Matsuno *et al*., 1996).

Evidence for an endogenous trimetazidine-displacing substance

Because [^{3}H]-trimetazidine binding sites appeared specific and some analogues showed high affinity, it was tempting to investigate the possible presence of an endogenous ligand which was able to recognize these binding sites. To this end we isolated a cytosolic fraction devoid of organelles and membranes and evaluated increasing concentrations of this fraction on [^{3}H]-trimetazidine binding (Figure 6). Complete inhibition of binding was seen at high concentrations. This persisted when the cytosol was boiled for 15 min and centrifuged to eliminate precipitated proteins. The presence of 5 mM EGTA in the isolation medium ruled out a possible action of Ca^{2+}, and EGTA alone was without effect.

Discussion

Trimetazidine is an antiischaemic drug effective in various experimental models, but its mechanism of action is not fully understood. Numerous data indicate that mitochondria could be its main target, and the recent demonstration of specific trimetazidine binding sites located on liver mitochondria has reinforced this hypothesis (Morin *et al*., 1998). Although we have provided evidence that the low-affinity trimetazidine binding sites may be involved in the regulation of the mitochondrial permeability transition pore, the implication of these sites in the mechanism of action of trimetazidine needed to be established.

The physiological relevance of these sites is strengthened, using the cytosol of liver cells, by the present demonstration of the presence of an endogenous substance which is able to displace [^{3}H]-trimetazidine from its binding sites. The nature of this compound is not yet established, but it does not seem to be a protein since it was not affected by boiling for 15 min. The demonstration of the selectivity of this substance towards trimetazidine sites, and its purification, will be one of the main objectives of future studies.

The pH strongly influenced [^{3}H]-trimetazidine binding. This drug has two p*K*a values and its binding was closely related to its protonation state. [^{3}H]-Trimetazidine binding increased with pH. The p*K*a of the more basic group in trimetazidine is 9.14 ± 0.02, which seems incompatible with the apparent p*K*a of 7.6 giving the best concordance between the binding and protonation curves in Figure 4A. However, it must be remembered that the apparent aqueous p*K*a of a base partitioning in an organic solvent experiences a shift to lower values (p*K*a shift) which is related to the partition coefficient of the base. The well-known equation describing this phenomenon is:

$$P = \frac{10^{(pKa - pK_oa)} - 1}{r} \quad (4)$$

where P is the solvent/water partition coefficient of the base, pK_oa is its aqueous p*K*a in the biphasic system, and r is the ratio of volumes of the organic and aqueous phases (Kaufman *et al*., 1975; Clarke & Cahoon, 1987; Avdeef, 1992).

Assuming that the protonation curve in Figure 4A is indeed that of trimetazidine in the mitochondria/water system, we have a $pKa - pK_oa \approx 1.5$, i.e. $P \approx (10^{1.5} - 1)/r \approx 33/r$. Assuming r values of 1/100 or 1/1000, we obtain log P = 3.5 or 4.5, respectively, for the mitochondria/water partitioning of neutral trimetazidine. This suggests that the binding curve of trimetazidine can indeed be interpreted to indicate that neutral trimetazidine has about 10 fold higher affinity than protonated trimetazidine for its binding sites on mitochondria. The fact that the fit between the two curves is far from perfect is suggestive of additional equilibria and the drop in specific binding beyond pH = 8.5 could well be due to ionic changes in the mitochondrial membranes.

Divalent cations were more potent in inhibiting [^{3}H]-trimetazidine binding than monovalent cations. However, the high concentrations needed to inhibit [^{3}H]-trimetazidine binding suggest that only minimal displacement would occur in the extra- or intra-mitochondrial environment under normal conditions. Only under pathological conditions could this effect be of relevance. Indeed, it is generally thought that during ischaemia-reperfusion the binding of Ca^{2+} to an internal site of the mitochondrial membrane is a key event in the sequence of events leading to the opening of the mitochondrial transition pore (MTP; Bernardi *et al*., 1993; Zoratti & Szabo', 1995). The opening of the MTP results in mitochondrial and thus, cellular injury (Bernardi, 1996). We recently observed that trimetazidine is able to inhibit MTP opening and that a relation exists between the ability of different drugs to inhibit MTP opening induced by Ca^{2+} in the presence of a prooxidant and the displacement of [^{3}H]-trimetazidine bound to mitochondria (Morin *et al*., 1998). This effect may result from an interaction between Ca^{2+} and the drug, the former being displaced by the drug. Indeed, our present data show that the regulation of [^{3}H]-trimetazidine binding by Ca^{2+} is characterized by a competitive inhibition reflected by (1) a monophasic inhibition (nH = 1) observed in the presence of increasing concentrations of Ca^{2+} and (2) a decrease in the density of the medium affinity sites without any modification either of the number of high affinity sites or of the

affinity of S-16950 for these sites. This interaction could explain the inhibition of MTP opening by trimetazidine.

A major obstacle in understanding the role for these binding sites was the low affinity of the labelling drug and the diversity of the sites. In order to facilitate their identification and molecular characterization, we examined trimetazidine derivatives as molecular probes for [^{3}H]-trimetazidine binding sites. This changed the binding profile of trimetazidine to become more heterogeneous since up to three binding sites were now detected in the presence of the N-benzyl derivative S-16950. It should be noted that the binding is highly dependent on the chemical structure of the drugs since a small substitution can induce a large variation in affinity. Indeed, replacement of a 2-methoxy group (S-64329) by a phenolic group (S-16950) confered a higher affinity and shifted the inhibition curve from a two-site to a three-site interaction (see Figure 3). S-16950 is the only drug tested up to now permitting the identification of three families of binding sites.

From a pharmacological point of view, it is interesting to note that the most effective inhibitors of [^{3}H]-trimetazidine binding are drugs known to interact with sigma receptors, especially sigma$_1$ receptors which are abundant in the liver (Dehaven-Hudkins *et al*., 1994). Moreover, the rank order of affinity pentazocine > opipramol > perphenazine > haloperidol > DTG > prenylamine > dextromethorphan etc... is consistent with binding to sigma sites (Klein & Musacchio, 1989; Basile *et al*., 1992; Dehaven-Hudkins *et al*., 1994). In addition, S-16950 is structurally related to N-substituted 4-benzylpiperidines, such as ifenprodil and eliprodil, which exhibit high affinity for sigma$_1$ receptors (Karbon *et al*., 1990; Whittemore *et al*., 1997), and its chemical structure is close to that of SA4503 which has recently been shown to be a potent and selective sigma$_1$ receptor agonist (Matsuno & Mita, 1998). It should be pointed out that ifenprodil also displaced [^{3}H]-trimetazidine binding with a high affinity.

Collectively, these data suggest that one of the mitochondrial sites labelled by [^{3}H]-trimetazidine could be a sigma receptor. This raises the question whether these receptors are located on mitochondria. Little is available to answer this question. Most studies were performed with brain preparations, showing an enrichment of sigma receptors in the microsomal and myelin fractions (McCann & Su, 1990). McCann *et al*. (1994) concluded that localization of these receptors in mitochondria is highly unlikely but Itzhak *et al*. (1991) and Dehaven-Hudkins *et al*. (1994) demonstrated a significant binding of sigma ligands to the mitochondrial fraction. Such results seem to depend on the radioligand used but cross-contamination is also a problem. However, our mitochondrial preparations were highly purified and the activities of representative markers of microsomes and plasma membranes were undetectable. Taken together, these data suggest that sigma receptors are indeed present on mitochondria.

The possible interaction of trimetazidine with sigma receptors would be of particular interest as several ligands of these receptors have been reported to have neuroprotective effects in *in vitro* and *in vivo* models of ischaemia (Matsuno & Mita, 1998). Although no clear pharmacological definition of a sigma agonist or antagonist and no clear correlation between sigma receptor affinity and neuroprotective potency have yet been established (Maurice & Lockhart, 1997), our hypothesis could contribute to a better understanding of the mechanism of action of trimetazidine.

In conclusion, the data presented in this study confirm the heterogeneity of [^{3}H]-trimetazidine binding sites. The sites of medium affinity are regulated by Ca^{2+} and could explain the inhibition of MTP opening by trimetazidine. A trimetazidine derivative, S-16950, showed a very high affinity for trimetazidine binding sites. Various data indicate that these sites located on mitochondria could represent a sigma receptor; a detailed binding analysis of S-16950 is in progress to evaluate its possible interaction with these receptors. The demonstration of the possible existence of an endogenous ligand able to modulate trimetazidine binding reinforced the hypothesis that these binding sites could have physiological relevance.

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