Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier

John C.W. Hildyard\textsuperscript{a}, Carina Āmmälā\textsuperscript{b}, Iain D. Dukes\textsuperscript{b}, Stephen A. Thomson\textsuperscript{b}, Andrew P. Halestrap\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK
\textsuperscript{b}GlaxoSmithKline Research and Development, 5 Moore Drive, Research Triangle Park, NC 27709, USA

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Abstract

Two novel thiazolidine compounds, GW604714X and GW450863X, were found to be potent inhibitors of mitochondrial respiration supported by pyruvate but not other substrates. Direct measurement of pyruvate transport into rat liver and yeast mitochondria confirmed that these agents inhibited the mitochondrial pyruvate carrier (MPC) with \( K_i \) values \(<0.1 \text{ \mu M} \). Inhibitor titrations of pyruvate-dependent respiration by heart mitochondria gave values (±S.E.) for the concentration of inhibitor binding sites (pmol per mg protein) and their \( K_i \) (nM) of 56.0±0.9 and 0.057±0.010 nM for the more hydrophobic GW604714X; for GW450863X the values were 59.9±4.6 and 0.60±0.12 nM. [\(^{3}\text{H}\)]-methoxy-GW450863X binding was also used to determine the MPC content of the heart, kidney, liver and brain mitochondria giving values of 56, 40, 26 and 20 pmol per mg protein respectively. Binding to yeast mitochondria was <10\% of that in rat liver mitochondria, consistent with the slow rate of pyruvate transport into yeast mitochondria. [\(^{3}\text{H}\)]-methoxy-GW450863X binding was inhibited by GW604714X and by the established MPC inhibitor, UK5099. The absorbance spectra of GW450863X and GW604714X were markedly changed by the addition of \( \beta \)-mercaptoethanol suggesting that the novel inhibitors, like \( \alpha \)-cyanocinnamate, possess an activated double bond that attacks a critical cysteine residue on the MPC. However, no labelled protein was detected following SDS-PAGE suggesting that the covalent modification is reversible. GW604714X and GW450863X inhibited \( \ell \)-lactate transport by the plasma membrane monocarboxylate transporter MCT1, but at concentrations more than four orders of magnitude greater than the MPC.

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1. Introduction

The transport of pyruvate into the mitochondria is essential for glucose oxidation, lipogenesis and gluconeogenesis, and is also required for the metabolism of some amino acids \([1,2]\). The existence of a specific mitochondrial pyruvate carrier (MPC) was unequivocally demonstrated in this laboratory by the discovery of the specific and potent inhibitor of transport, \( \alpha \)-cyan-4-hydroxycinnamate (CHC) \([3]\). This agent, and its more potent analogues such as \( \alpha \)-cyano-\( \beta \)-(1-phenylindol-3-yl)-acyrate (UK5099), inhibit the MPC by specifically modifying a thiol group on the carrier \([4]\). The same compounds also inhibit the plasma membrane monocarboxylate transporters (MCTs), but with \( K_i \) values some two or three orders of magnitude higher than those for the inhibition of the MPC \([3,5]\). Subsequent work in this and other laboratories led to a detailed description of the kinetic properties and substrate and inhibitor specificity of the MPC \([6–10]\). These studies revealed that the carrier also plays an important role in the transport of ketone bodies such as acetoacetate and \( \beta \)-hydroxybutyrate across the inner membrane.
mitochondrial membrane. Quantification of the level of MPC expression in heart and liver mitochondria was estimated from inhibitor titrations and radioactive binding studies to be in the range of 50 to 100 pmol per mg protein [11,12]. Very recently we have identified the mitochondrial pyruvate carrier (MPC) in yeast as a 42 kDa member of the mitochondrial carrier family [13]. The mammalian MPC has yet to be identified, but searching the human and mouse genomic database has revealed three candidate genes encoding members of the mitochondrial family with 30% sequence identity [13].

During the development of novel KATP channel agonists for use as potential antidiabetic drugs, some compounds were discovered that exerted metabolic effects similar to those previously observed in response to CHC [14] and thus consistent with an inhibition of mitochondrial pyruvate transport. In this paper we demonstrate that these compounds are extremely potent inhibitors of the MPC with \( K_i \) values of <1 nM, whilst being more than four orders of magnitude less potent as inhibitors of the plasma membrane monocarboxylate transporter, MCT1. We have also used these novel inhibitors to determine the concentration of the MPC in mitochondria from a range of rat tissues.

2. Experimental procedures

All reagents were obtained from Sigma (Poole, UK) unless otherwise stated. The structures of GW604714X and GW450863X are shown in Fig. 1. GW450863X was custom-labelled with tritium gas by Amersham Biosciences UK Ltd (Little Chalfont, Bucks, HP7 9NA, UK) to yield \([3H]\)-methoxy-GW450863X with specific activity of 3.03 TBq mmol\(^{-1}\) and 99% purity.

![Fig. 1. The chemical structures of GW604714X, GW450863X and \(\alpha\)-cyanocinnamate derivatives. The arrows show potential sites of Michael addition across an activated double bond by thiol groups.](image-url)

2.1. Preparation of mitochondria

Mitochondria were prepared from various rat tissues following homogenisation in isolation buffer (300 mM sucrose, 10 mM Tris HCl, 2 mM EGTA, 5 mg/ml bovine serum albumin, pH 7.4) using a Polytron PT10 (heart) or Dounce Potter (other tissues) homogeniser, and purified by Percoll gradient centrifugation [15,16]. For the preparation of brain mitochondria the first crude mitochondrial pellet was subject to an additional homogenisation in 5 ml BSA-free isolation buffer supplemented with 0.1% (w/v) digitonin in order to release mitochondria from synaptosomes, using a protocol adapted from the method of Anderson and Sims [17]. Mitochondria from *Saccharomyces cerevisiae* were prepared from spheroplasts obtained by lyticase treatment of yeast cells [13].

2.2. Measurement of mitochondrial pyruvate transport

As an indirect measure of mitochondrial pyruvate transport, pyruvate-dependent respiration by rat heart mitochondria was determined at 30 °C using a Clarke-type oxygen electrode as described previously [7]. A mildly hypotonic buffer containing 50 mM KCl, 20 mM Mops, 10 mM Tris, 2 mM KPi and 0.5 mM EGTA (pH 7.2) was employed to give maximal rates of respiration [18] and was supplemented with 2 mM L-malate and 1.0 mM ADP. Mitochondria were added at 0.25–0.5 mg protein per ml and pyruvate-dependent respiration was initiated by the addition of 0.5 mM pyruvate. Further additions were made as indicated in the figures.

For the direct determination of mitochondrial pyruvate transport, both radiotracer and fluorescent pH sensitive dye techniques were employed. The uptake of \([2-{\text{14C}}]\)-pyruvate into liver and yeast mitochondria was measured at 4 °C using an ascorbate/TMPD-generated proton gradient as described previously [13]. The pH-sensitive fluorescent dye \(2',7'\)-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to monitor the change in matrix pH changes associated with the proton-linked uptake of pyruvate and its analogue dichloracetate into mitochondria as follows. Isolated heart mitochondria were resuspended in isolation buffer at approximately 40 mg protein per ml and incubated with 25 μM BCECF-acetoxymethyl ester (BCECF-AM) for 7 min at 30 °C, before the removal of excess BCECF-AM by centrifugation. De-esterified BCECF remains entrapped within the mitochondrial matrix. Dye-loaded mitochondria were washed once, resuspended at about 20 mg protein per ml in hypotonic sucrose buffer (100 mM sucrose, 20 mM Tris HCl, 2 mM MgCl\(_2\), pH 7.4) and stored on ice. For transport studies mitochondria (300–500 μg protein) were added to 3.5 ml uptake buffer (100 mM sucrose, 2 mM MOPS, pH 6.8 containing 1 μg/ml each of oligomycin, rotenone and antimycin A) in 4 ml fluorescence cuvettes. Other inhibitors such as GW604714X or UK5099 were added as required and the BCECF fluorescence was...
monitored continuously at 20 °C using a Cairn spinning wheel fluorescence apparatus (Cairn Research Ltd, Faversham, Kent, ME13 8UP, UK). Excitation was at 440 nm and 490 nm and emission at >535 nm. Transport was initiated by the addition of the required monocarboxylate and was detected as a rapid increase in the 440/490 fluorescence ratio that reflects the decrease in matrix pH associated with proton-linked transport [19].

2.3. Measurement of inhibitor binding

Mitochondria isolated from yeast (2 mg protein) or rat heart, kidney, liver and brain (250–500 µg protein) were added to 1.5 ml microcentrifuge tubes containing 1 ml isolation buffer supplemented with GW450863X at the required concentration (0–500 nM) and 74 kBq of [³H]-methoxy-GW450863X. The specific activity of the [³H]-methoxy-GW450863X was determined by assaying the [³H] content of a small sample of the incubation medium prior to the addition of mitochondria. Following incubation at room temp for 5 min to allow inhibitor binding to reach equilibrium, mitochondria were collected by centrifugation and solubilized in 200 µl 5% (w/v) SDS. Values for free and bound concentrations of [³H]-methoxy-GW450863X were calculated from the ³H-content of the mitochondrial pellet and supernatant determined by scintillation counting. Non-specific binding of [³H]-methoxy-GW450863X was determined by performing parallel experiments in which mitochondria had been pre-incubated for 10 min with excess (5 µM) unlabelled GW450863X. In order to calculate the concentration of GW450863X binding sites (Et) and the dissociation constant (Kd) for the bound GW450863X, data were fitted by non-linear least-squares regression analysis using PFit software (Biosoft, Cambridge, UK) to the following equation:

\[ [I]_{\text{bound}} = [Et]_{\text{max}}* [I]_{\text{free}}/(K_i + [I]_{\text{free}}) + [I]_{\text{free}}*\text{NSB} \]

where \([I]_{\text{bound}}\) is the amount of bound GW450863X (in pmol), \([I]_{\text{free}}\) is the free concentration of GW450863X (nM), and NSB the correction for the non-specific binding of large hydrophobic aromatic inhibitors such as GW450863X to lipid membranes.

3. Results and discussion

3.1. GW604714X and GW450863X inhibit the MPC

As shown in Fig. 1, the chemical structures of GW604714X and GW450863X are quite distinct from those of the established inhibitors of the MPC, α-cyano-4-hydroxycinnamate and UK5099 [20]. In order to test this possibility directly, we determined the effect of β-mercaptoethanol on the absorbance spectrum of the two novel inhibitors. The addition of 50 mM β-mercaptoethanol caused the absorbance peak at about 350 nm to be almost completely abolished as shown in Fig. 2. Thus it is likely that, although quite distinct in structure, both inhibitors may act by attacking a specific cysteine residue on the MPC.

As an initial test of whether GW604714X and GW450863X inhibit mitochondrial pyruvate transport, their effects on the rate of ADP-stimulated pyruvate oxidation by rat heart mitochondria were investigated using an oxygen electrode as described previously [7]. As shown in Fig. 3, the addition of either compound at 1 µM gave a time dependent inhibition of pyruvate oxidation that became total within 1 min. Similar results were obtained with UK5099, an established inhibitor of the MPC. No inhibition of succinate- (not shown) or glutamate-dependent respiration was observed, indicating that inhibition was mediated either at the level of pyruvate transport or pyruvate dehydrogenase. It should be noted that the inhibition of pyruvate oxidation by low (nM) concentrations of GW604714X and GW450863X developed more slowly, requiring 2–5 min to reach completion, with GW604714X exhibiting a significantly longer latency than GW450863X does. This behaviour has been noted previously for α-cyanoacinnmate derivatives and has been shown to reflect the need for these

\[ \text{Fig. 2. The effects of mercaptoethanol on the absorbance spectrum of GW604714X and GW450863X.} \]

The compounds were present at 50 µM in 100 mM Tris HCl at pH 8.0, and where indicated (dotted traces) were preincubated with 50 mM β-mercaptoethanol for 15 min.
inhibitors to be transported (either by the carrier or by diffusion) into the mitochondria where they subsequently bind to a hydrophobic pocket on the matrix face of the carrier [10].

Confirmation that GW604714X and GW450863X inhibit the MPC was provided by the direct measurement of their effects on monocarboxylate transport into the mitochondria. This was achieved using two techniques. First, dichloracetate uptake into BCECF-loaded heart mitochondria was monitored by continuous measurement of the matrix pH as shown in Fig. 4. In these experiments, it was found to be important to avoid the presence of sodium in the medium, probably because of the high activity of the mitochondrial Na⁺/H⁺ antiporter [21] that is capable of modulating pH changes. It was for this reason that dichloracetate was used in place of pyruvate since the latter is only commercially available as the sodium salt. Following the addition of BCECF-loaded mitochondria (stored at pH 6.9) into uptake buffer (pH 7.4), there was a continuous acidification of the matrix (increase in the 440/490 fluorescence ratio) representing an equilibration of pH. However, the addition of 2 or 10 mM dichloracetate gave a rapid and transient increase in the rate of acidification that could be partially blocked by the addition of 1 μM UK5099 or GW604714X (data not shown). The extent of inhibition was much less at 10 mM dichloracetate than at 2 mM, consistent with the inhibitor-insensitive component being mediated by free diffusion of the undissociated acid as described previously [7,10]. In order to obtain some indication of the potency of GW604714X and GW450863X, a range of concentrations of each was employed and this revealed that a near-total inhibition occurs with concentrations as low as 20–50 nM.

Fig. 3. GW604714X and GW450863X inhibit pyruvate-dependent respiration by isolated rat heart mitochondria. Rat heart mitochondria (250 μg protein) were incubated in 1 ml respiration buffer containing 2 mM L-malate and 1.0 mM ADP in an oxygen electrode chamber. Pyruvate-dependent respiration was initiated by the addition of 0.5 mM pyruvate (P) followed by 1 μM GW450863X, GW604714X or UK5099 as indicated. Traces are also shown for mitochondria without inhibition, and mitochondria oxidising 5 mM L-glutamate plus 2 mM L-malate followed by addition of 1μM GW604714X. Further details are given under Experimental procedures.

Fig. 4. Inhibition of dichloracetate transport into heart mitochondria by GW604714X and GW450863X determined using BCECF fluorescence. BCECF-loaded mitochondria (500 μg protein in 3.5 ml transport buffer containing inhibitor as required) were incubated in the cuvette of the fluorimeter at 20 °C, and the 440/490 nm fluorescence ratio constantly monitored (2 data points per second). Dichloracetate (2 mM or 10 mM) was added at time zero and data were analysed by first order regression analysis as described under Experimental procedures to give the fitted line shown. The addition of the inhibitor shown preceded the addition of dichloracetate by 2 min. Inhibitor concentrations used were 0 nM (□), 10 nM (● and dashed line) and 50 nM (△).
However, under the constraints of this assay, the inhibitors could only be added for 60–100 s before the substrate, raising the possibility that given longer incubations these inhibitors may be effective at even lower concentrations.

The second technique employed the measurement of [2-\(^{14}\)C]-pyruvate uptake into mitochondria from rat liver or yeast (S. cerevisiae) and these data, shown in Fig. 5, confirm the data obtained with the BCECF-loaded mitochondria. For the experiments shown in Fig. 5, uptake was driven by a pH gradient generated using ascorbate in the presence of TMPD [13]. However, similar data (not shown) were obtained when the pH gradient was created using the same technique as employed in the BCECF experiments of Fig. 4, although the absolute amounts of uptake were smaller as reported previously [13]. Time courses from individual experiments are shown for rat liver (a) and yeast (b) mitochondria in the presence and absence of 5 \(\mu\)M UK 5099 and 1 \(\mu\)M GW604714X, whilst in Fig. 5c, mean data (±S.E. of 3 experiments) for the initial rate of transport are presented. It should be noted that the rate of transport into yeast mitochondria was <15% of that in liver mitochondria, suggesting the presence of considerably less MPC.

Since existing inhibitors of the MPC, such as CHC and UK 5099, can also inhibit the plasma membrane monocarboxylate transporter MCT1 with \(K_i\) values about three orders of magnitude less than for inhibition of the MPC [22], we tested the ability of GW604714X and GW450863X to inhibit the transport of 0.5 mM \([^{14}\text{C}]\)L-lactate into rat red blood cells, mediated by the monocarboxylate transporter MCT1. At 10 \(\mu\)M the two novel inhibitors only reduced the initial rate of uptake to 30% and 70% of control values respectively. This represents a selectivity for the MPC over MCT1 of over four orders of magnitude, substantially greater than for UK5099, the most potent \(\alpha\)-cyanocinnamate analogue.

### 3.2. Dissociation constants and number of binding sites for GW604714X and GW450863X

Two techniques were employed to determine the inhibitor binding affinity and number of binding sites per milligram protein. First, using hypotonic rat heart mitochondria, titrations with GW604714X and GW450863X of pyruvate-dependent ADP-stimulated respiration were performed as previously described for UK5099 [11]. Data are presented in Fig. 6. In these experiments, the first additions of inhibitor diminished the rate of respiration very little but after a threshold concentration was reached, pyruvate-dependent respiration became totally inhibited over a very narrow range of inhibitor concentration. A similar profile for the concentration dependence of pyruvate oxidation inhibition has been observed previously for UK5099 [11]. This is thought to reflect the minimal control that pyruvate transport into the mitochondria exerts on its rate of oxidation in the absence of inhibitor. However, with increasing additions of inhibitor, the transport becomes more rate limiting and rates of respiration declined sharply, reaching a basal level when transport is totally inhibited. This basal rate of respiration represents the oxidation of L-malate, endogenous substrates
characteristic of an inhibitor whose mitochondrial protein concentration. This behaviour is which significant inhibition develops is dependent on the of the carrier via free diffusion.

As shown for GW604714X in Fig. 6a, the threshold at which significant inhibition develops is dependent on the mitochondrial protein concentration. This behaviour is characteristic of an inhibitor whose \( K_i \) value for inhibition of the MPC is significantly less than the concentration of the target protein. Under such conditions, each inhibitor molecule added effectively blocks a single carrier molecule, as we have shown previously for UK5099. In Appendix A we provide a mathematical analysis of the effects of such a tight binding inhibitor of the MPC on the rate of pyruvate-dependent respiration under conditions where the MPC can transport pyruvate much faster than it is metabolised. The data for GW604714X can be fitted to the theoretical equation extremely well as shown in Fig. 6a and this allows the total number of inhibitor binding sites and their \( K_i \) values to be determined. Derived values for GW604714X were 61.1 ± 1.4 pmol per mg protein and 0.034 ± 0.013 nM respectively. In Fig 6b we present data from two separate experiments in which both GW604714X and GW450863X titrations were performed on the same mitochondrial preparations in order to allow direct comparison of their affinity for the MPC. Derived values for the number of binding sites for GW604714X and GW450863X were 56.0 ± 0.9 and 59.9 ± 4.6 pmol per mg protein respectively with corresponding \( K_i \) values of 0.057 ± 0.010 nM and 0.60 ± 0.12 nM respectively. The higher affinity of GW604714X compared to GW450863X may reflect the greater hydrophobicity of the former, since the potency of \( \alpha \)-cyanocinnamate derivatives also increases with their hydrophobicity [7]. It should be noted that the concentration of MPC determined in these experiments (60 pmol per mg protein) is in good agreement with previous values estimated from UK5099 titration of mitochondrial pyruvate metabolism and binding studies with \([^{14}C]\)-\( \alpha \)-cyanocinnamate [11,12].

An alternative and more direct method was also used to determine the number of binding sites for GW450863X and their \( K_d \) values in mitochondria from several rat tissues. For this purpose, the binding of \([^{3}H]\)-methoxy-GW450863X to isolated mitochondria was determined and the data analysed by non-linear least-squares regression to obtain values for the number of inhibitor binding sites per milligram of protein and their \( K_d \) data for rat heart, kidney, liver and brain mitochondria are shown in Fig. 7a and the derived parameter values summarised in Table 1. The calculated number of inhibitor binding sites was 56, 40, 26 and 20 pmol per mg protein for the heart, kidney, liver and brain mitochondria respectively. As might be predicted, the derived \( K_d \) value (about 15 nM) was similar for all mitochondria. For heart the number of binding sites derived this way is almost identical to that derived from the oxygen electrode studies presented in Fig. 6 and similar to values reported previously from UK5099 titrations of pyruvate-dependent respiration [11] and from \([^{14}C]\)-\( \alpha \)-cyanocinnamate binding studies [12]. However, the \( K_d \) value of 15 nM for \([^{3}H]\)-methoxy-GW450863X is substantially higher than the \( K_i \) value for the inhibitor derived from the oxygen electrode studies (0.6 nM). This difference may reflect either a higher affinity of the MPC for the inhibitor when it is actively translocating substrate, or a difference in affinity between GW450863 and \([^{3}H]\)-methoxy-GW450863X. From the difference in the rates of pyruvate transport between liver and yeast mitochondria (Fig. 5c) a value of 3–4 pmol of inhibitor binding sites per mg of mitochondrial protein would be predicted and this would be hard to detect experimentally against the background of non-specific binding. This was found to be the case as
shown in Fig. 7b. However, if the data are fitted assuming 4 pmol per mg protein, as shown in Fig 7b, this gives a calculated $K_d$ of 133±54 nM. We confirmed that GW604714X and GW450863X bind to the same sites of the MPC by demonstrating that the binding of $[^3H]$-methoxy-GW450863X to rat liver mitochondria could be greatly reduced by preincubation with 500 nM GW604714X as shown in Fig. 7c. Preincubation with 500 nM UK5099 also reduced the binding of $[^3H]$-GW450863X, although to a lesser extent than 500 nM GW604714X, suggesting that there may be some overlap in the site of occupancy of the inhibitors, or that the inhibitors can induce different conformations of the carrier. Reassuringly, the binding of $[^3H]$-methoxy-GW450863X to yeast mitochondria was also reduced by GW604714X and UK5099 (Fig. 7b, open symbols). However, because the amount of specific binding relative to the non-specific binding is small, the effect was barely detectable.

4. Conclusions

The data we present in this paper identify a new class of thiazolidine inhibitors of the mitochondrial pyruvate transporter, with $K_i$ values of about 1 nM for GW450863X and <0.1 nM for GW604714X. These $K_i$ values are substantially lower than the value of 5–10 nM for UK5099, which is the most potent of the known α-cyanocinnamate derivatives known to date [11]. Unlike the cyanocinnamates, these novel inhibitors bear no obvious structural resemblance to a monocarboxylate, and thus might bind to a different site. However, in common with the cyanocinnamates they possess an activated double bond that can react with thiol groups (Fig. 2). Furthermore, competition studies (Fig. 7c) imply that there is either some overlap with the binding site for UK5099 or that the two inhibitors produce mutually

![Fig. 7. Binding of $[^3H]$-methoxy-GW450863X to mitochondria from various rat tissues and yeast. Mitochondria (0.5 mg) from rat heart, kidney, liver and brain or yeast (2 mg) were incubated for 5 min with increasing concentrations of $[^3H]$-methoxy-GW450863X and the bound and free concentrations determined as described under Experimental procedures. Data were fitted by least squares regression analysis to a hyperbolic binding curve corrected for non-specific binding (increases linearly with inhibitor concentration). Derived values for the number of binding sites (pmol per mg protein) and their dissociation constants (nM) are given in Table 1 for mitochondria from rat tissues rat. For the yeast mitochondria (b) data are given from 2 control experiments (△,○) with corresponding data obtained in the presence of 500 nM GW604714X (△,□) or UK5099 (□). The line shown was fitted assuming 4 pmol per mg protein and generated a $K_d$ of 133±54 nM. In (c) data are presented for the amount of $[^3H]$-methoxy-GW450863X bound specifically to liver mitochondria in the presence and absence of 500 nM UK5099 or 500 nM GW604714X determined as described in Fig. 7a. Mitochondria (1.5mg) were incubated with 250 nM $[^3H]$-methoxy-GW450863X in the presence of DMSO vehicle, 500 nM UK5099 or 500 nM GW604714X. Data are presented as Means±S.E. of 3 separate incubations.

![Table 1](https://example.com/table1.png)

Table 1

<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>Concentration of binding sites (pmol per mg protein)</th>
<th>Dissociation constant (nM)</th>
<th>Non-specific binding constant (ml mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>25±2.6</td>
<td>21±1.8</td>
<td>0.068</td>
</tr>
<tr>
<td>Kidney</td>
<td>30±3.2</td>
<td>14±5.4</td>
<td>0.064</td>
</tr>
<tr>
<td>Liver</td>
<td>26±3.8</td>
<td>13±2.1</td>
<td>0.062</td>
</tr>
<tr>
<td>Brain</td>
<td>20±0.6</td>
<td>11±0.8</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Parameter values were obtained from least squares regression analysis of the data for $[^3H]$-methoxy-GW450863X binding to mitochondria shown in Fig. 7a.
exclusive conformations of the carrier, as is the case for carboxyatractyloside and bongkrekic acid binding to the adenine nucleotide translocase [23]. Another feature of the novel inhibitors that is similar to α-cyanocinnamate derivatives is that they take time to inhibit to their maximal extent, and both their potency and latency increases with their hydrophobicity. We have suggested previously that this may reflect the presence of a hydrophobic binding pocket on the matrix surface of the carrier. The inhibitors may reach this site by binding to the outer face of the carrier, which then undergoes a conformational change translocating the inhibitor into a hydrophobic matrix facing binding pocket [2,10]. Alternatively, the inhibitor may be sufficiently lipid soluble to accumulate within the bilayer and then diffuse laterally into a binding site on the carrier.

The number of binding sites for GW450863X is greatest for the heart (55–60 pmol per mg protein) that requires especially rapid transport of pyruvate into the mitochondria to fuel the citric acid cycle for the provision of large amounts of ATP required for heart contraction. The kidney (40 pmol per mg protein) is another metabolically active tissue that requires mitochondrial pyruvate transport for both the provision of ATP and for gluconeogenesis. In liver (26 pmol per mg protein), the major requirement for mitochondrial pyruvate transport is probably in gluconeogenesis, where pyruvate is converted to oxaloacetate by pyruvate carboxylase within the mitochondrial matrix. Surprisingly, in view of the total dependence of brain on glucose and ketone body oxidation for ATP production, the amounts of the carrier in brain mitochondria are relatively low (20 pmol per mg protein). One explanation may be the lack of any rapid biosynthetic pathways that require pyruvate transport.

The low (1–2 pmol per mg protein) levels of carrier in yeast are consistent with the relatively slow rates of pyruvate oxidation by yeast mitochondria and the low growth rate of S. cerevisiae on pyruvate as a carbon source (own observations). This may reflect the metabolic versatility of yeast that allows this organism to utilise a great variety of substrates, but with varying levels of efficiency.

The availability of a novel, specific and extremely potent inhibitor of mitochondrial pyruvate transport provides a useful tool for investigating the role of this process in a variety of metabolic pathways. It is especially important to note that the potency of inhibition of the plasma membrane monocarboxylate transporter (MCT1) by these novel inhibitors is at least four orders of magnitude less than for inhibition of the MPC. In this regard they are substantially more selective than the cyanocinnamates. Furthermore, the potency of GW604714X as an inhibitor of the MPC is similar to the affinity of carboxyatractyloside (CAT) for the adenine nucleotide translocase (ANT). CAT has played an essential role in the identification, purification and, most recently, the crystallisation and 3D-structural determination of the ANT [24]. It is to be hoped that GW604714X may enable similar progress to be made with the MPC.

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Appendix A. Analysis of the effects of mitochondrial pyruvate transport inhibitors on pyruvate-dependent respiration

The net rate of pyruvate transport into the mitochondria (v) is given by the difference between the influx and efflux rates and can be expressed:

\[ v = Vt / [(1 + K_{mp}/[Pyr]_{out}) - Vt / (1 + K_{mp}/[Pyr]_{in})] \]  

(1)

Where \( K_{mp} \) and \( Vt \) are the \( K_m \) and \( V_{max} \) for the mitochondrial pyruvate carrier and \([Pyr]_{out} \) and \([Pyr]_{in} \) are the extramitochondrial and matrix pyruvate concentrations, respectively. It is assumed that both \( K_{mp} \) and \( Vt \) are the same for influx and efflux of pyruvate on the MPC which appears to be the case [10]. At steady state the net rate of pyruvate transport into the mitochondria (v) must also equal the rate of pyruvate oxidation via pyruvate dehydrogenase which is given by:

\[ v = Vp / (1 + K_{mpDH}/[Pyr]_{in}) \]  

(2)

Where \( K_{mpDH} \) and \( Vp \) are the \( K_m \) and \( V_{max} \) respectively for pyruvate dehydrogenase.

Combining Eqs. (1) and (2) to eliminate \([Pyr]_{in}\) allows \( v \) to be expressed in terms of \([Pyr]_{out} \) only (now termed \([Pyr]\)):

\[ v^2*(K_{mpDH} + (K_{mpDH}*[Pyr]/K_{mp}) - [Pyr] + K_{mp}) \]
\[ + v^p*(Vt*(K_{mpDH} + [Pyr]) + Vp*(K_{mp} + [Pyr])) \]
\[ - [Pyr]^p*Vp*Vt = 0 \]

(3)

Vt can be expressed as:

\[ Vt = k*Et \]  

(4)

Where \( k \) is \( k_{CAT} \) and Et is the concentration of the mitochondrial pyruvate carrier (MPC).

However at each inhibitor concentration the active MPC (E) is given by:

\[ E = (Et - EI) \]  

(5)

Where EI is the concentration of carrier-inhibitor complex. Et can be calculated from the dissociation constant for the inhibitor (\( K_i \)) and the FREE concentration of inhibitor ([I]). However, for a tight binding inhibitor the free concentration is less than the total concentration added, because a significant proportion is bound to the MPC. This can be calculated as described previously [25] and allows
calculation of the concentration of the EI complex from the total $[I]$ and $K_i$ as follows:

$$[EI] = \left(\left([I]+Et+K_i\right) - \sqrt{\left(\left([I]+Et+K_i\right)^2 - 4Et*I]\right)}\right)/2$$

(6)

From this the maximum rate of the MPC (Vt) at any inhibitor concentration can be calculated as:

$$Vt = k_{CAS}*(Et - [EI])$$

(7)

Combining Eqs. (3), (6) and (7) and solving the resulting quadratic we obtain:

$$v = (-B^*([KmPDH]*[Pyr]/K_{mPDH}+[Pyr]+[KmPi]; +B^*([KmPi]*[Pyr]) + (K_{mPi}[Pyr]) + (K_{mPi}[Pyr])) \frac{A}{2}$$

Where: $A=(K_{mPDH}^*)+([KmPDH]*[Pyr]/K_{mPDH}+[Pyr]+[KmPi]; +B^*([KmPi]*[Pyr]) + (K_{mPi}[Pyr]) + (K_{mPi}[Pyr])$ (this assumes both $K_m$ values are equal—see [1,2]); $C=4* A * [Pyr]$. $[Pyr]$ is set at 0.5mM and $K_{mPDH}$ and $K_{mPi}$ are assumed to be 0.1 mM. This gives values of $A=0.2$, $B=0.6$ and $C=0.4$.

For the measurement of the rate of pyruvate oxidation using the oxygen electrode all rates are expressed as nmol O per min. In order to account for differences in protein concentration, Et and Vp are replaced by Et*P and Vp*P where P is the mitochondrial protein concentration in mg/mL. Et is the concentration of MPC expressed as pmoles per mg protein and Vp is the maximal rate of mitochondrial pyruvate metabolism expressed as nmol O uptake per min per mg protein. In addition, some oxygen uptake occurs in the absence of pyruvate and is insensitive to MPC inhibitors. This is expressed as NI*P. Thus the total rate of oxygen uptake at any protein concentration and any inhibitor concentration is given by Eq. (9) below:

$$v = (-B^*([K^*(Et*P - ([I]+Et*P + K_i)\right. \left. + Vp*P) + \sqrt{\left(\left([I]+Et*P + K_i\right)^2 - 4Et*P*P]\right)}\right)/2)$$

+ $Vp*P) + \sqrt{\left(\left([I]+Et*P + K_i\right)^2 - 4Et*P*P]\right)}\right)/2)$$

(9)

Experimental data can be fitted to this equation by least squares regression analysis using Pfit software (Biosoft, Cambridge UK) to generate values (± S.E.) for $K_i$ and Et.

References


