Inhibition of the mitochondrial permeability transition pore (MPTP) by the novel inhibitor GNX-4975 was characterized. Titration of MPTP activity in de-energized rat liver mitochondria allowed determination of the number of GNX-4975-binding sites and their dissociation constant ($K_i$). Binding sites increased in number when MPTP opening was activated by increasing $[\text{Ca}^{2+}]$, phenylarsine oxide (PAO) or KSCN, and decreased when MPTP opening was inhibited with bongkrekic acid (BKA) or ADP. Values ranged between 9 and 50 pmol/mg of mitochondrial protein, but the $K_i$ remained unchanged at $\sim 1.8 \text{nM}$ when the inhibitor was added before Ca$^{2+}$. However, when GNX-4975 was added after Ca$^{2+}$ it was much less potent with a $K_i$ of $\sim 140 \text{nM}$. These data imply that a protein conformational change is required to form the MPTP complex and generate the GNX-4975-binding site. Occupation of the latter with GNX-4975 prevents the Ca$^{2+}$ binding that triggers pore opening. We also demonstrated that GNX-4975 stabilizes an interaction between the adenine nucleotide translocase (ANT), held in its ‘c’ conformation with carboxyatractyloside (CAT), and the phosphate carrier (PiC) bound to immobilized PAO. No components of the $F_1F_o$-ATP synthase bound significantly to immobilized PAO. Our data are consistent with our previous proposal that the MPTP may form at an interface between the PiC and ANT (or other similar mitochondrial carrier proteins) when they adopt novel conformations induced by factors that sensitize the MPTP to $[\text{Ca}^{2+}]$. We propose that GNX-4975 binds to this interface preventing a calcium-triggered event that opens the interface into a pore.

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INTRODUCTION

The mitochondrial permeability transition pore (MPTP) is a large non-specific channel in the inner mitochondrial membrane (IMM) whose opening is triggered by high matrix $[\text{Ca}^{2+}]$ to which it can be sensitized by a variety of factors. These include elevated $[\text{P}_i]$, adenine nucleotide depletion, agents such as carboxyatractyloside (CAT) that stabilize the adenine nucleotide translocase (ANT) in its cytoplasmic facing (‘c’) conformation, mild chaotropic agents such as KSCN and oxidative stress or its chemical mimic phenylarsine oxide (PAO) [1]. It is now widely accepted that MPTP opening plays a central role in the cell death that underlies several pathological conditions including ischaemia/reperfusion injury [2–5]. However, the exact molecular composition of the MPTP remains unresolved [1,4,6,7]. There is general agreement that the matrix peptide prolyl cis–trans isomerase cyclophilin D (CyP-D) acts as key regulator of the MPTP, enhancing its sensitivity to $[\text{Ca}^{2+}]$. Indeed, CyP-D is the site of action of two potent inhibitors of MPTP opening, cyclosporin A (CsA) and sanglifehrin A (SfA), both of which protect cells from death mediated by MPTP opening such as in ischaemia/reperfusion injury [3]. However, the molecular composition of the IMM components of the MPTP, with which CyP-D interacts, remains controversial.

Studies from our and other laboratories have implicated the ANT and the phosphate carrier (PiC), both of which can bind CyP-D [8–11] and whose ligands modulate MPTP opening [10,12–14]. Indeed the ANT, when reconstituted into phospholipid membranes, does demonstrate calcium-activated pore formation that can be modulated by CyP-D [9,15,16]. However, knockdown of either the PiC or ANT does not prevent MPTP opening, although the response to calcium and ANT ligands is severely attenuated [11,17,18]. More recently, others have proposed a role for the $F_1F_o$-ATP synthase which can also interact with CyP-D [19], although there are competing hypotheses as to which subunits are involved. Data from the laboratories of Pinton [20] and Jonas [21] have implicated the c subunits of the $F_1$ ATP synthase, whereas Bernardi and colleagues propose a role for dimers of the $F_1F_o$-ATP synthase, perhaps involving subunits associated with the c-ring in the membrane such as a, e, f, g and A6L moving to produce the pore [4,22]. We and others have expressed reservations about such a role for the $F_1F_o$-ATP synthase since a considerable body of evidence is better explained by interactions of pore regulators with the ANT and PiC [1,6,23]. Nevertheless, it remains possible that an interaction between the ANT, PiC and ATP synthase is involved in MPTP formation and such interactions may occur in the ATP synthasome [1,6,24,25]. Most recent evidence has been presented for the mitochondrial AAA protease SPG7 (spastic paraplegia 7) being essential for MPTP opening [26], although others have questioned these conclusions [27].

The role of CyP-D in mediating MPTP opening was first demonstrated in our laboratory by performing CsA inhibitor titrations of MPTP opening and the pepidyl prolyl cis–trans isomerase activity of CyP-D. These demonstrated that the $K_i$ for CsA inhibition of both actions was the same ($\sim 5 \text{nM}$) and that

**Quantification of active mitochondrial permeability transition pores using GNX-4975 inhibitor titrations provides insights into molecular identity**

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the high affinity of binding allowed calculation of the number of CsA-binding sites which were also identical for both processes [28,29]. Recently, Congenia has developed a new class of highly potent cinnamic aniline-based inhibitors of MPTP opening that protect against ischaemia/reperfusion injury of the heart [30] and amyotrophic lateral sclerosis [31]. Although the generic structure of these inhibitors is available [31,32], the exact structure of GNX-4975 is not in the public domain. MPTP inhibition by these agents does not involve CyP-D, implying that they must interact with another component of the MPTP. In the present study, we explore the binding site of the inhibitors in more detail using GNX-4975 which we demonstrate to have a $K_i$ for MPTP inhibition of $\sim 2$ nM under a variety of conditions. Importantly, we show that the number of binding sites for GNX-4975 increases when MPTP opening is activated by PAO or KSCN, and decreases when MPTP opening is inhibited with bongkrekic acid (BKA) or ADP. These data imply that a protein conformational change is required to form the MPTP and that this generates the binding site for GNX-4975. We further demonstrate that GNX-4975 enhances the association of ANT to the PAO-bound PiC. This is consistent with our previous proposal that an interface between the PiC and ANT may occur when these proteins adopt novel conformations induced by factors that sensitize the MPTP to [Ca$^{2+}$] [1]. We propose that GNX-4975 binds to this interface preventing a calcium-triggered event that opens the interface into a pore.

**EXPERIMENTAL**

**Materials**

Unless otherwise stated, all chemical and biochemicals were obtained from Sigma–Aldrich or Fisher Scientific. Antibodies against the C-terminus of the ANT and PiC were raised either in-house or commercially as described in [10]. The use of rats conforming to the U.K. Animals (Scientific Procedures) Act 1986 and was approved by the appropriate University of Bristol ethics committee (UB/09/012).

Mitochondria were prepared from livers of 250–275 g male Wistar rats by Dounce Potter homogenization in sucrose isolation buffer (ISB: 300 mM sucrose, 10 mM Tris/HCl and 2 mM EGTA, pH 7.4) followed by differential centrifugation and Percoll density-gradient centrifugation as described previously [28]. Preparation of beef and pig heart mitochondria involved initial tissue disruption with a Polytron 10-35 GT homogenizer [10] with subsequent steps being the same as for liver mitochondria. For measurement of MPTP opening in de-energized rat liver mitochondria, they were routinely prepared 1 day before they were used to assay MPTP opening and stored overnight on ice. This avoided changes to the [Ca$^{2+}$] sensitivity of the MPTP caused by progressive loss of total adenine nucleotides over the first few hours of storage on ice [12,32].

**Methods**

**Measurement of MPTP opening in de-energized mitochondria**

This was performed by one of two techniques. A swelling assay was used in which pore opening was triggered by addition of Ca$^{2+}$ [12,28]. In addition a shrinkage assay was employed in which pre-swollen mitochondria were incubated with the required [Ca$^{2+}$] to open the MPTP, the extent of which was then determined by the rate of shrinkage induced by addition of polyethylene glycol (PEG) [32]. In both cases the initial rate of decrease (swelling) or increase (shrinkage) in light scattering (monitored as $A_{340}$) was used to determine the extent of MPTP opening. For the swelling assay, mitochondria were incubated at 25°C and 0.5–1 mg of protein/ml in de-energized assay buffers containing either 150 mM KSCN or 125 mM KCl plus 2.5 mM potassium phosphate as the osmotic support and 20 mM MOPS, 10 mM Tris/HCl, 2 mM nitrolotriacetic acid (NTA), 0.5 µM rotenone, 0.5 µM antimycin A and 2 µM A23187 at pH 7.2. MPTP opening was initiated by addition of [Ca$^{2+}$] (usually 50 µM free) and $A_{340}$ was monitored continuously in a spectrophotometer with computerized data acquisition. When GNX-4975 was included, it was added 2 min prior to the addition of Ca$^{2+}$, whereas incubations with other drugs or reagents were carried out for 4 min prior to the addition of Ca$^{2+}$.

For the shrinkage assay, mitochondria (2 mg/ml) were pre-swollen by incubation for 20 min at 30°C in standard de-energized KSCN buffer (as above) but without added NTA or A23187 and with addition of 1 mM CaCl$_2$. Any residual swelling was terminated by an addition of 1.2 mM EGTA, which also resealed swollen mitochondria. The resulting swollen mitochondria were collected by centrifugation at 12 000 g for 10 min and resuspended at 2 mg/ml in de-energized KSCN buffer without added NTA or A23187. In order to ensure equilibrium of matrix with the buffer, the swollen mitochondria were incubated again at 30°C supplemented with 1 mM CaCl$_2$. After 3 min, 1.2 mM EGTA was added to reseal the mitochondria before centrifugation at 12 000 g for 10 min. The swollen mitochondria were then resuspended at 30 mg/ml in de-energized KSCN buffer containing 2 mM NTA and 2 µM A23187 and stored on ice. The extent of MPTP opening in these pre-swollen mitochondria was determined following incubation at 0.33 or 0.67 mg/ml for the stated time in KSCN or KCl buffer containing, where indicated, the required [GNX-4975] and free [Ca$^{2+}$] (calculated as described in [31]). Shrinkage was initiated by rapid addition of 0.5 ml of 50% (w/v) PEG (to give a final PEG concentration of 7%, w/v) and was continuously monitored (ten data points per second) as an increase in $A_{340}$.

**Determination of the number of binding sites and $K_i$ for GNX-4975 inhibition of the MPTP**

The initial rate of swelling or shrinking of mitochondria pre-incubated with increasing concentrations of GNX-4975 was determined by differentiation of the time course of $A_{340}$ change. As described previously [34,35], the data were then fitted using FigSys (BioSoft) to the equation for the inhibition of rate by a tight binding inhibitor:

$$V = \frac{k \cdot E_i \cdot (B - \sqrt{B \cdot B - 4 \cdot C})}{2}$$

where $B = E_i \cdot P + I + K_i$

and $C = E_i \cdot P \cdot I$

$E_i$ represents the number of GNX-4975-binding sites in pmol/mg, $P$ is the mitochondrial protein concentration (mg/ml), $K_i$ is the dissociation constant for GNX-4975 (nM) and $k$ is the rate constant for swelling ($\Delta A_{340} \cdot s^{-1} \cdot nM^{-1}$). Unless otherwise stated, for each titration, a separate $E_i$, $K_i$ and $k$ were derived and the mean value ± S.E.M. calculated for several mitochondrial preparations ($n = 3–6$ as indicated). In order to correct for variations in the rates of swelling or shrinking determined using different mitochondrial preparations on different days, rates at any particular inhibitor concentration were usually calculated as a percentage of the maximal rate of swelling in the absence of inhibitor or other MPTP modulating reagents. In this case, the $k$ value becomes 100/$E_i$ and so its value is not presented.
Measurement of MPTP opening in energized mitochondria

This was performed by simultaneously measuring extra-mitochondrial [Ca\(^{2+}\)] with Fura-FF fluorescence, membrane potential with Rhodamine 123 fluorescence and mitochondrial swelling with light scattering at 490 nm as described previously [14]. The incubation medium consisted of 125 mM KCl, 10 mM Tris/HCl, 20 mM MOPS, 2.5 mM potassium phosphate, 5 mM L-glutamate, 2 mM L-malate, 20 μM EGTA, 0.1 μM Fura-FF and 100 nM Rhodamine-123, pH 7.2, at 30°C. MPTP opening was induced by sequential additions of Ca\(^{2+}\) as indicated in the figures.

Preparation and solubilization of inner mitochondrial membranes

Centrifugation steps in this process were carried out using a benchtop Eppendorf 5415R centrifuge at 4°C. Mitochondria were washed in ISB and centrifuged at 12 000 g for 5 min before resuspension in ISB containing protease inhibitors (4 μg/ml each of antipain, pepstatin A and leupeptin plus 0.5 mM benzamidine) and insoluble material removed by centrifugation at 4000 g on a rotary mixer, and the resulting mitoplasts collected by centrifugation at 9000 g for 10 min before resuspension in ISB containing protease inhibitors and PEG ether W1 (Sigma P7516, formerly known as Lubrol) at 0.16 mg/mg of mitochondrial protein. The mixture was incubated at 4°C for 15 min on a rotary mixer and insoluble material removed by centrifugation at 4000 g for 30 s. The supernatant was centrifuged at 125 000 g and 4°C for 30 min in a Beckman Optima™ TLX ultracentrifuge (TLA-55 rotor) to yield the IMM pellet. Where required, pre-treatment of mitochondria with drugs or reagents was performed in ISB at a protein concentration of 2 or 5 mg/ml for 10 min at room temperature with constant agitation. The treated mitochondria were collected by centrifugation at 12 000 g for 5 min and resuspended in ISB containing protease inhibitors (as above), prior to digitonin treatment. Where indicated, drugs or reagents were present throughout the duration of the isolation, including the Lubrol treatment.

Binding of solubilized IMM proteins to immobilized phenylarsine oxide

An immobilized PAO matrix was synthesized by coupling 4-aminophenylarsine oxide to Affi-gel 10 (Bio-Rad Laboratories 153-6099) as described previously [10,12]. The resin was washed twice in 10 volumes of PAO column buffer (PCB: 150 mM Na\(_2\)SO\(_4\), 50 mM HEPES and 1 mM EGTA, pH 7.2) containing 0.5% (v/v) Triton X-100 and then resuspended to give a 50% (v/v) slurry ready for use. IMM fractions from mitochondria incubated with the required reagents were resuspended in PCB containing 1% (v/v) Triton X-100 and protease inhibitors (as above) and solubilized for 1 h on a rotary mixer at 4°C. Insoluble material was removed by centrifugation at 16 000 g for 12 min and the supernatant transferred to prepared aliquots of pre-washed 50% slurry of PAO beads (200 μl/1 mg of solubilized protein) and incubated for 1 h on a rotary mixer at 4°C. Beads were collected by centrifugation at 200 g for 1 min and were washed five times with 1 ml of PCB containing 3% (v/v) Triton X-100 to remove nonspecifically bound proteins. Specifically bound proteins were then eluted by incubating in 80 μl of 25 mM DTT in PCB containing 1% (v/v) Triton X-100 for 30 min on a rotary mixer at 4°C. Beads were collected by centrifugation at 2000 g for 3 min and the supernatant (70 μl) was added to an equal volume of SDS/PAGE sample buffer. Samples were analysed by SDS/PAGE and Western blotting or analysed by Orbitrap MS in the University of Bristol Proteomics Facility. Development of Western blots was performed using ECL with film detection of the emitted light and analysis of the scanned film with ImageJ software (http://imagej.nih.gov/). The integrated optical density for each band was calculated as the product of the ‘area’ and ‘mean grey value’ with subtraction of a background signal obtained for an equivalent adjacent area lacking any band. In one set of experiments comparative analysis was performed using direct detection of ECL with an ODDESEY Fc (LI-COR) and analysis of the bands performed using Image Studio (LI-COR). The band intensities were determined by the software and corrected for background by subtracting the mean signal of five pixels above and below each band of interest.

RESULTS

GNX-4975 is a tight binding inhibitor of the MPTP

We first confirmed that GNX-4975 is a potent inhibitor of MPTP opening in energized liver mitochondria by simultaneously measuring extra-mitochondrial [Ca\(^{2+}\)] (Fura-FF fluorescence), membrane potential (Rhodamine 123 fluorescence) and mitochondrial swelling (light scattering at 490 nm) as described previously [14]. As shown in Supplementary Figure S1, sequential additions of Ca\(^{2+}\) were taken up by the mitochondria until the MPTP opened, at which time the accumulated Ca\(^{2+}\) was released, the membrane potential was dissipated and the light scattering rapidly decreased as the mitochondria swelled. In control mitochondria, this occurred after addition of 70–80 μM Ca\(^{2+}\), whereas in the presence of 0.2 μM GNX-4975 MPTP opening required 200–240 μM Ca\(^{2+}\).

In order to determine an accurate concentration-dependence of MPTP inhibition by GNX-4975, we employed de-energized mitochondria in the presence of a Ca\(^{2+}\) ionophore (A23187), since this avoids any complications that might be caused by secondary effects of the drug on mitochondrial energization or calcium transport. We also performed the assays in medium containing 150 mM KSCN since we have previously shown that this mildly chaotrope buffer promotes the active conformation of the MPTP and generates very consistent data [32]. GNX-4975 was added at the required concentration to the mitochondrial suspension 2 min before Ca\(^{2+}\) was added to initiate MPTP opening whose extent was determined from the initial rate of swelling (decrease in A\(_{490}\)) quantified by taking the first derivative of each time course. Figure 1 shows mean data (+S.E.M.) for such inhibitor titrations on six separate mitochondrial preparations each used at two different concentrations of mitochondrial (0.5 and 1 mg of protein/ml). It is immediately apparent that more GNX-4975 was required to give 50% inhibition at the higher protein concentration as is predicted for a very tight binding inhibitor whose K\(_i\) value is less than the concentration of binding sites. This was observed previously for CsA inhibition of MPTP opening and using the appropriate equation for inhibition by a tight binding inhibitor this allowed calculation of the true K\(_i\) (dissociation constant) for inhibitor binding and the number of inhibitor-binding sites (E\(_i\)) [28,29]. We successfully analysed the data of Figure 1 in this way and determined E\(_i\) to be 12.6 ± 1.7 pmol/mg and the dissociation constant for drug binding (K\(_i\)) to be 1.95 ± 0.17 nM (values presented as means ± S.E.M. for six separate mitochondrial preparations). It should be noted that absolute values of light scattering are not linearly related to mitochondrial protein concentration and can also vary from day to day. Thus, to analyse the data from several experiments, the rates of swelling in the absence of inhibitor at 0.5 and 1 mg/ml mitochondrial protein were set at 50 and 100 respectively.
and values at each concentration of GNX-4975 were calculated relative to the control value.

**MPTP activation increases the number of GNX-4975-binding sites without affecting their $K_i$**

The data shown in Figure 2A compare the inhibition profile of GNX-4975 in KSCN medium, which activates the MPTP, with data obtained in a more physiological KCl-based medium containing 125 mM KCl and 2.5 mM potassium phosphate. It is immediately apparent that rates of swelling at zero inhibitor are less in the KCl medium (set at 100 %) than in KSCN medium, whereas the derived $K_i$ values (mean ± S.E.M. for five separate experiments) were similar, being 1.68 ± 0.13 and 2.29 ± 0.17 nM respectively. However, the calculated number of GNX-4975-binding sites in the KSCN buffer (19.2 ± 2.2 pmol/mg) was significantly higher ($P < 0.01$) than in the KCl buffer (9.43 ± 1.4 pmol/mg). It should be noted that in these experiments, in order to ensure that rates of swelling in KCl medium were sufficiently rapid to allow accurate inhibitor titrations, we activated MPTP opening with 150 μM Ca2+ and 2.5 mM potassium phosphate. It is immediately apparent that rates of swelling at zero inhibitor are less in the KCl medium (set at 100 %) than in KSCN medium, whereas the derived $K_i$ values (mean ± S.E.M. for five separate experiments) were similar, being 1.68 ± 0.13 and 2.29 ± 0.17 nM respectively. However, the calculated number of GNX-4975-binding sites in the KSCN buffer (19.2 ± 2.2 pmol/mg) was significantly higher ($P < 0.01$) than in the KCl buffer (9.43 ± 1.4 pmol/mg). It should be noted that in these experiments, in order to ensure that rates of swelling in KCl medium were sufficiently rapid to allow accurate inhibitor titrations, we activated MPTP opening with 150 μM Ca2+. As discussed further below, this explains the higher value of $E_i$ than determined in Figure 1 where only 50 μM Ca2+ was employed. These data suggest that activation of the MPTP by KSCN involves an increase in the active conformation of the pore to which GNX-4975 binds. It should be noted that it is not possible to relate the rates of swelling directly to the number of binding sites because many additional factors can affect the absolute value of $A_{20}$ as noted above.

Activation of the MPTP can also be achieved by exposing the mitochondria to oxidative stress and this can be mimicked by the vicinal thiol reagent PAO [12,36], which acts, at least in part, by cross-linking thiol groups on the ANT and PiC [10,12,37]. In Figure 2B we show that, following a short pre-incubation with 20 μM PAO, MPTP opening was activated as expected and that this led to an increase in the number of binding sites for GNX-4975 from 11.9 ± 0.92 to 17.2 ± 1.3 pmol/mg (means ± S.E.M. for six separate mitochondrial preparations; $P < 0.01$) without any significant change in $K_i$ (1.93 ± 0.21 and 2.56 ± 0.14 nM respectively). Conversely, BKA, an inhibitor of the ANT that traps the carrier in its matrix facing (‘m’) conformation, inhibits MPTP opening as does ADP which also favours the ‘m’ conformation [12,13,38,39]. In Figure 3A, we show that mitochondrial pretreatment with 5 μM BKA decreased the number of GNX-4975-binding sites from 13.2 ± 0.64 to 9.43 ± 0.47 pmol/mg (means ± S.E.M. for six separate mitochondrial preparations; $P < 0.001$), again without any change in $K_i$ (1.45 ± 0.23 and 1.46 ± 0.16 nM respectively). In Figure 3B, we show similar data for mitochondria pre-treated with 20 μM ADP which decreased the number of GNX-4975-binding sites from...
Characterization of GNX-4975 inhibition of the MPTP

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Figure 3 The ‘m’ conformation of the ANT reduces the number of GNX-4975-binding sites

Experiments were performed exactly as described for Figure 2B but with 3 min of pre-treatment of either 5 μM BKA (A) or 20 μM ADP (B) prior to addition of 50 μM Ca²⁺ to initiate swelling. Mean data ± S.E.M. (error bars) for six separate mitochondrial preparations are given. The rate of swelling in the absence of both GNX-4975 and ADP or BKA was set at 100 and all other rates calculated relative to this. Data fitted as described in Figure 1 to derive mean values (± S.E.M.) for Kᵢ of 5.3 pmol/mg (P < 0.05), whereas Kᵢ values remained unchanged (1.45 ± 0.23 and 1.46 ± 0.16 nM respectively). In (B), the derived values for Eᵢ in the absence and presence of ADP were 14.1 ± 0.83 and 11.0 ± 0.97 pmol/mg (P < 0.05) with Kᵢ values of 1.57 ± 0.24 and 1.45 ± 0.15 nM respectively.

14.1 ± 0.83 to 11.0 ± 0.97 pmol/mg (means ± S.E.M. for six separate mitochondrial preparations; P < 0.05), also without any change in Kᵢ (1.57 ± 0.24 and 1.44 ± 0.15 nM respectively).

The number of GNX-4975-binding sites is increased when higher [Ca²⁺] is used to induce MPTP opening

Increased [Ca²⁺] within the mitochondrial matrix is a key trigger for MPTP opening and, under de-energized conditions, the extent of MPTP opening can be increased progressively with increasing [Ca²⁺] [12,32]. In Figure 4, we investigate the effect of increasing the [Ca²⁺] used to trigger MPTP opening on the number of GNX-4975-binding sites. When MPTP opening was triggered with 150 μM [Ca²⁺], initial rates of swelling were 200 ± 7.9% of the rates at 50 μM [Ca²⁺] and the corresponding number of GNX-4975-binding sites increased from 13.2 ± 2.4 to 20.9 ± 1.6 pmol/mg (means ± S.E.M. for five separate mitochondrial preparations; P < 0.05) without any significant change in Kᵢ (1.91 ± 0.35 and 2.15 ± 0.26 nM respectively). These data imply that [Ca²⁺] may trigger MPTP opening by inducing the open conformation to which GNX-4975 binds.

When swelling was employed to measure the extent of MPTP opening, the assay is initiated by addition of Ca²⁺. This places limitations on studying the interaction between [Ca²⁺] and GNX-4975 binding since the inhibitor must always be added before or at the same time as Ca²⁺. This limitation can be overcome by using a different assay of MPTP opening in which mitochondria are pre-swollen with Ca²⁺ and then caused to shrink by addition of PEG 2000 that is too large to permeate the MPTP. The PEG exerts an osmotic pressure on the IMM that shrinks the mitochondria at a rate (measured as an increase in Δψᵢ) proportional to the extent of MPTP opening [40,41]. In this assay, the MPTP is opened by addition of Ca²⁺ prior to addition of PEG 2000 which allows GNX-4975 to be added either before or after Ca²⁺ prior to determination of the extent of MPTP opening. We first demonstrated that the shrinkage assay could be used to determine the number and Kᵢ of GNX-4975-binding sites on the active MPTP. Data are presented in Figure 5A where the concentration-dependence of MPTP inhibition by GNX-4975 at two protein concentrations is determined using this assay. The data were fitted in exactly the same manner as the swelling data of Figure 1 and gave values of the number of binding sites for GNX-4975 of 46.9 ± 5.3 pmol/mg with a Kᵢ of 2.08 ± 0.30 nmol (mean ± S.E.M. for three separate experiments). The number of GNX-4975-binding sites is substantially higher than that observed in the swelling data of Figure 1 (12.6 ± 1.7 pmol/mg), and this probably reflects the formation of additional active MPTP complexes during the pre-swelling of mitochondria induced by addition of 1 mM [Ca²⁺] in KSCN buffer which is accompanied by the loss of intramitochondrial ATP and ADP, both of which inhibit MPTP opening [12,13]. Importantly, however, the Kᵢ value for GNX-4975 was very similar to that obtained in the swelling assay (1.95 ± 0.17 nM).

We also used the shrinkage assay to compare the concentration-dependence of GNX-4975 inhibition of MPTP opening in binding in KCl and KSCN buffers to see whether the activation by KSCN
and increase in GNX-4975-binding sites seen in the swelling assay (Figure 2A) was also observed in the shrinkage assay. In all cases, the initial 20 min of pre-swelling was carried out in KSCN buffer and the mitochondria were maintained in KSCN buffer after their isolation by centrifugation. However, the mitochondria were then resuspended in the appropriate buffer (KSCN or KCl) for the shrinkage assay. Data are shown in Figure 5B and, in agreement with the swelling data (Figure 2A), the number of GNX-4975-binding sites (pmol/mg; mean ± S.E.M. for three separate preparations) increased from 29.6 ± 2.3 in the KCl buffer to 49.0 ± 2.8 in KSCN buffer (P < 0.01) without a significant change in K_i (0.81 ± 0.34 and 1.13 ± 0.37 nM respectively).

Having validated the shrinkage assay for measurement of GNX-4975 binding to the MPTP, we employed it to see whether the order of addition of Ca^{2+} and GNX-4975 affected the potency of the inhibitor. The data of Figure 6A show that when 0.1 μM GNX-4975 was added to pre-swollen mitochondria after 50 μM Ca^{2+}, it was much less potent at inhibiting MPTP opening (3.8 ± 0.37 nM) for 2 mg of protein respectively. For comparison, data from Figure 5B (closed triangles) have been included to illustrate that GNX-4975 is much more effective when added before Ca^{2+}.
Table 1 Collated $E_i$ and $K_i$ values for GNX-4975 binding

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Figure</th>
<th>n</th>
<th>$K_i$ (nM)</th>
<th>$E_i$ (pmol/mg)</th>
<th>$E_i$ (pmol/mg) at fixed $K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Swelling assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>KCl, 150 μM Ca$^{2+}$</td>
<td>2A</td>
<td>5</td>
<td>1.68 ± 0.13</td>
<td>9.43 ± 1.4</td>
<td>9.10 ± 1.5</td>
</tr>
<tr>
<td>KSCN, 50 μM Ca$^{2+}$, 5 μM BKA</td>
<td>3A</td>
<td>6</td>
<td>1.46 ± 0.16</td>
<td>9.43 ± 0.47</td>
<td>8.56 ± 1.7</td>
</tr>
<tr>
<td>KSCN, 50 μM Ca$^{2+}$, 20 μM ADP</td>
<td>3B</td>
<td>6</td>
<td>1.45 ± 0.15</td>
<td>11.0 ± 0.07</td>
<td>11.0 ± 1.1</td>
</tr>
<tr>
<td>KSCN, 50 μM Ca$^{2+}$</td>
<td>2A, 2B, 3A, 3B, 4</td>
<td>23</td>
<td>1.71 ± 0.13</td>
<td>13.1 ± 0.60</td>
<td>12.9 ± 0.59</td>
</tr>
<tr>
<td>KSCN, 50 μM Ca$^{2+}$, 20 μM PAO</td>
<td>2B</td>
<td>6</td>
<td>2.56 ± 0.14</td>
<td>17.2 ± 1.3</td>
<td>19.5 ± 1.6</td>
</tr>
<tr>
<td>KSCN, 150 μM Ca$^{2+}$</td>
<td>2A</td>
<td>10</td>
<td>2.22 ± 0.15</td>
<td>20.1 ± 1.3</td>
<td>21.4 ± 1.4</td>
</tr>
<tr>
<td><strong>Shrinking assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl, 50 μM Ca$^{2+}$</td>
<td>5B</td>
<td>3</td>
<td>0.81 ± 0.34</td>
<td>29.6 ± 2.3</td>
<td>22.1 ± 3.8</td>
</tr>
<tr>
<td>KSCN, 50 μM Ca$^{2+}$</td>
<td>5B</td>
<td>3</td>
<td>1.13 ± 0.37</td>
<td>49.0 ± 2.8</td>
<td>45.7 ± 5.2</td>
</tr>
</tbody>
</table>

GNX-4975 binds only to the active conformation of the MPTP

In Table 1, we present data for the calculated number of GNX-4975-binding sites and the associated $K_i$ values under all the conditions used in the present study. Reassuringly, differences in the calculated $K_i$ values are small. We confirmed that these differences in the calculated $K_i$ values could not account for any differences in calculated $E_i$ values by re-analysing the data for each experimental condition using a fixed value of $K_i$ (1.8 nM – the mean of the values obtained in the swelling experiments). These data are also included in Table 1. In Figure 7, we plot the calculated mean number of GNX-4975-binding sites determined under each condition with the mean absolute rate of swelling determined under the same conditions and demonstrate a very significant linear correlation (two-tailed Pearson’s correlation coefficient ($r$) of 0.743; $P < 0.001$). This strongly supports the hypothesis that GNX-4975 binds to the open pore complex and that a variety of agents that enhance or reduce MPTP opening do so by increasing or decreasing this active conformation of the MPTP. By contrast, titration of MPTP opening with CsA always produces the same number of binding sites irrespective of the degree of activation because it inhibits by interacting with CyP-D in its native conformation, preventing its binding to the membrane component(s) of the MPTP [28,29,41]. Since the number of GNX-4975-binding sites varies according to the activation state of the MPTP, it seems probable that this reflects a small fraction of a membrane protein (or proteins) undergoing a conformation change to form the pore.

GNX-4975 stabilizes an interaction between the ANT and the PiC in heart IMM membranes

We have suggested previously that both the ANT and PiC may be implicated in MPTP formation, with the pore forming at the interface of these two proteins in a novel conformation [1]. If this were the case, then GNX-4975 might bind at this interface and stabilize the interaction between the two proteins. In order to investigate this possibility, we made use of the observation that, for solubilized beef heart IMMs, both proteins bind to immobilized PAO. Binding of both proteins is prevented by the ubiquinone (UQ) analogues, UQ0 and Ro 68-3400, that inhibit MPTP opening, whereas binding of the ANT is specifically prevented by CAT that traps the ANT in its ‘c’ conformation [10,12,42]. Since CAT is associated with enhanced MPTP opening, it would be predicted that some ANT might remain bound to the PAO column in the presence of GNX-4975 to stabilize its interaction with the PiC. The data of Figure 8 confirm this to be the case for solubilized IMMs from both pig and beef heart mitochondria. As expected, treatment of heart mitochondria with 10 μM CAT prior to IMM isolation and solubilization totally prevented ANT binding to the PAO column. However, when either beef or pig heart mitochondria were treated with 10 μM CAT followed by 1 μM GNX-4975 prior to IMM isolation and solubilization, some ANT remained bound to the PAO column, consistent with the inhibitor stabilizing an interaction between the ‘c’ conformation of the ANT and the PiC or another IMM protein bound to the column. Underneath each blot, we give the integrated optical density for
Table 2  Orbitrap MS analysis of proteins specifically eluted from immobilized PAO

<table>
<thead>
<tr>
<th>Molecular mass (kDa)</th>
<th>Description</th>
<th>Protein score</th>
<th>Percentage of total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most abundant proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.9</td>
<td>ADP/ATP translocase 1</td>
<td>34.78</td>
<td>11.77</td>
</tr>
<tr>
<td>35.0</td>
<td>Phosphate carrier protein</td>
<td>11.97</td>
<td>4.05</td>
</tr>
<tr>
<td>111.3</td>
<td>2-Oxoglutarate dehydrogenase</td>
<td>8.11</td>
<td>2.75</td>
</tr>
<tr>
<td>9.3</td>
<td>NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 4</td>
<td>7.34</td>
<td>2.48</td>
</tr>
<tr>
<td>47.9</td>
<td>Keratin, type I cytoskeletal 18</td>
<td>7.31</td>
<td>2.47</td>
</tr>
<tr>
<td>36.7</td>
<td>NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 10</td>
<td>7.12</td>
<td>2.41</td>
</tr>
<tr>
<td>36.7</td>
<td>NDUFA10 protein (fragment)</td>
<td>5.49</td>
<td>1.86</td>
</tr>
<tr>
<td>33.2</td>
<td>Δ-β-Hydroxybutyrate dehydrogenase</td>
<td>5.14</td>
<td>1.74</td>
</tr>
<tr>
<td>55.2</td>
<td>ATP synthase subunit α</td>
<td>4.73</td>
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<td>51.6</td>
<td>ATP synthase subunit β</td>
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<td>1.59</td>
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<tr>
<td>30.3</td>
<td>ATP synthase subunit γ</td>
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<td>1.59</td>
</tr>
<tr>
<td>24.7</td>
<td>ATP synthase subunit b</td>
<td>4.70</td>
<td>1.59</td>
</tr>
<tr>
<td>20.9</td>
<td>ATP synthase subunit OSCP (oligomycin-sensitivity-conferring protein)</td>
<td>4.20</td>
<td>1.42</td>
</tr>
<tr>
<td>18.7</td>
<td>ATP synthase subunit d</td>
<td>4.16</td>
<td>1.41</td>
</tr>
<tr>
<td>15.1</td>
<td>ATP synthase subunit δ</td>
<td>4.16</td>
<td>1.41</td>
</tr>
</tbody>
</table>

ATP synthase subunits

Table 2 Orbitrap MS analysis of proteins specifically eluted from immobilized PAO. Binding of solubilized bovine heart mitochondria to immobilized PAO was carried out as described in the Experimental section. Specifically bound proteins were eluted using DTT, run 1 cm into a 12% acrylamide gel and analysed by Orbitrap MS as outlined in the Experimental section. The protein area column uses the three peak method of quantification to determine the relative amount of each protein in the sample. To calculate each individual protein contribution towards the total eluted protein as a percentage, each protein area score was standardized to the sum of all area scores for all of the protein hits eluted (295-43). Data for all 664 proteins detected are available online in a Supplementary Excel file.

Figure 8  The effects of GNX-4975 on the binding to immobilized PAO of the ANT and PIC from control and CAT-treated heart mitochondria

Binding of solubilized beef and pig heart IMM proteins to immobilized PAO was carried out as described in the Experimental section. Identical amounts of solubilized IMM proteins were added to the immobilized PAO in each case and specifically bound proteins were eluted using DTT and analysed by Western blotting using ANT- and PIC-specific antibodies as indicated. Only the section of the blot of ~30 kDa is shown since no other significant bands were detected. Two separate experiments are shown for both beef and pig mitochondria and the values beneath each blot represent the integrated optical density for each band visualized on film and scanned. This was determined as described in the Experimental section and expressed as a percentage of the value for the untreated sample. The bottom blots represent samples from Experiment 1 re-analysed using an ODDESEY Fc (LI-COR) to directly detect the ECL signal. Lanes are: (1) untreated, (2) 1 μM GNX-4975 treated, (3) 10 μM CAT treated, (4) treated with 10 μM CAT followed by 1 μM GNX-4975.

As a percentage of the total ANT bound in the absence of CAT is 4.51 ± 0.33% (mean ± S.E.M., n = 4 comprising the two beef and two pig experiments shown). This value is likely to be an overestimate because the amount of ANT remaining after CAT in the presence of GNX 4975 is very small and thus requires a long film exposure to visualize the band. This causes the intensity of the ANT band in the absence of CAT to exceed the linear response of the film. Indeed, in the bottom panel, we have re-analysed the blots for one set of pig and one set of beef mitochondria using an ODDESEY Fc (LI-COR) to directly detect the ECL signal. These data suggest that the overestimate is by a factor of ~1.65 in both the pig and beef samples. From these data, we can estimate the amount of ANT whose binding is enhanced by GNX-4975 to be ~2.7% of the total.

In order to establish whether other proteins that have been proposed to be part of the MPTP, such as subunits of the F1F0-ATP synthase, might act as a binding partner for the ANT, we performed a proteomic analysis using Orbitrap MS to identify all IMM proteins from beef heart mitochondria that bound to the PAO column. Full data are provided as a Supplementary Excel file but Table 2 presents the 12 most abundant proteins (each >1% of total protein bound) and, in addition, those components of the F1F0-ATP synthase that were detected (α, β, γ, b, oligomycin-sensitivity-conferring protein (OSCP), d and δ subunits). As reported previously, the dominant proteins were the ANT (11.8%) and PIC (4.1%), whereas the F1F0-ATP synthase subunits bound at levels similar to a range of other IMM proteins (<0.3%). This makes them less attractive candidates for the MPTP than the PIC and ANT and very unlikely targets for the site of PAO activation on the pore.

DISCUSSION

The exact molecular composition of the membrane component of the MPTP remains uncertain with evidence presented for a regulatory or structural involvement of the ANT, PIC, different...
GNX-4975 binds only to the active MPTP at the Ca\(^{2+}\) activation site

Our data demonstrate that GNX-4975 is a potent MPTP inhibitor \((K_i \sim 1.8\) nM\) that binds only to the active (open) form of the pore. The data, summarized in Table 1 and Figure 7, show that activators of the MPTP such as PAO, KSCN and higher \([Ca^{2+}\)] increase the number of inhibitor-binding sites, whereas the BKA and ADP that reduce MPTP opening decrease the number of GNX-4975-binding sites. None of these effectors caused a major change in the \(K_i\) of GNX-4975 for inhibition of MPTP opening provided the inhibitor was added before \(Ca^{2+}\) as in the swelling assay for MPTP opening. However, using the shrinkage assay, it was shown that if \(Ca^{2+}\) was added prior to GNX-4975 the \(K_i\) was greatly elevated (Figure 6). The simplest explanation for these data would be that \([Ca^{2+}\)] and GNX-4975 compete for the same binding site on the active conformation of the MPTP, but that once GNX-4975 is bound its dissociation is so slow that \(Ca^{2+}\) cannot displace it within the timeframe of the experiment. Such behaviour is well documented for tight binding inhibitors \([47]\), and a low off rate constant \((k_{off})\) has also been observed for CsA binding \([41,48]\). The \(k_{off}\) could be determined from the \(K_i\) if the rate constant for inhibitor binding \((k_a)\) was known, since \(K_i = k_{off}/k_a\). Values of \(k_a\) for tight inhibitor binding to soluble enzymes have been shown to range between 10\(^{4}\) and 10\(^{6}\) mol\(-1\) s\(^{-1}\) and can be substantially lower where inhibitor binding produces a conformation change \([47]\). For a \(k_a\) of 10\(^{3}\) mol\(-1\) s\(^{-1}\) the calculated \(k_{off}\) is 2 x 10\(^{-4}\) s\(^{-1}\) implying that it would take nearly 1 h for 50\% dissociation of the inhibitor and thus that \(Ca^{2+}\) would not displace the GNX-4975 during the time of the assay. This would explain the low \(K_i\) values observed when the inhibitor was added prior to \(Ca^{2+}\), but considerably higher values when the order of addition was reversed.

The open MPTP is formed when a small fraction of an IMM protein adopts a novel conformation

Although CsA and GNX-4975 exhibit similar \(K_i\) values for inhibition of the MPTP, they act quite differently. CsA binds to CyP-D and prevents its association with a membrane component of the MPTP that facilitates the calcium-activated conformational change required for pore opening \([1,4,6,25]\). As such the number of CsA-binding sites determined from inhibitor titrations of MPTP opening always matches the amount of CyP present in the mitochondrial matrix \([28,29]\). Conversely, for GNX-4975 the number of binding sites varies depending on the activity of the MPTP. This can be explained if formation of the active MPTP requires a fraction of a native IMM protein (or proteins) to adopt a novel conformation to which GNX-4975 binds. The concentration of GNX-4975-binding sites determined in liver mitochondria varied between 9 and 20 pmol/mg of protein depending on conditions in normal de-energized liver mitochondria, and increased up to \(\sim 50\) pmol/mg of protein in pre-swollen mitochondria (Table 1). Thus the IMM component(s) that form the MPTP must be present in concentrations significantly higher than this which is true of the proposed candidate proteins, the PiC, ANT and subunits of the F\(_{1}\)F\(_{0}\)-ATP synthase as noted above.

GNX-4975 stabilizes an interaction between the PiC and the ANT in its ‘c’ conformation

We have previously proposed that the MPTP may be formed at the interface between the ANT and the PiC when one or both of these proteins is in a distinct conformation such as the ‘c’ conformation of the ANT and the PiC modified by oxidative stress or PAO \([1]\). This would be entirely consistent with the GNX-4975-binding studies discussed above and received further support from the effects of CAT and GNX-4975 on the binding of IMM proteins to immobilized PAO shown in Figure 8. We have previously shown that PAO activates the MPTP by binding to vicinal thiol groups on both the ANT and PiC \([10,12,37]\). If the MPTP is formed when such an interaction occurs between the PAO-bound PiC and the ANT in the ‘c’ conformation, then a small fraction of the ANT might remain attached to the PiC on the column if this interaction survived the solubilization process. This was observed in the presence of GNX-4975 but not in its absence (Figure 8 and Table 2), suggesting that inhibitor binding may stabilize the interaction between the two proteins. The relatively small amount (2.7\%) of the ANT bound compared with the total ANT bound in the absence of CAT is to be expected since the GNX-4975-binding data suggest only \(\sim 10\)–20 pmol/mg of protein of the active MPTP complex compared with a concentration of the ANT of 300 and 1200 nmol/mg in rat liver and bovine heart mitochondria respectively \([43]\). Other proteins that have been proposed to be important in MPTP formation, such as subunits of the F\(_{1}\)F\(_{0}\)-ATP synthase \([4,7]\) and the mitochondrial AAA protease SPG7 \([26]\), were either not detected in the protein fraction bound to the PAO or were present at extremely low levels (<0.3\%) compared with the ANT and PiC (11.8 and 4.1\% respectively) as shown in Table 2. Although this does not rule out their role in MPTP formation, it does suggest that these proteins are unlikely to contain the vicinal thiol groups whose binding of PAO activates the MPTP.

Insights into the molecular mechanism of the MPTP

The data we have obtained with GNX-4975 are entirely consistent with previous data from our laboratory, reviewed in \([1]\), that led us to propose that such conformational changes in both the ANT and PiC may induce an interface between these two membrane proteins, perhaps involving their tightly bound annular cardiolipin and co-ordinated by the F\(_{1}\)F\(_{0}\)-ATP synthase in the proposed ATP synthasome complex. The ANT provides the binding site for regulation by adenine nucleotide, ligands of the ANT and oxidative stress as has been demonstrated by the loss of such regulation in mitochondria lacking ANT1 and ANT2 \([17]\). Furthermore, the reconstituted ANT can form \(Ca^{2+}\)-activated channels that are regulated by CyP-D \([16,49]\). Involvement of the PiC can account for the activation of the MPTP by P\(_{i}\) and its inhibition by low concentrations of \(N\)-ethylmaleimide, as well as providing an additional site of action of oxidative stress and PAO \([10,14]\). We have further proposed that \(Ca^{2+}\) may act through its binding to annular cardiolipin at the interface between the...
In their cytosol-facing (‘c’) conformation, such as when stabilized by CAT or oxidative stress, IMM candidate proteins for the MPTP such as the ANT and PIC can undergo a further conformation change that is enhanced by KSCN and Pi, and to which CyP-D can bind. This produces an interface between the two proteins, perhaps involving cardiolipin that represents the MPTP in its closed state. Binding of Ca\(^{2+}\) on the matrix face triggers a further conformation change, facilitated by CyP-D (thick arrows cf. thin arrows), to open the MPTP. GNX-4975 binds to the closed MPTP, stabilizing this conformation and so preventing Ca\(^{2+}\) from triggering pore opening which is reversible. The equilibrium between open and closed states is determined by the \([Ca^{2+}]\) whose action is competitively inhibited by GNX-4975 binding. However, the rate of dissociation of GNX-4975, once bound, is very low and thus not readily overcome by increasing \([Ca^{2+}]\).

PiC and the ANT [1]. Indeed, cardiolipin is one of the few calcium-binding molecules that can distinguish between Sr\(^{2+}\) and Ca\(^{2+}\) [50] which may explain why the MPTP is activated by Ca\(^{2+}\), but not Sr\(^{2+}\) [40]. Our present data suggest that GNX-4975 binds to the active form of the MPTP in competition with Ca\(^{2+}\) at this interface. This is illustrated schematically in Figure 9.

Despite this evidence in favour of our model, it cannot be ignored that knockdown of either the ANT or the PIC only attenuates MPTP activity and modulates its regulatory properties. These data demonstrate that neither the PIC nor the ANT is indispensable for MPTP activity, implying that other proteins can form the MPTP. However, the PIC and ANT are members of the 53-strong mitochondrial carrier family (MCF) which share a common structure in which six transmembrane helices form the substrate translocation pathway that involves conformational changes between inward- and outward-facing conformations [51]. Thus it is possible that any member of the MCF can enter a conformation in which they form an interface with another MCF member to form the MPTP. Annular cardiolipin is likely to surround all MCF members and thus could provide a common Ca\(^{2+}\)-binding site whatever MCF members form the pore. Nevertheless, because the ANT and PIC are by far the most prevalent MCF members and may already be in close association through the ATP synthasome, they will usually form the MPTP and account for its regulation by adenine nucleotide, ANT ligands, PAO, oxidative stress and Pi, [1].

**AUTHOR CONTRIBUTION**

Andrew Richardson performed the experiments, analysed the data and generated the Figures while Andrew Halestrap directed the research and wrote the paper with the assistance of Andrew Richardson.

**ACKNOWLEDGEMENTS**

We thank Dr Kate Heesom of the University of Bristol Proteomics Facility for assistance with analysis of the proteomics data and Congenia S.r.l. (Via Privata Giovannino De Grassi, 11 20123, Milan, Italy) for providing us with GNX-4975.

**FUNDING**

This work was supported by a British Heart Foundation Ph.D. studentship [grant number FS/11/32/28814].

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Characterization of GNX-4975 inhibition of the MPTP

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