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Identification of adenine nucleotide translocase 4 inhibitors by molecular docking

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ABSTRACT

The protein adenine nucleotide translocase (ANT) is localized in the mitochondrial inner membrane and plays an essential role in transporting ADP into the mitochondrial matrix and ATP out from the matrix for cell utilization. In mammals there are four paralogous ANT genes, of which ANT4 is exclusively expressed in meiotic germ cells. Since ANT4 has been shown essential for spermatogenesis and male fertility in mice, inhibition of ANT4 appears to be a reasonable target for male contraceptive development. Further, in contrast to ANT1, ANT2 and ANT3 that are highly homologous to each other, ANT4 has a distinguishable amino acid sequence, which serves as a basis to develop a selective ANT4 inhibitor. In this study, we aimed to identify candidate compounds that can selectively inhibit ANT4 activity over the other ANTs. We used a structure-based method in which ANT4 was modeled then utilized as the basis for selection of compounds that interact with sites unique to ANT4. A large chemical library (>100,000 small molecules) was screened by molecular docking and effects of these compounds on ADP/ATP exchange through ANT4 were examined using yeast mitochondria expressing human ANT4. Through this, we identified one particular candidate compound, [2,2′-methanediylbis(4-nitrophenol)], which inhibits ANT4 activity with a lower IC_{50} than the other ANTs (5.8 μM, 4.1 μM, 5.1 μM and 1.4 μM for ANT1, 2, 3 and 4, respectively). This newly identified active lead compound and its chemical structure are expected to provide new opportunities to optimize selective ANT4 inhibitors for contraceptive purposes.

1. Introduction

The world's population now exceeds 7 billion, and it is estimated that half of all human conceptions are unplanned and half of the resulting pregnancies are unwanted [13,16]. Therefore, there is a demand for increased access to existing contraceptives, improved contraceptive education, and additional contraceptive options. Since choices and preferences of contraceptive methods vary widely across different cultures, religions, and socio-economical status, it is particularly important to develop additional contraceptive options. Men currently account for a third of all contraceptive use; however, male-directed contraceptive options are extremely limited to only condoms or vasectomy [25,27]. Hormonal contraceptives for men are under development, but overall efficiency is not yet as reliable as hormonal contraceptives for women [1]. An alternative chemical male contraceptive with safety, efficiency and better cost-performance is needed.

Adenine nucleotide translocase (ANT), also called ADP/ATP carrier (AAC), is one of the solute carrier transporters expressed on the inner mitochondrial membrane [18]. ANT exclusively transports adenine nucleotides, such as ADP and ATP, by an antiport mechanism [2,18]. Under aerobic conditions, an outgoing mitochondrial matrix ATP^{4-} passes through ANT in exchange for an incoming cytoplasmic ADP^{3-}. Thus, ANT plays an essential role in aerobic energy metabolism in eukaryotic organisms. To date, all eukaryotes investigated have multiple paralogous genes encoding ANT. The ANT proteins are differentially expressed depending on extracellular oxygen concentrations, cellular growth rates, or in a tissue-dependent manner [14].

Most mammalian species including humans have four independent ANT family genes encoding ANT1, ANT2, ANT3 and ANT4 [18,20]. ANT1 is predominantly expressed in skeletal muscle and heart and is thought to be optimized for large and rapid ATP requirement in muscle metabolism [10]. ANT2 and ANT3 are essentially ubiquitously expressed throughout somatic tissues. ANT2 is inducible upon cell proliferation, whereas ANT3 is expressed in a constitutive manner [28]. ANT4 is the fourth and most recently identified member of the mammalian ANT genes [7,17,26], and its expression is exclusively found in testis among all adult organs both in humans and mice [17,26]. Although one original paper indicated

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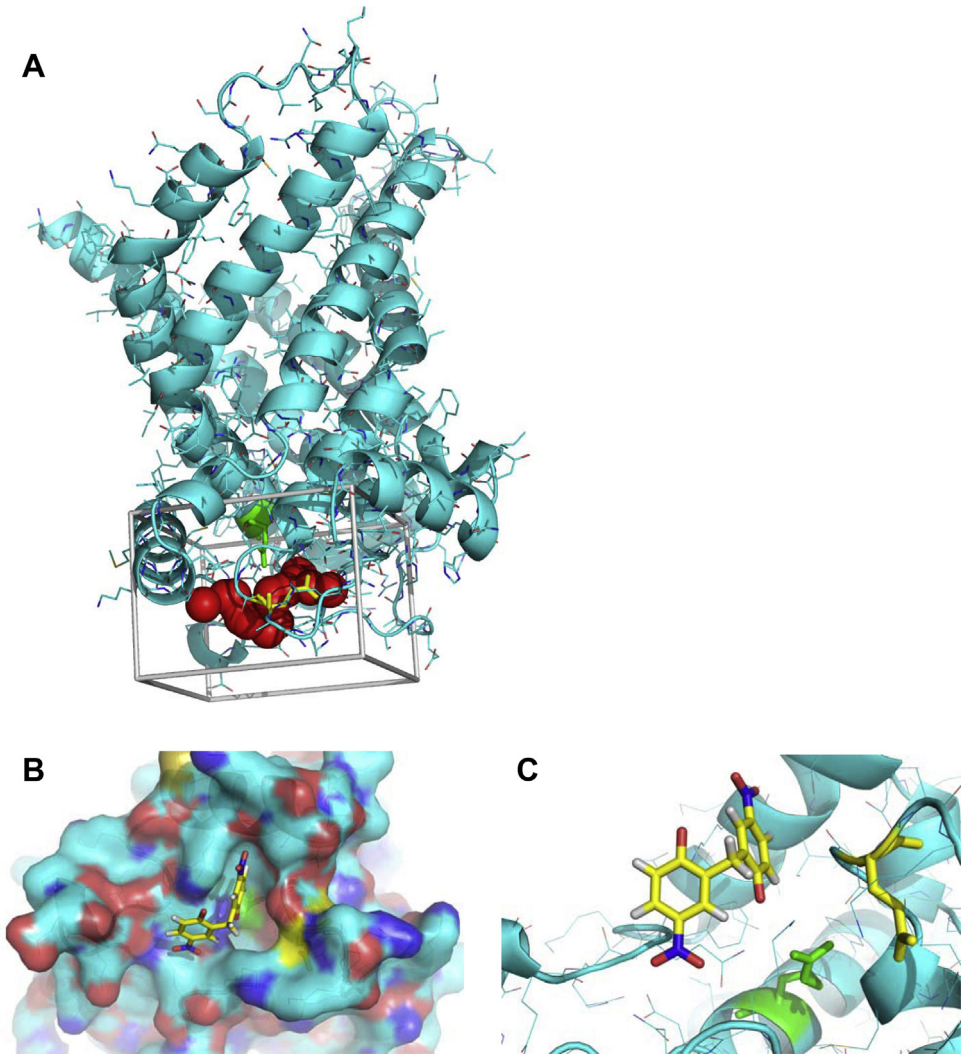

Fig. 1. Identification of 2,2′-methanediylbis(4-nitrophenol) (MDBNP) as a putative ANT4 inhibitor by molecular docking. (A) A ribbon diagram showing a homology model of human ANT4 is shown in cyan. The molecule is viewed from a horizontal side of the mitochondrial inner membrane. The top is oriented toward the inter-membrane space while the bottom is oriented toward the mitochondrial matrix. Arg152, an ANT4 specific position, is shown as green sticks. Glu256 is shown as yellow sticks. The red spheres represent the site selected for molecular docking. The gray box represents the boundary of the scoring grid used for molecular docking. Color codes for atoms are: carbon (cyan), oxygen (red), nitrogen (blue) and sulfur (amber). The molecule is viewed from a horizontal side of the mitochondrial inner membrane. The top is oriented toward the inter-membrane space while the bottom is oriented toward the mitochondrial matrix. The selected cleft used for molecular docking locates to the matrix side (shown with a predicted chemical binding). (B and C) The structural pocket selected for molecular docking is shown oriented with the mitochondrial matrix face toward the reader (rotated 90° about a horizontal axis in the plane of the page compared to panel A). The molecular surface of ANT4 is colored: carbon (cyan), oxygen (red), nitrogen (blue) and sulfur (amber). The molecular surface of Arg152 is shown in green and Glu256 is highlighted in yellow. MDBNP is depicted as sticks (yellow for carbon, red for oxygen, blue for nitrogen, and white for hydrogen). The compound is predicted to bind 4.0 Å from Arg152 (closest contact) and 5.1 Å from Glu256. The molecular surface of ANT4 is omitted in panel C for clarity.

a minor expression of ANT4 in human brain and liver as well [7], we did not see traces of ANT4 mRNA expression in any human adult somatic organs (unpublished observations) as shown by Kim et al. [17]. Upon closer examination, ANT4 is particularly expressed in meiotic germ cells [4] and essential for spermatogenesis and subsequent male fertility in mice [3,4]. Taking the selective expression pattern of ANT4 into account, ANT4 may be a good target for developing male contraceptives.

ANT4 has a unique gene structure and primary amino acid sequence compared to other somatic ANTs. ANT1, ANT2 and ANT3 have an identical exon/intron structure and are highly homologous to each other (approximately 90% amino acid identity by a whole peptide sequence alignment) [4,20]. In contrast, the exon/intron configuration of ANT4 is different from that of ANT1, ANT2 and ANT3, perhaps reflecting an alternate evolutionary origin [4,20]. ANT4 has a lower sequence similarity to other ANTs (approximately 70% amino acid identity by a whole peptide sequence alignment) and has a unique amino acid sequence including extended N-terminal and C-terminal regions when compared to other ANTs [17,20]. The differential structure provides a basis to identify a selective inhibitor of ANT4. Natural small compound inhibitors of ANTs exist. These include carboxyatractyloside and bongkrekic acid, which were originally isolated from a poisonous plant and a bacterium, respectively [8,18,29]. Both compounds specifically and potently inhibit ADP/ATP exchange of ANT. However, these compounds inhibit all known ANT members equally and are toxic as they obstruct cellular respiration and energy transfer in many cell types.

In order to develop selective inhibitors for ANT4, we utilized a molecular docking approach. A target binding pocket was selected by two criteria. The first criterion was that the small molecule must be positioned in close proximity to amino acids essential for the ANT activity. The second criterion was that the binding cleft contains a unique amino acid which exists only in ANT4 but not in

ANT1, ANT2 or ANT3. Candidate compounds were identified by *in silico* screening for the most favorable intermolecular interaction with this unique ANT4 binding pocket, and then subjected to a biological screening for inhibitory activity on ADP/ATP exchange of human ANT4 expressed in yeast mitochondria. Further, the specificity in ANT4 inhibition was tested by comparing the effect on other human ANTs.

2. Materials and methods

2.1. Molecular docking

We utilized SWISS-MODEL, an Automated Comparative Protein Modeling Server, to generate an atomic model of human ANT4 based on the most similar solved structure, bovine Ant1/Adt1 (PDB 1okc) [24]. We utilized DOCK6.1 (UCSF) to carry out molecular docking simulations [19,22]. We used ZINC [15] to download the coordinates for 139,735 compounds representing the NCI plated 2007 molecules set, pH 6–8.The small molecules were docked into a structural feature of the modeled human ANT4 protein selected by the program SPHGEN_CPP, a modified form of the DOCK suite program Sphgen that permits input of large proteins (http://dock.compbio.ucsf.edu/Contributed_Code/sphgen_cpp.htm). We selected a structural pocket for molecular docking that contains an isoform specific amino acid at position 152 (Arg for ANT4). Spheres within 8 Å were selected for molecular docking. The scoring grid was set to extend 5 Å beyond the selected spheres. Each small molecule was positioned in the selected site in 1000 orientations. DOCK6.1 was set to score two types of interactions: van der Waals contacts (non-polar interactions) and electrostatic interactions (polar interactions). These grid-based scores were summed (for each compound) to generate overall energy scores. The scores were used to rank the selected compounds for functional testing. Distances were measured using Coot [6].

2.2. Chemical compounds

The compounds identified through molecular docking were acquired from the NCI/DTP. The compounds were dissolved and diluted in DMSO for use in the ADP/ATP exchange assays. Atractyloside (ATR) was obtained by Sigma and dissolved in distilled water.

2.3. Yeast strains and media

Endogenous ANT (AAC) genes of *Saccharomyces cerevisiae* were replaced with human ANT1, ANT2, ANT3 or ANT4 as we described previously [11]. For ANT4, an additional mutation (A30V) was required for successful expression in yeast mitochondria as described. The strains used in this study were: yNhANT1 (*MATα ura3–52 leu2–3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::6xHis-yNhANT1 aac3-Δ1::hisG [r+, TRP1]*), yNhANT2 (*MATα ura3–52 leu2–3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::6xHis-yNhANT2 aac3-Δ1::hisG [r+, TRP1]*), yNhANT3 (*MATα ura3–52 leu2–3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::6xHis-yNhANT3 aac3-Δ1::hisG [r+, TRP1]*), and yNhANT4 A30V (*MATα ura3–52 leu2–3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::6xHis-yNhANT4(A30V) aac3-Δ1::hisG [r+, TRP1]*).

Complete glucose medium (YPD) contained 2% glucose, 2% bacto peptone, 1% yeast extract, 40 mg/L adenine, and 40 mg/L tryptophan. Complete ethanol–glycerol medium (YPEG) contained 3% glycerol, 3% ethanol, 2% bacto peptone, 1% yeast extract, 40 mg/L adenine, and 40 mg/L tryptophan.

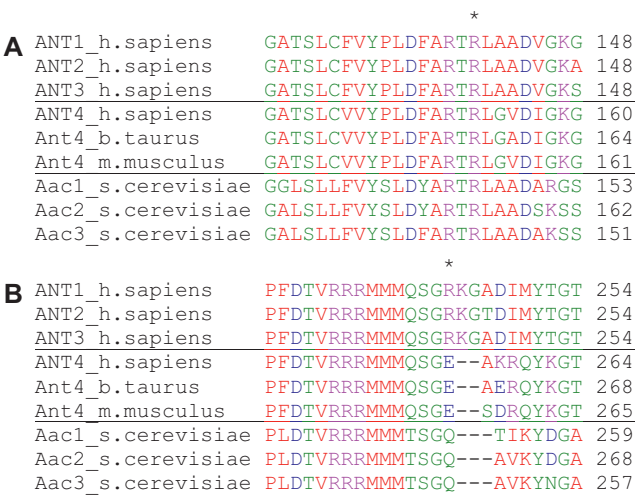

Fig. 2. Amino acid alignment of ANTs around Arg152 (A) and Glu256 (B) of human ANT4 peptide. The amino acid sequences of human (*Homo sapiens*) ANT1, 2, 3 and 4, cow (*Bos taurus*), mouse (*Mus musculus*) Ant4 and yeast (*Saccharomyces cerevisiae*) Aac1, 2 and 3 are aligned. Asterisks indicate R152 (A) and E256 (B) in human ANT4 and corresponding amino acids in other ANTs.

2.4. Mitochondria isolation

Mitochondria were isolated from yeast spheroplasts as described previously [11]. Briefly, yeast mitochondria were energized by cultivating yeast strains in a non-fermentable carbon source prior to mitochondria isolation. Yeast strains were treated with Zymolyase (Seikagaku America) to generate spheroplasts, and then sheared with a Dounce homogenizer. Mitochondria were collected by differential centrifugation.

2.5. ADP/ATP exchange assay

ADP/ATP exchange assay was performed by our standardized assay [11]. Briefly, freshly isolated mitochondria were added to reaction buffer (0.6 M mannitol, 0.1 mM EGTA, 2 mM $MgCl_2$, 10 mM KPi, 5 mM α-ketoglutarate, 0.01 mM Ap5A, 10 mM Tris-HCl, pH 7.4) containing the ATP detection system (2.5 mM glucose, hexokinase (2 E.U.), glucose-6-phosphate dehydrogenase (2 E.U.), 0.2 mM NADP). The exchange reaction was initiated by adding 2 mM ADP (14 μM as free ADP). The ATP efflux rate was monitored as the rate of NADPH formation (increase in absorbance at 340 nm). The initial linear part of the kinetic curve (initial 3.5 min) was used as the initial velocity. The reactions volume was 200 μl, and reactions were carried out in 96 well microtiter plates and read by Synergy HT plate reader (BioTek). The IC_{50} value for each inhibitor was obtained by 4-parameter logistic fitting using Gen5 Data analysis software (BioTek).

3. Results

3.1. Molecular docking strategy

In order to identify selective ANT4 inhibitors, we utilized a molecular docking approach to locate sites that differ among ANT isoforms. The human ANT4 structure was modeled on the most similar structure in the protein data bank, bovine Ant1/Adt1 (1okc) [24], which has 71% amino acid identity to human ANT4. The predicted structure of human ANT4 is shown in Fig. 1A. Binding pockets were selected by two criteria. The first criterion was that the small molecule must be positioned in close proximity (less than 4.5 Å) to amino acids essential for the ANT activity. In mutagenesis studies

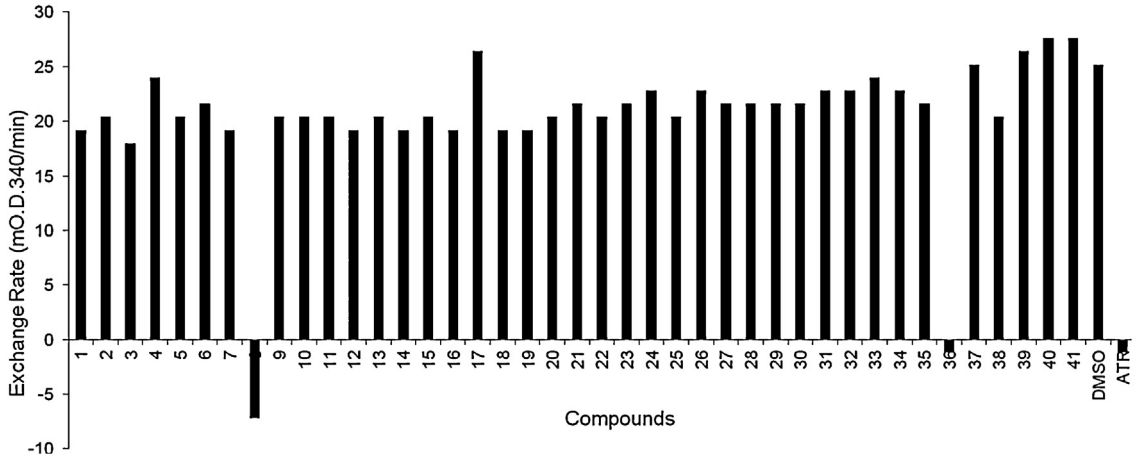

Fig. 3. The effects of candidate compounds on ANT4 inhibition. The top 41 high scoring compounds by molecular docking were obtained through NCI/DTP. The candidate compound (#1 through #41, 100 μM each), a vehicle control (DMSO, 0.1%) or atractyloside (ATR, 10 μM) was tested for their inhibitory effect on ADP/ATP exchange through human ANT4 expressed in yeast mitochondria. 2,2′-Methanediylbis(4-nitrophenol) (MDBNP) (#8) and disulfoacetic acid (#36) showed a clear inhibitory effect in the assay.

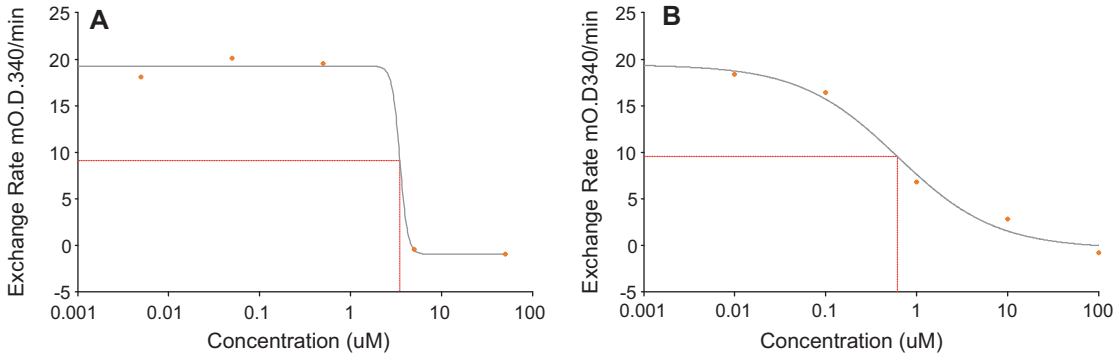

Fig. 4. The dose response of disulfoacetic acid (A) and MDBNP (B). The effects of the compounds on the ADP/ATP exchange through human ANT4 were examined at the range of 5×10^{-10} M to 5×10^{-5} M. The red-dot line indicates the concentration of 50% inhibition (IC_{50}). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in other species, more than 10 amino acids were determined to be essential for the ADP/ATP exchange activity of ANT [23]. Substitution of these amino acids inactivates ANT completely. The amino acids corresponding to these sites were mapped onto the three-dimensional homology model of human ANT4. The second criterion was that the binding cleft contains a unique amino acid which exists only in ANT4 but not in ANT1, ANT2 or ANT3.

By these criteria, we selected a binding pocket on the surface of ANT4 that contacts the mitochondrial matrix as shown in Fig. 1B and C. The binding pocket is comprised of both Arg152 (highlighted in green) and Glu256 (highlighted in yellow). Arg152 is conserved in all previously identified ANTs throughout species, and was determined to be an essential amino acid for transport activity [12] (Fig. 2A). On the other hand, Glu256 is uniquely conserved only among mammalian ANT4 sequences, and differs markedly from the corresponding amino acid in ANT1, 2 and 3 (Fig. 2B).

We utilized DOCK6.1 (UCSF) to conduct molecular docking simulations. The coordinates for 139,735 compounds were used as the ligand database for molecular docking utilizing the site selected in the human ANT4 structure. The 41 highest-scoring compounds were selected for functional analysis based on previous molecular studies using this chemical library [9,21].

3.2. ADP/ATP exchange assay for candidate compounds

In our previous study [11], we generated yeast strains expressing human ANT1, ANT2, ANT3 or ANT4 after deleting all endogenous yeast ANTs (AACs). Thus, mitochondria of these yeast strains utilize solely one human ANT for their ADP/ATP exchange. Yeast mitochondria expressing human ANT4 were used in an initial biological screening study for candidate compounds. When we examined the effects of compounds (100 μM) on the ADP/ATP exchange through ANT4, two out of the 41 candidate compounds demonstrated inhibition of the exchange (Fig. 3).

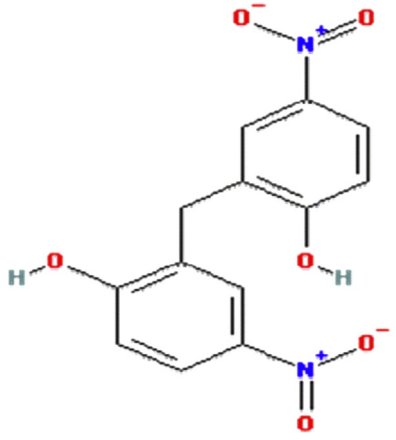

Fig. 5. The chemical structure of MDBNP.

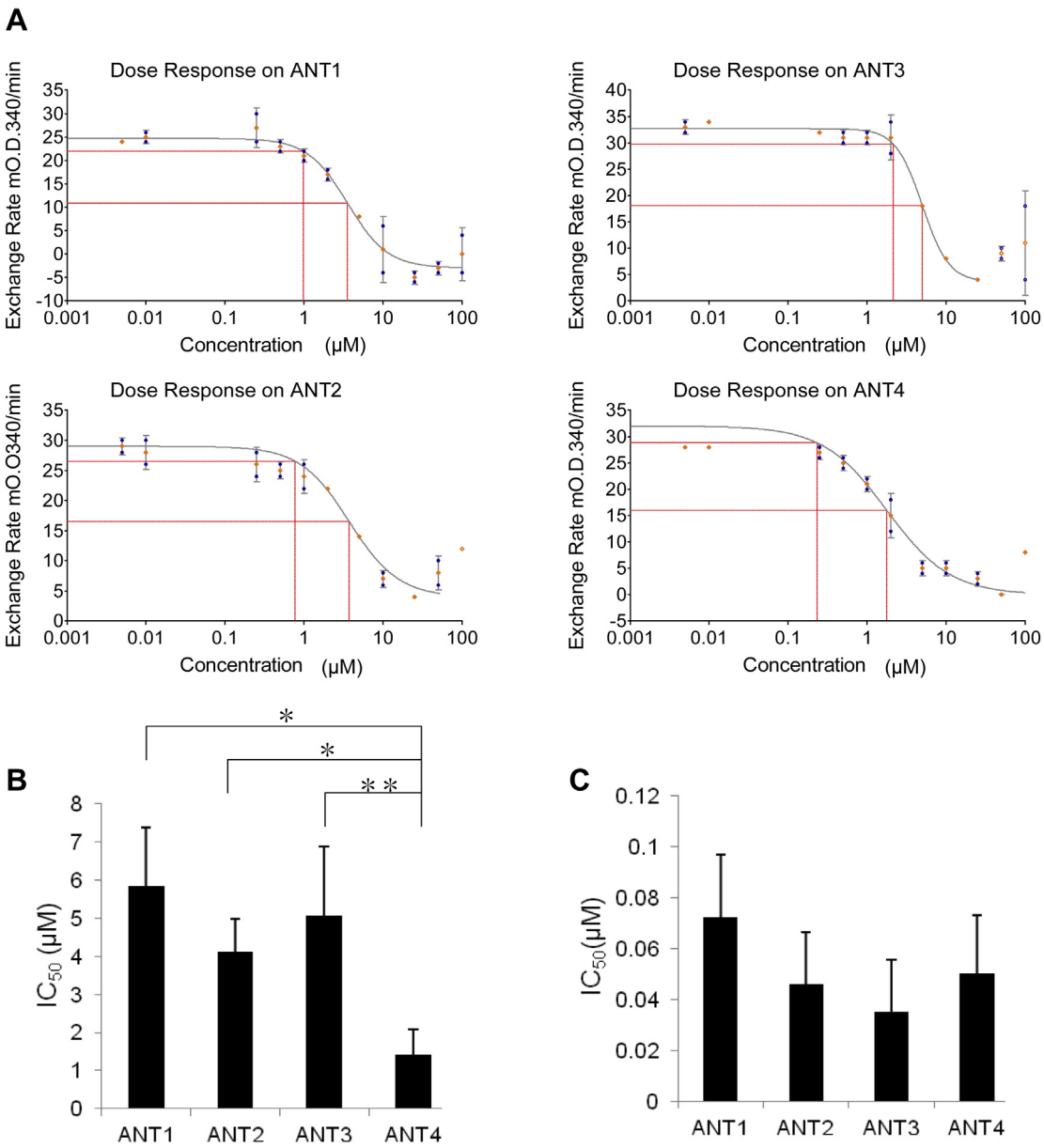

Fig. 6. (A) The dose response of MDBNP on the ADP/ATP exchange through either human ANT1, ANT2, ANT3 or ANT4 is shown individually. (B) IC_{50} values of MDBNP for ANT1, 2, 3 and 4. Average values were calculated from four independent experiments. The IC_{50} values of ANT1, 2 and 3 (5.8 μM, 4.1 μM, and 5.1 μM, respectively) were significantly higher than that for ANT4 (1.4 μM). $^{*}P < 0.005$, $^{**}P < 0.01$. (C) As a control, average values of IC_{50} values of ATR on human ANT1, 2, 3 and 4 are shown. There were no significant differences.

Compound #36 (NSC243618) was ranked third by *in silico* molecular docking and was identified as disulfoacetic acid. Disulfoacetic acid failed to completely dissolve in DMSO. A dose response analysis with six different concentrations of the compound, ranging from 5×10^{-10} M to 5×10^{-5} M, is shown in Fig. 4A with an IC_{50} of 3.5 μM. However, the dose response curve showed an atypical artificial inhibition pattern likely caused by aggregation of the small molecule compound. Therefore, further analysis of this disulfoacetic acid was not pursued.

In contrast, the dose response curve for compound #8 (NSC25062) showed a typical inhibition curve demonstrated in Fig. 4B with an IC_{50} of 0.62 μM. Compound #8 was ranked 21 out of the 41 highest-scoring compounds *in silico* and was identified as 2,2′-methanediylbis(4-nitrophenol) (MDBNP), also known as bis(2-hydroxy-5-nitro-phenyl)methane. It has a chemical formula of $C_{13}H_{10}N_2O_6$ and a molecular weight of 290 g/mol. The chemical structure is shown in Fig. 5. The predicted interactions of MDBNP with ANT4 are depicted in Fig. 1A, B, and C. It should be noted that the IC_{50} value of MDBNP on ANT4 inhibition is more than 10 fold higher when compared to the known potent ANT inhibitor atractyloside (ATR) (IC_{50}: ~0.05 μM), as shown below in Fig. 6C.

3.3. Inhibition of ANT4 by MDBNP

In order to investigate whether MDBNP selectively inhibits ANT4, we examined the effect of the compound on the ADP/ATP exchange through other human ANTs using yeast mitochondria expressing ANT1, ANT2, or ANT3. Individual dose response curves for each ANT isoform are presented in Fig. 6A. Fig. 6B shows the average IC_{50} values of MDBNP for each ANT isoform of four independent experiments. The average IC_{50} values of the compound on ANT1, ANT2, ANT3 and ANT4 were 5.8 μM, 4.1 μM, 5.1 μM and 1.4 μM, respectively. The IC_{50} values for ANT1, ANT2 and ANT3 were significantly higher than that for ANT4 ($P < 0.01$).

In contrast, there were no statistical differences among the IC_{50} values for ANT1, ANT2 and ANT3. In order to exclude a possibility that the identified compound inhibits any enzymatic activity non-specifically (e.g. by aggregation), we added MDBNP to the hexokinase- and glucose-6-phosphate dehydrogenase-based ATP detection assay in the presence of control ATP. MDBNP (up to 25 μM) did not inhibit the rate of NADPH formation (data not shown).

Adenine nucleotide translocase activity is known to be indiscriminately inhibited by a natural poison atractyloside (ATR) [5,8,23]. In contrast to MDBNP, the average IC_{50} values of ATR exhibited no statistical difference among ANT isoforms (Fig. 6C).

4. Discussion

Although molecular docking is considered a useful approach to identify putative binding candidates, the method is dependent on selection of appropriate structural features for targeting. To determine the best pocket to screen selective ANT4 inhibitors, we postulated two essential criteria. First, the pocket should be proximal to the sites critical for adenine nucleotide transporter activity; thus a small chemical association would have a higher possibility of interfering with the activity. Second, the pocket should include a unique amino acid to ANT4; thus a binding compound might preferentially associate with ANT4 over other ANTs. Following these criteria within the predicted human ANT4 structure, we first identified the amino acid residues that have been determined essential for the ADP/ATP exchange activity in mutagenesis studies in other species [23]. Indeed, many of these amino acid residues are known to be critical for substrate binding (adenine nucleotide) and/or associated with the previously identified specific inhibitor, carboxyatractyloside in the crystallography study of bovine ANT1 [24]. These amino acid residues are primarily located in the central portion of ANT. Secondly, we marked the amino acid residues unique to ANT4 that are considerably different from the corresponding residues in somatic ANTs in terms of size and polarity. Most of these differential amino acid residues are located outside the central portion of the ANT4 structure. In the end, there were few potential binding pockets detected within ANT4 that contained residues meeting both criteria.

However, one binding pocket located on the surface of ANT4 (the surface in contact with the mitochontrial matrix, shown facing down in Fig. 1A) appeared to be a plausible target. This binding pocket is formed by at least one of the amino acids essential for exchange activity (R152), as well as one of the amino acids unique to ANT4 (E256). MDBNP is predicted to be 4.0 Å from R152. R152 is located at the end of the 3rd alpha-helix domain of ANT4, and this arginine is conserved in all previously identified ANT molecules throughout species. The R152 of human ANT4 corresponds to R145 in *N. crassa* AAC which was determined to be essential for the AAC activity by a mutagenesis study [12]. Conversely, E256 resides proximal to the ANT RRRMMM signature at the end of the 5th alpha-helix and is unique to mammalian ANT4. MDBNP is predicted to be 5.1 Å from E265. Other mammalian ANTs (ANT1, ANT2 and ANT3) have either arginine or lysine at the corresponding site, so ANT4 bears the opposite charge in the pocket (Fig. 2B). Thus, the pocket proximal to E256 may serve as a good candidate to identify small chemicals uniquely associating with ANT4.

This approach identified MDBNP as an inhibitor of the ADP/ATP exchange activity through ANT in the low micromolar range. To the best of our knowledge, this is a first study that identified an ANT inhibitor using a molecular docking approach. Indeed, there are two well-known ANT inhibitors: carboxyatractyloside (CATR) (and its derivative atractyloside, ATR) and bongkrekic acid (BA). Both CATR and BA are poisonous agents isolated from nature [8,18,29]. Although both poisonous agents potently inhibit the ADP/ATP exchange activity of ANT, their mechanisms of binding and biological consequences of the two molecules are quite different. CATR-binding locks ANT in the conformation facing the cytoplasm (c-state) and can induce cellular apoptosis. BA-binding locks ANT in the conformation facing the matrix (m-state) and protects cells from apoptosis [8,18,29]. A third inhibitor of ANT may be useful for gaining a better understanding of the structure–function relationship in ANT and its biochemical regulation.

It should be noted, however, there is no evidence from the current study alone that the identified inhibitor actually binds in the proposed binding pocket. Nevertheless, the fact that MDBNP shows a degree of selectivity toward ANT4 is potentially useful for the development of new contraceptives. Although a modest difference in selectivity for ANT4 may not be optimal for a drug candidate as a male contraceptive in humans, the outcome of our study is encouraging because it demonstrates proof of concept for capability of generating a selective ANT4 inhibitor lead compound. MDBNP may serve as a lead compound to identify further potent and selective inhibitors, and the structure information obtained in the study could be useful for optimizing drugs *in silico*.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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