Design and Combinatorial Synthesis of N-Acyl Iminodiacetic Acids as Bongrekic Acid Analogues for the Inhibition of Adenine Nucleotide Translocase

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Abstract: The adenine nucleotide translocase (ANT) mediates ADP/ATP exchange in mitochondria and is also a critical component of the mitochondrial permeability transition (MPT) pore. Opening of this physiological pore has been implicated as a key step in initiating cell death, hence agents that prevent MPT pore opening may be of potential therapeutic value. The natural product bongrekic acid is a potent ANT inhibitor that is reported to block MPT opening. We present the design and synthesis of N-acyl iminodiacetic acids as novel inhibitors of ANT-1, using bongrekic acid as an initial lead. The identification of potent ANT-1 inhibitors from a primary binding assay and the preliminary structure–activity relationship based on these new leads are discussed.

Key words: bongrekic acid, mitochondria, library design, combinatorial synthesis, N-acyl iminodiacetic acids

Adenine nucleotide translocase (ANT) is an abundant integral mitochondrial inner membrane protein, whose key function is the selective exchange of cytosolic ADP for mitochondrial matrix ATP across the otherwise impermeable inner mitochondrial membrane. By providing the mitochondrial oxidative phosphorylation machinery with substrate for ATP production, and by supplying the cell with a high-energy phosphate source, the ANT-facilitated exchange of ADP and ATP is the most active transport system in aerobic cells and is a critical component in maintaining cellular energy metabolism.1–3 ANT is also an important component of a protein complex called the mitochondrial permeability transition (MPT) pore, the opening of which has been recognized as a key step in initiating cell death.4 This relatively non-specific pore can open under pathologic conditions, such as elevated Ca2+ concentration, oxidative stress, and depletion of adenine nucleotides, which are associated with events such as stroke or myocardial infarction. Pore opening allows cytoplasmic solutes less than 1500 daltons in mass to equilibrate across the inner mitochondrial membrane, causing collapse of mitochondrial membrane potential, swelling and rupture of the outer membrane, and release of toxic macromolecules that induce apoptosis.5–7 Thus, agents that prevent MPT pore opening may provide a potentially efficacious strategy for the treatment of ischemia/reperfusion injury and for certain neurodegenerative diseases.

Two different conformations of ANT have been demonstrated on the basis of interactions with specific ligands,8 namely the inhibitors atractyloside (ATR) and bongrekic acid (BKA) (Figure 1). These high-affinity ligands bind to ANT in an asymmetric fashion, either from the matrix (m) or from the cytosolic (c) side of the inner mitochondrial membrane, and they can mutually displace each other. Atractyloside binds to ANT in the c-conformation and induces the permeability transition, while bongrekic acid interacts with ANT in the m-conformation and inhibits the permeability transition in response to a variety of apoptotic stimuli.9 This finding suggests that different small molecule ligands of ANT may possess a spectrum of activities, i.e. they may act as cell protective agents targeted for acute tissue insults or degenerative diseases, or as cytotoxic agents for hyperproliferative diseases.

BKA is a natural toxin produced by the microorganism Pseudomonas cocovenans.10 When protonated, it diffuses through the lipid phase of the inner mitochondrial membrane and is a potent inhibitor of ANT-facilitated ADP/ATP exchange (IC50 =2 × 10–8 M). A stereocontrolled, convergent total synthesis of BKA has been reported which involved 33 steps.11 This makes it impractical to synthesize BKA in large amounts or its analogues for structure activity relationship (SAR) investigations. As a result, limited SAR studies around this molecule have been described to date.12 Moreover, large quantities of

Figure 1
BKA are difficult to obtain by fermentation. Importantly, although BKA inhibits MTP by binding to ANT, it can also manifest toxicity by interfering with the critical ANT-mediated transport of ADP and ATP across the inner mitochondrial membrane. Thus, compounds that inhibit MTP without affecting ADP/ATP translocation would be highly desirable. In this report, we describe the design and combinatorial synthesis of N-acyl iminodiacetic acids as bongkrekic acid analogues. The identification of potent ANT-1 inhibitors from a primary binding assay and the preliminary SAR are discussed.

It is plausible that the tight binding of BKA to ANT is due to electrostatic interactions between the terminal carboxylate moieties of BKA and positively charged amino acid residues of ANT, and/or hydrophobic interactions between the partially unsaturated middle segment of BKA and ANT. To verify these hypotheses, N-acyl iminodiacetic acids, represented by the general structure in Figure 2, were designed as BKA analogues. In these derivatives, the N-acyl iminodiacetic acid moiety serves as a substitute for the di-carboxylic acid end of BKA. The N-acyl iminodiacetic acid and the unsaturated di-acid end of BKA have in common a planar feature. Incorporation of an R1 substituent at the α-position allowed us to further define the conformation of the di-acid end.

![Figure 2](image)

As BKA is a linear flexible molecule, it is difficult to predict its three-dimensional conformation when bound to ANT. In order to ascertain the preferred conformation, various aminophenyl carboxylic acids were introduced in the middle of the molecule to restrict the number of possible conformations. These aminophenyl carboxylic acids can be derived from the corresponding nitrophenyl carboxylic acids. The amino moiety serves as an attachment point for mimetics of the mono-acid end of BKA (R3). Critical considerations for our synthetic strategy were that these N-acyl iminodiacetic acids could be synthesized from readily available building blocks, and that the chemical transformations would be amenable to solid phase organic synthesis techniques. This synthetic strategy allows access to a large number of compounds with various permutations to probe the SAR space in a rapid fashion.

As illustrated in Scheme 1, synthesis of the N-acyl iminodiacetic acid derivatives was carried out in a combinatorial fashion to quickly explore the various structural and physicochemical parameters. The building blocks are listed in Table 1. Bromoacetic acid was coupled to Wang resin using diisopropylcarbonodimide (DIC) and a catalytic amount of DMAP in DMF to give 1. Bromoaetate 1 was aliquoted in equal portions for subsequent steps. Each portion was allowed to react with each corresponding amino acid derivative (R1 building blocks, Table 1) in the presence of diisopropylethylamine (DIEA) in DMSO to yield the masked iminodiacetic acid segment 2. Each intermediate 2 was divided and acylated with the nitrophenyl-carboxylic acids or nitro-phenacyl chlorides (R2 building blocks, Table 1) to furnish 3. Acylation reactions with nitro-carboxylic acids were carried out using DIC, DIEA and catalytic amounts of DMAP in NMP. With nitro-phenacyl chlorides, the reactions were carried out in the presence of DIEA in NMP. The nitro group in 3 was converted into corresponding amine 4 by reduction using tin(II) chloride dihydrate in DMF. Amine 4 was washed, dried and divided in equal portions for the subsequent transformation. Each portion was allowed to react with an acyl-chloride or anhydride (R3 building blocks,
Table 1) to furnish 5. The products were then cleaved off the resin using TFA to give 6. In this step, the tert-butyl esters are deprotected and released as iminodiacetic acids from the resin. For the mono-benzyl ester derivatives ($R = \text{Bn}$), half of the amount of 6 was converted into the corresponding dicarboxylic acid 7 via Pd-catalyzed hydrogenolysis. The methyl esters and amides were screened in their original forms.

Table 1  List of Building Blocks for the Library Synthesis of $N$-Acyl Iminodiacetic Acid Derivatives

<table>
<thead>
<tr>
<th>$R^1$ component</th>
<th>$R^2$ component</th>
<th>$R^3$ component</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine methyl ester</td>
<td>2-nitrobenzoyl chloride</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>glycine amide</td>
<td>3-nitrobenzoyl chloride</td>
<td>benzoyl chloride</td>
</tr>
<tr>
<td>glycine tert-butyl ester</td>
<td>4-nitrobenzoyl chloride</td>
<td>glutaric anhydride</td>
</tr>
<tr>
<td>aspartic acid di-tert-butyl ester</td>
<td>2-nitrophthalic acid</td>
<td>heptanoyl chloride</td>
</tr>
<tr>
<td>glutaric acid di-tert-butyl ester</td>
<td>3-nitrophthalic acid</td>
<td>decanoyl chloride</td>
</tr>
<tr>
<td>alanine benzyl ester</td>
<td>4-nitrophthalic acid</td>
<td>methyl suberyl chloride</td>
</tr>
<tr>
<td>valine benzyl ester</td>
<td>2-nitrophenoxyacetic acid</td>
<td>methyl sebacoyl chloride</td>
</tr>
<tr>
<td>leucine benzyl ester</td>
<td>3-nitrophenoxyacetic acid</td>
<td></td>
</tr>
<tr>
<td>phenylalanine benzyl ester</td>
<td>4-nitrophenoxyacetic acid</td>
<td></td>
</tr>
<tr>
<td>2-nitrocinnamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-nitrocinnamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-nitrocinnamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-(2-nitrophenyl)-2-furoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-(3-nitrophenyl)-2-furoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-(4-nitrophenyl)-2-furoic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These $N$-acyl iminodiacetic acid derivatives were evaluated for their affinity to ANT using a binding assay against a radioiodinated atractyloside derivative.\(^\text{16}\) The assay employed bovine cardiac mitochondria, which are known to predominantly express the ANT-1 isoform of ANT.\(^\text{17}\) All compounds were screened at a concentration of 10 $\mu$M for an initial assessment of ANT-1 binding activity. Compounds that were shown to displace >50% of the radioligand were then fully characterized using 10-point dose response studies (5 nM–30 $\mu$M). Several compounds with EC\(_{50}\) value in the low $\mu$M range were identified from these libraries, and their structures and EC\(_{50}\) values are summarized in Table 2. Large hydrophobic substitutions at the $\alpha$-position of the di-acid terminus were found to be crucial for binding to ANT-1, as analogues derived from glycine, alanine, valine, aspartic acid and glutaric acid building blocks did not yield any active compounds in the screening assay. No compounds with acetyl, benzoyl, alkyl acid or alkyl esters at the $R^3$ position showed significant binding to ANT-1. This indicates hydrophobic long chain alkyl substituents are preferred at this position. It is not possible to draw any reliable conclusions on the middle segment based on the limited number of hits. Interestingly, the comparable binding affinities of MITO-3207 and MITO-3331, despite the presence of a benzyl ester in the latter compound, suggest that alteration of the central portion of the general structure in Figure 2 may compensate for the loss of an ionic interaction. However, it is also conceivable that hydrophobic interactions of the mono-benzyl ester moiety play a role in the binding of MITO-3207. Clearly, further SAR exploration of the middle segment is necessary to reveal the feasibility of using conformational constraints to yield more potent ANT-1 inhibitors.

Table 2  EC\(_{50}\) Values of Selected BKA Analogues

<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound</th>
<th>EC(_{50}) ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BKA</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>MITO-3152</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>MITO-3207</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>MITO-3331</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>MITO-3332</td>
<td>12.1</td>
</tr>
</tbody>
</table>

In conclusion, we have described the design and combinatorial synthesis of a library of easily accessible small molecules as analogues of bongkrekic acid, a complex natural product. Competitive displacement of a radiolabeled ANT-1 ligand was used to identify new lead compounds with good binding characteristics for the transporter. The preliminary SAR information generated from this investigation provides a guide for refining the design and synthesis of additional bongkrekic acid analogues using our
facile library synthesis approach. Such compounds will allow us to further explore the binding features necessary for modulating the conformation of ANT to prevent mitochondrial permeability transition and also preserve the translocase activity of the target.

All intermediates and final products were characterized using LC-MS by a SpectraSystem P4000 HPLC system coupled with a Finnigan LCD/Deca mass spectrometer employing electrospray ionization. HPLC was performed on a Keystone 5 μm betasil C8 column (100 x 2 mm) using a linear gradient of 5–95% solvent B over 5 min followed by 95% solvent B for 3 min and 3% solvent B for 2 min (solvent A: 0.05% TFA in H₂O; solvent B: 0.05% TFA in CH₃CN) at a flow rate of 0.30 mL/min. When necessary, final products were purified by preparative RP-HPLC using a Gilson 215 liquid handling system. HPLC was performed on a Keystone 5 μm betasil C18 column (150 x 20 mm) using H₂O with 0.05% TFA (solvent A) and CH₃CN with 0.05% TFA (solvent B) as eluents at a flow rate of 15 mL/min. The gradient was varied according to the polarity of the compound purified.

General procedures for the solid phase synthesis of compound purified.

**Coupling of Bromoacetic Acid to Wang Resin**

Polystereylene Wang resin (10.0 g, 1.25 mmol/g) was shaken at r.t. with bromoacetic acid (8.68 g, 62.5 mmol), DIC (9.79 mL, 62.5 mmol) and DMAP (10 mg) in CH₂Cl₂ (60 mL) in a polypropylene bottle for 4 h. The resin was collected via filtration. The resin was washed with DMF (3 x 40 mL), CH₃OH (3 x 40 mL), CH₂Cl₂ (3 x 20 mL), CH₃OH (3 x 20 mL), and air dried. The resulting bromoacetate resin 1 (12.0 g) was used in the next step without further analysis.

**Displacement of Bromide with Amino Esters**

Bromoacetate resin 1 (4.0 g) was shaken with glycine tert-butyl ester HOAc salt (3.82 g, 20.0 mmol) and DIEA (7.2 mL, 75 mmol) in DMSO (13 mL) in a 20 mL polypropylene syringe fitted with a polyethylene frit at r.t. for 24 h. The resin was washed with DMF (3 x 20 mL), CH₃OH (3 x 20 mL), DMF (3 x 40 mL), methanol (3 x 40 mL), CH₂Cl₂ (3 x 40 mL), methanol (3 x 40 mL), and air dried. The resulting bromoacetate resin 1 (12.0 g) was used in the next step without further analysis.

**Coupling of Nitrophenyl Acids**

Resin 2 (1.5 g) was shaken with 2-nitrophenylacetic acid (1.25 g, 6.9 mmol), DIEA (2.0 mL, 11.5 mmol), and bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrop) (3.26 g, 7.0 mmol) in DMF (60 mL) at r.t. overnight. The resin was washed with DMF (3 x 40 mL), CH₃OH (3 x 20 mL), DMF (3 x 40 mL), methanol (3 x 40 mL), CH₂Cl₂ (3 x 40 mL), methanol (3 x 40 mL), and air dried. The resulting bromoacetate resin 1 (12.0 g) was used in the next step without further analysis.

**Reduction of Nitro Groups to Amines**

Resin 3 (1.7 g) was shaken with tin dichloride dihydrate (2.0 M, 20 ml) at r.t. overnight. The resin was washed with DMF (5 x 10 mL), CH₃OH (3 x 10 mL), DMF (3 x 10 mL), CH₃OH (3 x 10 mL), CH₂Cl₂ (3 x 10 mL), CH₃OH (3 x 10 mL), and air dried to yield resin 4. A small sample (ca. 50 mg) of the resulting resin 4 was treated with TFA–H₂O (95:5, 1.0 mL) for 1 h at r.t. The solution was collected via filtration. The resin was washed with HOAc (3 x 1 mL). The combined solution was lyophilized. The residue was analyzed by mass-spectrometry to confirm the identity and approximate purity of the intermediate.

**Coupling of R(C(=O)X) to Resin**

When a carboxylic acid was used, the coupling reaction was carried out in the presence of DIC. For example, resin 4 (0.275 g) was shaken with benzoic acid (0.31 g, 2.5 mmol), DIC (0.47 mL, 3.0 mmol), DIEA (0.87 mL, 5.0 mmol) and DMAP (10 mg) in DMF (50.0 mL) at r.t. overnight. The resin was washed with DMF (3 x 5 mL), CH₃OH (3 x 5 mL), DMF (3 x 5 mL), CH₃OH (3 x 5 mL), CH₂Cl₂ (3 x 5 mL), CH₃OH (3 x 5 mL), and air dried. The resulting resin 5 was treated with TFA–H₂O (95:5, 3.0 mL) for 1 h at r.t. The solution was collected via filtration. The resin was washed with HOAc (3 x 5 mL). The combined solution was lyophilized to give the desired product 6. Its purity and identity were assessed using HPLC-MS spectrometry.

When an acyl anhydride or chloride was used, the coupling reaction was carried out without DIC. For example, resin 4 (0.275 g) was shaken with acetic anhydride (0.24 mL, 2.5 mmol), DIEA (0.87 mL, 5.0 mmol) and DMAP (10 mg) in DMF (50.0 mL) at r.t. overnight. The resin was washed with DMF (3 x 5 mL), CH₃OH (3 x 5 mL), DMF (3 x 5 mL), CH₃OH (3 x 5 mL), CH₂Cl₂ (3 x 5 mL), CH₃OH (3 x 5 mL), and air dried.

**TFA Cleavage**

Resin 5 (0.305 g) was treated with TFA–H₂O (95:5, 3.0 mL) for 1 h at r.t. The solution was collected via filtration. The resin was washed with HOAc (3 x 5 mL). The combined solution was lyophilized to give the desired product 6. Its purity and identity were assessed using HPLC-MS spectrometry.

**Displacement Assays of the Adenine Nucleotide Translocase Radioligand from Isolated Mitochondria using BKA Analogues**

Competition binding assays were performed using bovine cardiac mitochondria, known to express the ANT-1 isoform, and an 125I-ATR derivative as ligand. One microgram aliquots of mitochondrial protein were incubated with 100 μL binding buffer (10 mM Tris, 120 mM KCl, 6 mM MgCl₂, 1 mM EDTA, pH 7.4) containing 0.5 nM of the radioligand and test compound at various concentrations. Mixtures were incubated for 1 h on ice, at the end of which unbound ligand was separated by centrifugation. Supernatants containing unbound radioligand were aspirated and discarded. Mitochondrial pellets were washed with four volumes of cold binding buffer and counted in a Micromedic 4/200 automatic gamma counter. Some assays were performed in a 96 well microtiter well format using a Brandel 96-well cell harvester. Unbound ligand was removed by filtration and 4 volumes washed through glass fiber filter mats (Whatman GF/B paper, catalogue # FPXR-196, Brandel, Inc, Gaithersburg, MD). Following a drying step at 60 °C for 1 h, scintillation fluid was added and the radioactivity associated with the mitochondrial pellets retained in the filter mat was determined in a 1450 Wallac MicroBeta TriLux liquid scintillation and luminescence counter (EG&G Wallac, Gaithersburg, MD). Full dose response curves for hits were generated with the derivatives using a compound concentration range of 5 nM–30 μM. EC₅₀ values were calculated using Prism software (GraphPad, San Diego, CA).

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