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***N*-Phenylbenzamides as Potent Inhibitors of the Mitochondrial Permeability Transition Pore**

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Abstract

Persistent opening of the mitochondrial permeability transition pore (PTP), an inner membrane channel, leads to mitochondrial dysfunction and renders the PTP a therapeutic target for a host of life-threatening diseases. Herein, we report our effort toward identifying small-molecule inhibitors against this target through structure-activity-relationship optimization studies, which led to the identification of several potent analogs around the *N*-phenylbenzamide compound series identified by high-throughput screening. In particular, compound **4**, (3-(benzyloxy)-5-chloro-*N*-(4-(piperidin-1-ylmethyl)phenyl)benzamide), displayed noteworthy inhibitory activity in the mitochondrial swelling assay (EC₅₀ of 280 nM), poor to very good physicochemical as well as in vitro pharmacokinetic properties, and conferred very high calcium retention capacity to mitochondria. From the data, we believe compound **4** in this series represents a promising lead for the development of PTP inhibitors of pharmacological relevance.

Calcium deregulation adjourned

Persistent opening of the mitochondrial permeability transition pore (PTP), which is a Ca²⁺-release megachannel, causes cell death. Here we describe the discovery of potent *N*-phenylbenzamide compounds as PTP inhibitors that confer very high calcium retention capacity to the mitochondria that also possess good-to-very good pharmacological profiles.

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Keywords

calcium retention capacity; mitochondria; mitochondrial swelling; permeability transition; *N*-phenylbenzamides

It has been appreciated for some time that the mitochondrial permeability transition pore (PTP), a channel of the inner mitochondrial membrane (IMM), can inappropriately open in a variety of human pathologies.^[1] Persistent opening of pore causes depolarization, release of matrix Ca^{2+} , cessation of oxidative phosphorylation and may result in mitochondrial swelling and rupture of outer mitochondrial membrane (OMM) culminating in cell death.^[2] The molecular nature of the PTP has been clarified only recently. Traditional models of PTP involving complexes of OMM and IMM proteins have been discounted on the basis of genetic studies showing the PTP can still form in the absence of each of these proteins.^[3–4] Other recent studies have suggested that dimers of the F_0F_1 ATP synthase (F-ATP synthase) are able to form channels with properties expected for the PTP.^[5] The precise nature of the molecular transition of this complex from a Mg^{2+} -dependent ATP synthetic device into a Ca^{2+} -dependent pore has yet to be determined. While progress has been made on the molecular identity of PTP, our ability to treat diseases in which inappropriate activation of the PTP plays a role has been limited to the use of cyclosporin A (CsA) and its analogs, Debio 025 and NIM811, which act on the pore through their matrix receptor and PTP modulator cyclophilin D (CyPD).^[6]

In vivo treatment has proven effective in models of collagen VI muscular dystrophy^[7–11] and provided encouraging results in a pilot trial on patients affected by Ullrich Congenital Muscular Dystrophy and Bethlem Myopathy.^[12] The limit of cyclophilin inhibitors is that their target, CyPD, modulates the pore indirectly, as shown by the fact that the PTP can still open when CyPD has been genetically ablated.^[13–16] Therefore, we^[17] as well as others^[18] have carried out programs aimed at identifying novel PTP inhibitors through the unbiased screening of compound libraries. Here, we report a novel series of potent small-molecule

inhibitors of the PTP that can be used as investigative tools, and possibly, developed into therapeutics for PTP-based diseases.

A high-throughput screen of the Molecular Libraries Small Molecule Repository (MLMSR) collection of 363,827 compounds revealed benzamide compounds **1** and **2** (Figure 1), among several other hits (data not presented), that were chosen as starting points for structure-activity-relationship (SAR) studies based on their biological activity and physicochemical properties.^[19] The two key assays that were used to identify the hits were: (i) Ca^{2+} -induced mitochondrial swelling, a light-scattering-based assay that depends on PTP opening that allowed us to identify the inhibitors and assess their concentration-response, and (ii) Rhodamine (Rh) 123-uptake, a counterscreen assay which allowed us to identify compounds that prevent Ca^{2+} uptake by interfering with development or maintenance of the IMM potential, thereby preventing mitochondrial swelling by preventing Ca^{2+} entry rather than by inhibiting the PTP. Indeed, Ca^{2+} uptake through the selective mitochondrial Ca^{2+} uniporter in respiring mitochondria is driven by the Ca^{2+} electrochemical gradient, which does not form if the test compound inhibits respiration and/or has uncoupling properties.

During the hit validation and SAR optimization stages of the project, in addition to the mitochondrial swelling and Rh123-uptake assays, we used the Ca^{2+} retention capacity (CRC) assay, a sensitive method that allows one to define precisely the effect of inhibitors on the Ca^{2+} -dependent propensity of the pore to open.^[20–21] To the best of our knowledge, this is the first report of the inhibitory activity of *N*-phenylbenzamide compounds toward the PTP.

The benzamide analogs were assembled in a two-step process (Scheme 1). The 3-hydroxybenzoic acid derivatives **5** were treated with either substituted or unsubstituted benzyl bromide along with potassium carbonate in DMF for 24 h to give the corresponding benzyl 3-(benzyloxy)benzoate derivatives. These benzoate esters were then saponified with 10 M potassium hydroxide in MeOH under reflux conditions to yield the corresponding carboxylic acids **6** in 50–80% yield. Subsequent PyBOP-mediated amide coupling reactions with various substituted anilines **7** in the presence of Hunig's base and DMF under microwave conditions afforded the corresponding analogs **8** of the benzamide series.

A SAR campaign was carried out around the hit molecules, **1** and **2**, that led to couple of very potent analogs, **3** and **4**, out of 82 analogs that were screened.^[22] Results for a representative SAR study that was mainly focused on investigating the 6-membered heterocyclic pendant attached to the eastern aryl ring (**12–15**) as well as on studying the effect of halogen substitution around the western half of the structure (**9–11** and **16–20**) have been summarized in Table 1. These studies revealed that replacement of the pendant piperidine with piperazines (**14** and **15**) in the eastern region was well-tolerated and afforded compounds with similar potency at preventing mitochondrial swelling that increased the CRC of mitochondria as well. All other changes failed afford compounds with any significant improvement in activity compared to **3** and **4** (Figure 1 and Table 2). As noted in Table 1, most of the analogs showed activity in the Rh123 uptake assay, suggestive of interference with the IMM, an untoward side effect. The strategy to replace chloro with a

fluoro substituent in the western aryl ring resulted in analogs **9** and **10** with a much-improved profile in the counterscreen and CRC assays.

Clearly compounds **3** and **4** (Figure 1 and Table 2) demonstrated the best activity in the swelling and CRC assays. Of the two compounds, **4** showed slightly superior activity and, hence, was chosen for extensive biological characterization using a variety of established in vitro assays. First, we examined PTP-dependent swelling in isolated mouse liver mitochondria following uptake of 50 μM Ca^{2+} . In a full concentration-response ranging from 12.2 nM to 1.56 μM , inhibition of swelling was demonstrated with an EC_{50} of 0.398 ± 0.025 μM (Figure 2A) which is in the same order of magnitude as for standard PTP inhibitors CsA and GNX-865,^[18] a cinnamic anilide identified in a high-throughput screen similar to the one employed here (Table 1). We next tested the CRC, which allows quantification of the amount of Ca^{2+} necessary to open the pore. At 12.5 μM a compound-to-solvent CRC ratio of 19 was generated, the highest reported in the literature to date (Figure 2B). We also observed that the maximum CRC ratios of isolated mouse liver mitochondria treated with **4** are about 4 times higher than ones treated with CsA, which implied that the compounds might be acting on different biological targets. To test this hypothesis, we investigated the threshold Ca^{2+} load required for the PTP to open in response to **4** in CyPD-null mouse liver mitochondria, which lack the mitochondrial CsA binding site. We observed a 7-fold increase in CRC in these mitochondria (which are already partially desensitized due to the absence of CyPD), suggesting that benzamides have a different molecular target. Maximal PTP inhibition by **4**, as assessed by both mitochondrial swelling and CRC assays, occurred at concentrations higher than those observed with diarylisoxazole-3-carboxamides, the other class of inhibitors that was identified in the high-throughput screen.^[17]

We also tested whether **4** is protective against known inducers of the PTP that trigger pore opening by inducing oxidative stress. Isolated mouse liver mitochondria were loaded with 10 μM Ca^{2+} (which is not able to induce PTP opening per se, Figure 2D *traces a*) and then challenged with reagents that modify distinct classes of redox-sensitive thiols ($-\text{SH}$) (*traces b*) which affect the PTP sensitivity to Ca^{2+} and trigger pore opening: (a) matrix $-\text{SH}$ groups that react with diamide and PhAsO_3 ,^[23] (b) inner membrane external thiols that react with copper (II) bis(1,10-phenanthroline) complex $[\text{Cu}(\text{OP})_2]$, and (c) outer membrane N-ethylmaleimide (NEM)-reactive thiols.^[24–25] In all cases, the PTP transition from the closed to open conformation was delayed by 3.12 μM CsA (*traces c*) and prohibited by the same concentration of **4** (*traces d*), as assessed in mitochondrial swelling assays (Figure 2D). Therefore, these inhibitors are effective in preventing PTP opening regardless of the methods used to induce it.

In all the above-mentioned studies, murine mitochondria were used for identifying and optimizing PTP inhibitors. However, due to the existence of species-specific PTP regulation,^[26–28] we deemed it essential to test whether an inhibitory effect could be also detected in human mitochondria. As demonstrated in Figure 2E, compound **4** induced a concentration-dependent increase in the CRC of permeabilized HeLa cells.

It has been suggested that the PTP forms from a unique conformation of dimers (or higher oligomeric forms) of F-ATP synthase.^[5] In light of these findings, we investigated whether **4** also affects ATP synthesis, which would be potentially an undesirable side effect.

Mitochondrial respiration was measured both in isolated mouse liver mitochondria and in intact HeLa cells in the presence or absence of **4**. No significant differences were observed in respiratory control ratios, FCCP-stimulated, and oligomycin-sensitive respiration in either isolated mouse liver mitochondria (Figure 2C and data not shown) or HeLa cells (Figure 2F) at low concentrations of **4** (i.e., 10 μ M). A decrease in the IMM potential (Figure 2A) and respiratory control ratio (Figure 2C) in isolated mouse liver mitochondria (reflecting a decreased ability to generate ATP) and of oxygen-consumption rate in HeLa cells was observed only at higher concentrations of **4**. These findings confirm that compound **4** shows no effect on ATP synthesis and HeLa cell proliferation at low concentrations (5 μ M and 10 μ M, respectively). However, at concentrations above 10 μ M it may cause cellular toxicity, as confirmed in HeLa (Figure 2G) and Fa2N-4 cells (Table 2, last entry).

We next examined the physicochemical and in vitro pharmacokinetic properties for the analogs **3** and **4** (Figure 1 and Table 2). Analogs **3** and **4** displayed promising physicochemical parameters, possessing a desirable number of hydrogen bond donors and acceptors, reduced topological polar surface area, molecular weight less than 500 Da, and moderately favorable ligand efficiency. Although the cLogP values were generally high for **3** and **4** (above 6), analogs **14** and **15** had much reduced cLogP values around 4.9. These key analogs were characterized further for in vitro pharmacology to create a baseline profile for future structure-property-relationship (SPR) optimization efforts. Overall, analogs **3** and **4** demonstrated poor-to-very good in vitro pharmacokinetic features, having poor-to-good aqueous solubility (pH dependent) and good chemical stability in the presence of excess dithiothreitol (DTT), confirming that these inhibitors do not possess reactive functionality. Compounds **3** and **4** demonstrated moderate plasma stability and very high plasma protein binding. A parallel artificial membrane permeability assay (PAMPA) was used as an in vitro model for passive transport, and blood-brain barrier (BBB) permeability was used to predict CNS penetration. Compounds **3** and **4** demonstrated very good permeability in both of these assays. Metabolic liability was apparent for both **3** and **4** after 1 h exposure, especially in mouse liver microsomes in the presence of NADPH. The key analogs showed some degree of toxicity towards Fa2N-4 immortalized human hepatocytes, having LC₅₀s of 30 and 32 μ M with 75-fold and 114-fold selectivity compared to the EC₅₀s for the mitochondrial swelling for **3** and **4**, respectively. Activity of these key analogs in the Rh123 uptake and cytotoxicity assays suggests a possible trend, which will be monitored during future studies.

In summary, SAR optimization studies around the *N*-phenylbenzamide scaffold led to the discovery of potent inhibitors of the PTP conferring mitochondria with a very high CRC, which is a robust measure of inhibition of the PTP. Compound **4** confers a CRC ratio of 19 (the highest reported for a PTP inhibitor to date) and showed promising inhibition of swelling, with an EC₅₀ of 280 nM. We carried out biological characterization of the PTP through a series of *in vitro* assays and found that compound **4** was protective against both Ca²⁺ and oxidative-stress-triggered pore opening, and that it inhibits both the mouse and human PTP. Moreover, we found that the biological target for this compound series is not

CyPD, and that no inhibition of F-ATP synthase is observed at concentrations that fully inhibit the PTP. Higher concentration ($>10\ \mu\text{M}$) of compound **4** showed interference with the IMM potential and cytotoxicity. Overall, this compound series, represented by compounds **3** and **4**, possesses a promising in vitro pharmacological profile, poor-to-good aqueous solubility (pH-dependent), and good permeability. Future studies will involve additional optimization in order to decrease compound toxicity and provide analogs suitable for in vivo testing for efficacy in relevant disease models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Compounds **3** and **4** derived from hits **1** and **2**.

Figure 2.

Effect of **4** on the PTP and cell viability. (A) *Full symbols*: **4** prevents mitochondrial swelling induced with 50 μM Ca²⁺ in a concentration-dependent manner; *open symbols*: interference with Rh123 uptake upon treatment with compound **4**; (B) Concentration-response of **4**-to-solvent CRC ratios of WT (*squares*) and CyPD KO (*triangles*) mitochondria; (C) respiratory control ratios (RCR) of **4** or solvent-treated mouse liver mitochondria; (D) Effect of **4** with known chemical inducers of the PTP. Mitochondria were supplemented with 10 μM Ca²⁺ only, traces a; 10 μM Ca²⁺ and with, as indicated, 7 μM PhAsO, 2 mM Diamide, 7 μM Cu(OP)₂ or 2 mM *N*-ethylmaleimide traces (b)–(d); in traces c and d 3.125 μM CsA or **4**, respectively, were also present; (A)–(D) assays were performed on isolated mouse liver mitochondria. (E) **4**-to-solvent CRC ratios of permeabilized HeLa cells (0.8 million/condition). (F) Oxygen-consumption rates (OCR) of HeLa cells, treatments were made as indicated. (G) Interference with HeLa cell proliferation after 24-hour treatment with indicated concentration of **4**. Data are a representative (D, F) and an average ± SEM of 4 experiments.

Scheme 1.

General procedure for synthesis of benzamide analogs.

Table 1

Structure-activity-relationship optimization of representative analogs

compound	CRC/CRC ₀ at 12.5 μ M	mitochondrial swelling EC ₅₀ (μ M)	Rh123 uptake EC ₅₀ (μ M)	compound	CRC/CRC ₀ at 12.5 μ M	mitochondrial swelling EC ₅₀ (μ M)	Rh123 uptake EC ₅₀ (μ M)
9	10.8 $\pm$ 0.6	0.521 $\pm$ 0.046	58.6 $\pm$ 2.9	16	6.50 $\pm$ 1.20	1.19 $\pm$ 0.33	23.2 $\pm$ 1.3
10	13.3 $\pm$ 1.0	0.372 $\pm$ 0.028	> 100.00	17	2.37 $\pm$ 0.41	4.00 $\pm$ 0.66	> 100
11	2.26 $\pm$ 0.11	3.60 $\pm$ 0.17	75.7 $\pm$ 1.8	18	5.91 $\pm$ 0.38	1.24 $\pm$ 0.39	24.7 $\pm$ 1.5
12	8.81 $\pm$ 0.39	0.403 $\pm$ 0.031	> 100	19	2.59 $\pm$ 0.54	1.39 $\pm$ 0.17	> 100
13	14.1 $\pm$ 1.1	0.423 $\pm$ 0.028	49.6 $\pm$ 4.3	20	10.1 $\pm$ 0.8	0.49 $\pm$ 0.08	28.6 $\pm$ 1.1
14	16.1 $\pm$ 1.5	0.286 $\pm$ 0.027	42.6 $\pm$ 3.1	GNX-865	7.67 $\pm$ 0.35	0.073 $\pm$ 0.007	> 100
15	18.3 $\pm$ 1.3	0.206 $\pm$ 0.021	46.4 $\pm$ 3.2	Cyclosporin A	4.40 $\pm$ 0.24	0.149 $\pm$ 0.009	> 100

Data are an average of 3 experiments $\pm$ SEM

Comparison of activity and physicochemical properties and summary of in vitro pharmacology for compounds **3** and **4**

parameter	comparative data			assessment	Result	
	compd 3	compd 4			compd 3	compd 4
CRC/CRC ₀ at 12.5 μ M	17.2 $\pm$ 0.6	19.5 $\pm$ 1.7		aqueous solubility in 1xPBS pH 5.0/6.2/7.4 μ g/mL [μ M] [<i>a</i>]	26.8/7.0/0.2 [61.6/16.1/0.5]	22.8/22.6/0.64 [52.5/52.1/1.5]
mitochondrial swelling EC ₅₀ (μ M)	0.398 $\pm$ 0.025	0.280 $\pm$ 0.024		chemical stability with 5x DTT, % parent remaining after 8 h [<i>a</i>]	83	91
Rh123 uptake EC ₅₀ (μ M)	36.5 $\pm$ 1.82	24.5 $\pm$ 1.12		aqueous stability (1:1 PBS/acetone), % parent remaining after 48 h [<i>b</i>]	89.3	93.4
molecular weight [<i>c</i>] (g/mol)	434.9	434.9		plasma protein binding, % bound for human; mouse (1 μ M / 10 μ M) [<i>a</i>]	99.0/99.0; 99.0/98.8	99.2/99.2; 99.3/98.3
topological polar surface area, tPSA [<i>c</i>]	41.5	41.5		plasma stability (% remaining after 3 h) human; mouse [<i>a</i>]	44; 57	39; 35
cLogP [<i>c</i>]	6.2	6.4		PAMPA permeability, Pe ($\times 10^{-6}$ cm/s) Donor pH: 5.0/6.2/7.4 Acceptor pH: 7.4 [<i>a</i>]	1281/1425/1276	1300/1339/und
hydrogen bond acceptors [<i>d</i>]	2	2		BBB PAMPA Permeability, Pe ($\times 10^{-6}$ cm/s) Donor pH: 7.4 Acceptor pH: 7.4 [<i>a</i>]	381	113
hydrogen bond donors [<i>d</i>]	2	2		hepatic microsome stability (% remaining after 1 h) human; mouse (+NADPH/-NADPH) [<i>a</i>]	65/75; 9.6/80	61/67; 30/74
heavy atoms [<i>e</i>]	31	31		toxicity towards Fa2N-4 immortalized human hepatocytes LC ₅₀ (μ M) [<i>a</i>]	30	32
ligand efficiency, LE	0.29	0.30				

[*a*] Data collected by David B. Terry at the *Conrad Prebys* Sanford Burnham Medical Research Institute.

[*b*] Data collected by Patrick Porubsky at the University of Kansas Analysis, Purification, and Compound Management Core, Specialized Chemistry Center.

[*c*] Data were generated using CambridgeSoft ChemBioDraw, version 12.

[*d*] Data were calculated using SYBYL 8.0, Tripos Associates, St. Louis, MO, 2010.

[*e*] Data were calculated using Marvin 15.3.23.0, 2015, ChemAxon.