ChemPubSoc Europe

DOI: 10.1002/cmdc.201500545

CHEMMEDCHEM Communications

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

Sudeshna Roy[+],*[a] Justina Šileikytė[+],[b] Benjamin Neuenswander,[a] Michael P. Hedrick,[d] Thomas D. Y. Chung,[e] Jeffrey Aubé,[a] Frank J. Schoenen,*[a] Michael A. Forte,*[c] and Paolo Bernardi*[b]

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 of this target through structure–activity relationship optimization studies, which led to the identification of several potent analogues 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 (EC_{50} = 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.

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 can 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_OF_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 cyclosporine A (CsA) and its analogues, 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 US National Institutes of Health Molecular Libraries Small Molecule Repository (MLSMR) collection of 363 827 compounds revealed benzamides **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 keys 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 123 (Rh123) uptake, a counter screen assay that allowed us to identify compounds that prevent Ca^{2+} uptake by interfering with develop-

[a] *Dr. S. Roy,[++] B. Neuenswander, Prof. J. Aubé,[++] Dr. F. J. Schoenen*
University of Kansas Specialized Chemistry Center
2034 Becker Drive, Lawrence, KS 66049 (USA)
E-mail: s.roy@unc.edu
schoenen@ku.edu

[b] *Dr. J. Šileikytė,[+] Prof. P. Bernardi*
CNR Neuroscience Institute and Department of Biomedical Sciences
University of Padova, Via Ugo Bassi 58/B, Padova, 35131 (Italy)
E-mail: bernardi@bio.unipd.it

[c] *Prof. M. A. Forte*
Vollum Institute, Oregon Health & Science University
3181 S.W. Sam Jackson Park Road, Portland, OR 97239 (USA)
E-mail: forte@ohsu.edu

[d] *Dr. M. P. Hedrick*
Conrad Prebys Center for Chemical Genomics
Sanford Burnham Prebys Medical Discovery Institute
10901 N. Torrey Pines Road, La Jolla, CA 92037 (USA)

[e] *Dr. T. D. Y. Chung*
Office of Translation to Practice, Mayo Clinic
Rochester, MN 55905 (USA)

[++] *Current address: Division of Chemical Biology and Medicinal Chemistry and the Center for Integrative Chemical Biology and Drug Discovery, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599 (USA)*

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201500545.

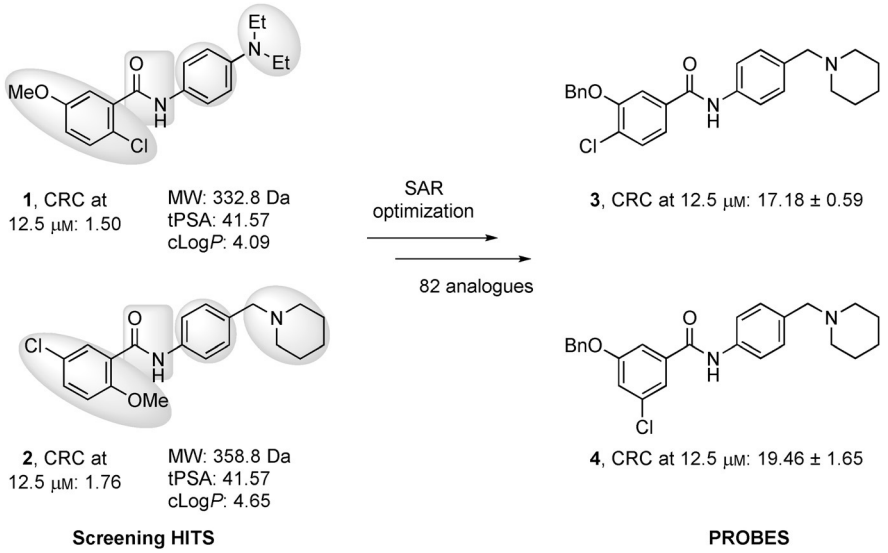


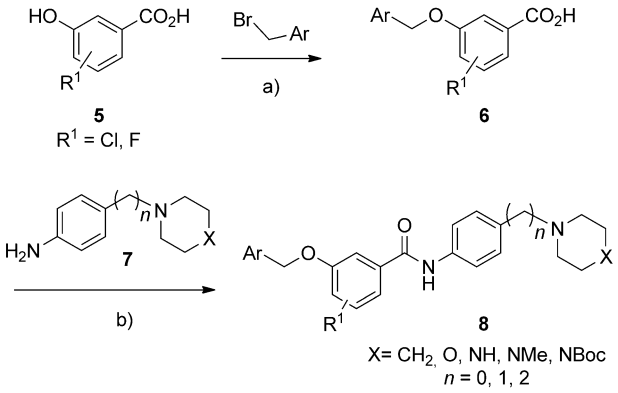
Figure 1. Probes **3** and **4** derived from screening hits **1** and **2**.

ment 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 analogues were assembled in a two-step process (Scheme 1). 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 methanol under reflux conditions to yield corresponding carboxylic acids **6** in 50–80% yield. Subsequent benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)-mediated amide coupling reactions with various substituted anilines **7** in the presence of Hünig's base and DMF under microwave conditions afforded the corresponding analogues (**8**) of the benzamide series.

An SAR campaign was carried out around the hit molecules, **1** and **2**, that led to the identification of a couple of very potent analogues, **3** and **4**, out of 82 analogues 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

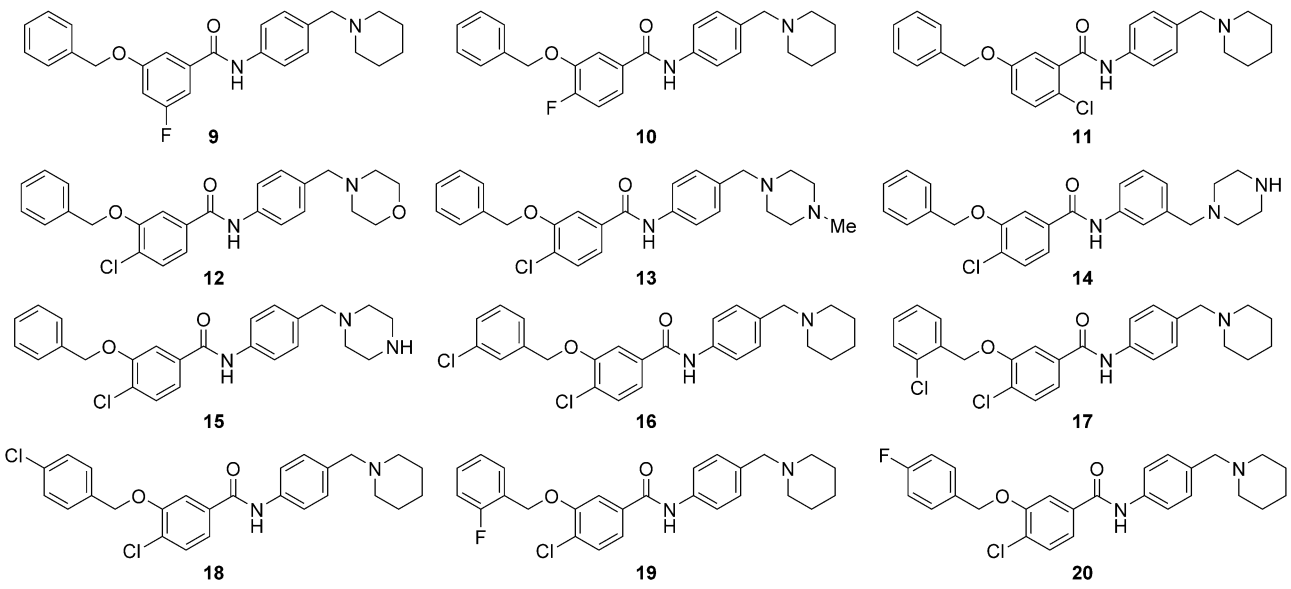


Scheme 1. General procedure for synthesis of the benzamide analogues. Reagents and conditions: a) 1. K_2CO_3, DMF, rt, 24 h; 2. 10 M aq. KOH, MeOH, 50 °C, 5 h (50–80% yield); b) PyBOP, *i*-Pr_2NEt, DMF, μW, 20–30 min (40–70% yield).

in Table 1. These studies revealed that replacement of the pendant piperidine with piperizines (**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 to afford compounds with any significant improvement in activity compared with **3** and **4** (Figure 1 and Table 2). As noted in Table 1, most of the analogues showed activity in the Rh123 uptake assay, suggestive of interference with the IMM, an untoward side effect. The strategy to replace the chloro with a fluoro substituent in the western aryl ring resulted in analogues **9** and **10** with a much-improved profile in the counter screen and CRC assays.

Clearly compounds **3** and **4** (Figure 1and 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 follow-

Table 1. Structure–activity relationship optimization of representative analogues.[a]



Compd	CRC/CRC$_0$ at 12.5 μM	EC$_{50}$ [μM] mitochondrial swelling	EC$_{50}$ [μM] Rh123 uptake	Compd	CRC/CRC$_0$ at 12.5 μM	EC$_{50}$ [μM] mitochondrial swelling	EC$_{50}$ [μM] Rh123 uptake
9	10.8 ± 0.6	0.521 ± 0.046	58.6 ± 2.9	**16**	6.50 ± 1.20	1.19 ± 0.33	23.2 ± 1.3
10	13.3 ± 1.0	0.372 ± 0.028	> 100.00	**17**	2.37 ± 0.41	4.00 ± 0.66	> 100
11	2.26 ± 0.11	3.60 ± 0.17	75.7 ± 1.8	**18**	5.91 ± 0.38	1.24 ± 0.39	24.7 ± 1.5
12	8.81 ± 0.39	0.403 ± 0.031	> 100	**19**	2.59 ± 0.54	1.39 ± 0.17	> 100
13	14.1 ± 1.1	0.423 ± 0.028	49.6 ± 4.3	**20**	10.1 ± 0.8	0.49 ± 0.08	28.6 ± 1.1
14	16.1 ± 1.5	0.286 ± 0.027	42.6 ± 3.1	GNX-865	7.67 ± 0.35	0.073 ± 0.007	> 100
15	18.3 ± 1.3	0.206 ± 0.021	46.4 ± 3.2	Cyclosporin A	4.40 ± 0.24	0.149 ± 0.009	> 100

[a] Data are the average ± SEM of ≥ 3 experiments.

Table 2. Comparison of activity and physicochemical properties, and summary of in vitro pharmacology of compounds **3** and **4**.

Parameter	Comparative data compd **3**	Comparative data compd **4**	Assessment	Result compd **3**	Result compd **4**
CRC/CRC$_0$ at 12.5 μM	17.2 ± 0.6	19.5 ± 1.7	Aqueous solubility in 1xPBS pH 5.0/6.2/7.4 μg mL^{-1} [μM][a]	26.8/7.0/0.2 [61.6/16.1/0.5]	22.8/22.6/0.64 [52.5/52.1/1.5]
EC$_{50}$ [μM] mitochondrial swelling	0.398 ± 0.025	0.280 ± 0.024	Chemical stability with 5 × DTT % parent remaining after 8 h[a]	83	91
EC$_{50}$ [μM] Rh123 uptake	36.5 ± 1.82	24.5 ± 1.12	Aqueous stability (1:1 PBS/acetonitrile) % parent remaining after 48 h[b]	89.3	93.4
MW[c] [Da]	434.9	434.9	Plasma-protein binding [% bound for human; mouse (1 μM/10 μM)][a]	99.0/99.0; 99.0/98.8	99.2/99.2; 99.3/98.3
tPSA[c]	41.5	41.5	Plasma stability (% remaining after 3 h) human; mouse[a]	44; 57	39; 35
cLog P[c]	6.2	6.4	PAMPA permeability, Pe (× 10^{-6} cm s^{-1}) Donor pH 5.0/6.2/7.4 Acceptor pH 7.4[a]	1281/1425/1276	1300/1339/und
HBA[d]	2	2	BBB PAMPA Permeability, Pe (× 10^{-6} cm s^{-1}) Donor pH 7.4 Acceptor pH 7.4[a]	381	113
HBD[d]	2	2	Hepatic microsome stability (% remaining after 1 h) human; mouse (+ NADPH/-NADPH)[a]	65/75; 9.6/80	61/67; 30/74
Heavy atoms[e]	31	31	Toxicity towards Fa2N-4 immortalized human hepatocytes LC$_{50}$ [μM][a]	30	32
LE[f]	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] Molecular weight (MW), topological polar surface area (tPSA) lipophilicity (cLog P) data were generated using CambridgeSoft ChemBioDraw, version 12. [d] Hydrogen-bond acceptors (HBA) and hydrogen-bond donors (HBD); data were calculated using SYBYL 8.0, Tripos Associates, St. Louis, MO (USA), 2010. [e] Data was calculated using Marvin 15.3.23.0, 2015, ChemAxon. [f] Ligand efficiency (LE).

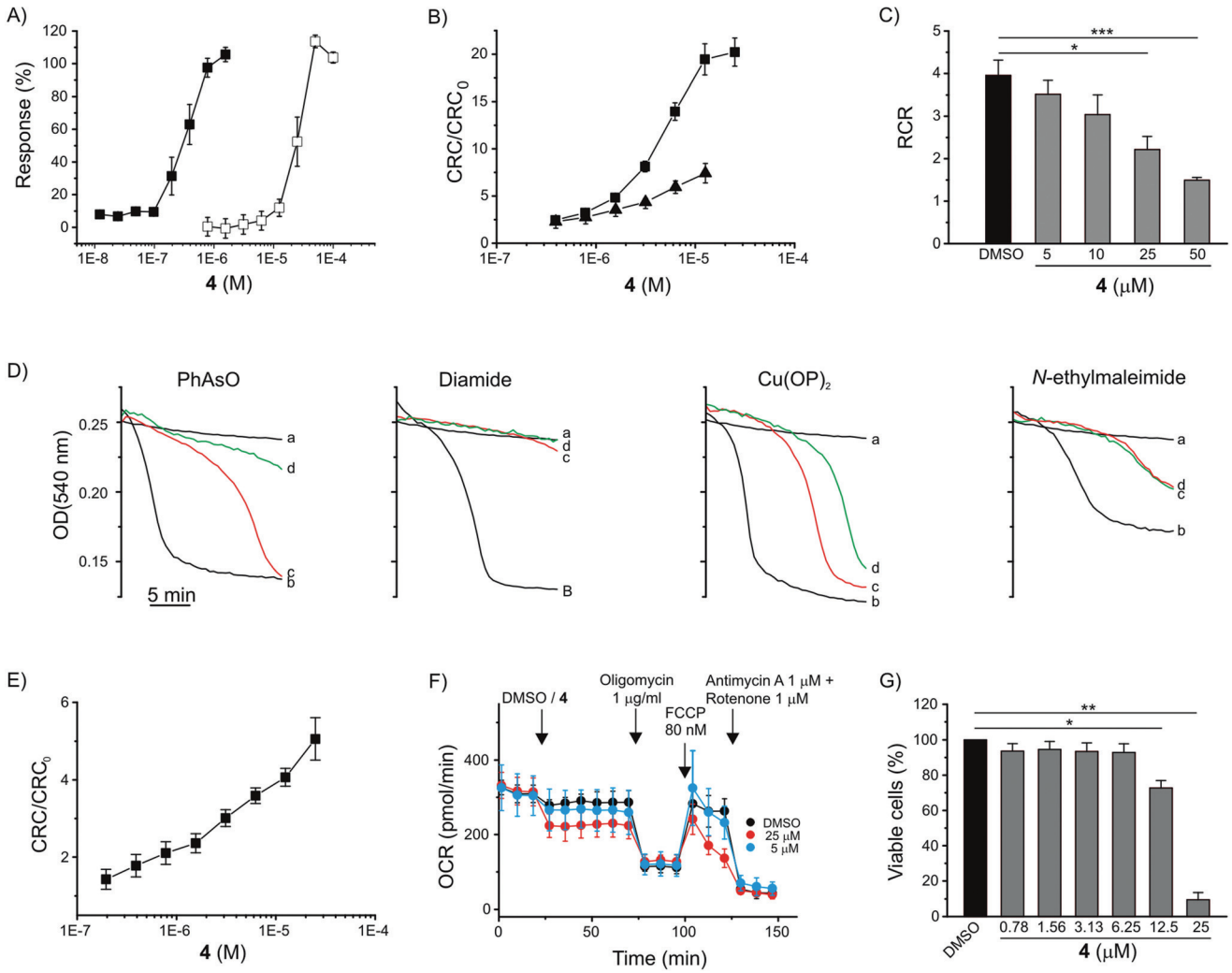


Figure 2. Effect of **4** on the permeability transition pore (PTP) and cell viability. (A) *Full symbols:* **4** prevents mitochondrial swelling induced with 50 μM Ca^{2+} in a concentration-dependent manner; *open symbols:* interference with Rh123 uptake upon treatment with compound **4**; (B) Concentration response of **4**-to-solvent CRC ratios of wild type (*squares*) and CyPD knock-out (*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 Ca^{2+} only, traces (a); 10 μM Ca^{2+} 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 h treatment with indicated concentration of **4**. Data are a representative (D, F) and an average ± SEM of ≥4 experiments.

ing uptake of Ca^{2+} (50 μM). In a full concentration–response study employing concentrations ranging from 12.2 nM to 1.56 μM, inhibition of swelling was demonstrated with an EC_{50} value of 0.280 ± 0.024 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 four 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 sevenfold 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 compound **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, traces a in Figure 2D) and then challenged with reagents that modify distinct classes of redox-sensitive

thiols (-SH) (traces b–d in Figure 2D), which affect the PTP sensitivity to Ca^{2+} and trigger pore opening: (i) matrix -SH groups that react with diamide and phenylarsine oxide (PhAsO),[23] (ii) inner membrane external thiols that react with copper(II) bis(1,10-phenanthroline) complex [$Cu(OP)_2$], and (iii) 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 in Figure 2D) and prohibited by the same concentration of **4** (traces d in Figure 2D), 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., $\leq$10 μM). 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 μM and $\leq$10 μM, respectively). However, at concentrations above 10 μM, it might cause cellular toxicity, as confirmed in HeLa (Figure 2G) and Fa2N-4 cells (last entry in Table 2).

We next examined the physicochemical and in vitro pharmacokinetic properties of analogues **3** and **4** (Figure 1 and Table 2). Analogues **3** and **4** displayed promising physicochemical parameters, possessing a desirable number of hydrogen-bond donors and acceptors, decreased topological polar surface area, a molecular weight of less than 500 Da and moderately favorable ligand efficiency. Although the cLog*P* values were generally high for **3** and **4** (above 6), analogues **14** and **15** had much decreased cLog*P* values around 4.9.

These key analogues were characterized further for in vitro pharmacology to create a baseline profile for future structure–property relationship (SPR) optimization efforts. Overall, analogues **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 central nervous system (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 analogues showed some degree of toxicity towards Fa2N-4 immortalized human hepatocytes, having LC_{50} values of 30 and 32 μM with 75-fold and 114-fold selectivity compared to the EC_{50} values for the mitochondrial swelling for **3** and **4**, respectively. Activity of these key analogues 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 EC_{50} value 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 Ca^{2+}- 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 μM) 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 analogues suitable for in vivo testing for efficacy in relevant disease models.

Acknowledgements

The authors gratefully acknowledge funding from the US National Institutes of Health (NIH) and Telethon–Italy. Chemistry efforts at the University of Kansas Specialized Chemistry Centerwere supported by NIH grant U54HG005031awarded to J. Aubé. Support for the University of Kansas NMR instrumentation was provided by an NIH Shared Instrumentation Grant (S10RR024664) and a US National Science Foundation (NSF) Major Research Instrumentation Grant (0320648). The authors thank Patrick Porubsky (University of Kansas) for compound management, and for aqueous and chemical stability data. Initial assay validation, high-throughput screening, and hit confirmation efforts at the Conrad Prebys Center for Chemical Genomics were supported by NIH grant U54HG005033 awarded to J.C. Reed. Funding for the biological assays was supported by NIH grants R03A033978 awarded to M. Forte, P. Bernardi and U54HG005031-05S1 awarded to J. Aubé, and by Telethon–Italy grant GGP14037 to P. Bernardi.

Keywords: calcium retention capacity · mitochondria · mitochondrial swelling · *N*-phenylbenzamides · permeability transition

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Received: November 20, 2015
Published online on December 23, 2015