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Parallel Synthesis, Evaluation, and Preliminary Structure−Activity Relationship of 2,5-Diamino-1,4-benzoquinones as a Novel Class of Bivalent Anti-Prion Compound

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A library of 11 entries, featuring a 2,5-diamino-1,4-benzoquinones nucleus as spacer connecting two aromatic prion recognition motifs, was designed and evaluated against prion infection. Notably, 6-chloro-1,2,3,4-tetrahydroacridine **10** showed an EC_{50} of 0.17 μM, which was lower than that displayed by reference compound BiCappa. More importantly, **10** possessed the capability to contrast prion fibril formation and oxidative stress, together with a low cytotoxicity. This study further corroborates the bivalent strategy as a viable approach to the rational design of anti-prion chemical probes.

Introduction

Transmissible spongiform encephalopathies, or prion diseases, are rapidly progressive, fatal, and untreatable neurodegenerative syndromes that can affect humans and animals. A key pathogenic event in prion diseases is the conversion of a host-encoded cellular prion protein, termed PrP^C,[a] into its abnormal amyloidogenic isoform, PrP^{Sc}, predominantly in the central nervous system of the infected host.[1,2] The cellular and molecular biology of the prion protein (PrP) remains enigmatic.[3] Several lines of evidence suggest that PrP^{Sc} acts as a conformational template by which PrP^C is converted to a new molecule of PrP^{Sc}, which in turn has a strong tendency to aggregate into insoluble amyloid fibrils.[2] The $PrP^C \rightarrow PrP^{Sc}$ conversion is interrelated with multiple molecular mechanisms involving reactive oxygen species (ROS) production, increased oxidation of lipids, proteins, and DNA and an imbalance of metal ions finally leading to neuronal death.[4,5] These mechanisms are found also in other similar conformational diseases.[6]

Despite the numerous efforts aimed at identifying compounds useful against prion diseases, there are still no therapies on the market. Therefore, it is of continued importance to identify chemical scaffolds to be exploited for the design of novel drugs.[7] Most of the anti-prion molecules that have been identified so far are derived from screening approaches. Structurally, diverse chemical compounds covering a broad range of the chemical space have been identified.[8] Intriguingly, most of them share a common bivalent structure. This is the case of the natural product curcumin,[9] the bis-acridine analogues,[10,11] the diphenylmethane derivative (GN8),[12] bebeerine,[13] bisepigallocatechin digallate,[13] 2,2′-bisquinolines,[14] 4,5-dianilinophthalimide,[15] analogues of Congo red,[16] and diketopiperazines (DKP).[17]

Although a structure−activity relationship is not easy to discern from such chemically unrelated compounds, we envisaged that bivalent ligands bearing lipophilic bi- or tri(hetero)cyclic scaffolds connected by a central core might possess anti-prion activity. Bivalency, and multivalency in general, is a well-known and efficient strategy widely used by medicinal chemists to enhance binding efficacy in molecular recognition processes.[18] Multivalent chemical probes, featuring multiple copies of an amyloid binding motif connected by a spacer, have been developed with the aim to simultaneously bind to several binding sites or several amyloid peptides, thus achieving higher potency.[19] In prion research, by joining two quinacrine moieties through a piperazine spacer, May et al. afforded the first bivalent antiprion ligand BiCappa (Figure 1SI), which was 100 times more potent than monomeric quinacrine.[10] Heterodimers incorporating recognition elements taken from quinacrine itself and imipramine with a piperazine unit were shown to improve the anti-prion efficacy of quinacrine up to a low nanomolar range.[20] Furthermore, assembling multiple acridine or curcumin moieties to a cyclopeptide scaffold has emerged as a promising strategy for the development of inhibitors against amyloid formation.[21,22]

Building on the bivalent approach, we have recently reported that a 2,5-diamino-1,4-benzoquinone (BQ) linked to

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[a] Abbreviations: BQ, 2,5-diamino-1,4-benzoquinones; CoQ, coenzyme Q; DKP, diketopiperazines; NQO1, NAD(P)H/quinone oxidoreductase 1; GT1, mouse hypothalamus cells; OS, oxidative stress; PPIs, protein−protein interactions; PrP, prion protein; PrP^C, normal cellular prion protein; PrP^{Sc}, infectious conformational form of prion protein; moPrP, recombinant mouse prion protein; PK, proteinase K; ROS, reactive oxygen species; ScGT1, scrapie-infected mouse hypothalamus cells; SFP, sulforaphane; TBARS, thiobarbituric acid-reactive substances; THA, 1,2,3,4-tetrahydroacridine.

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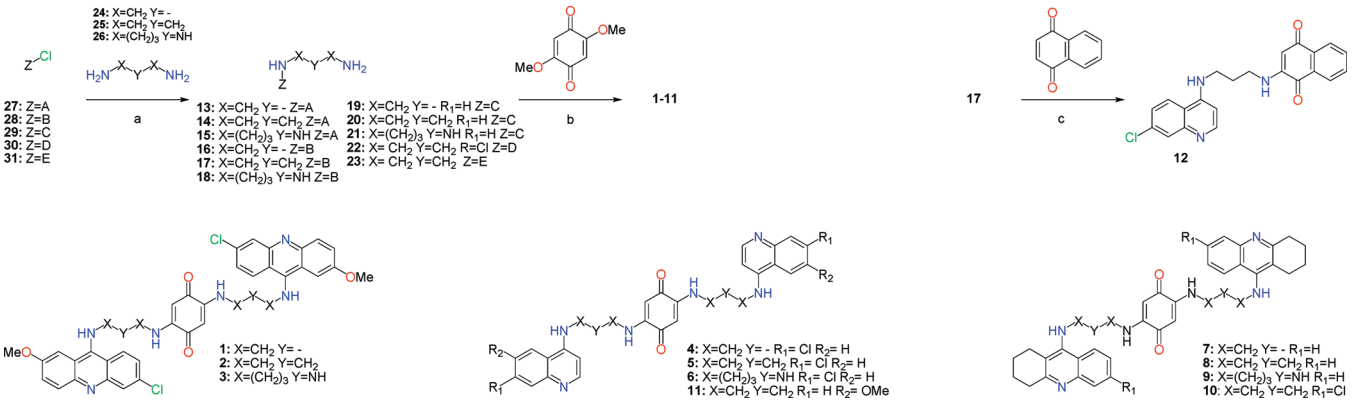
Scheme 1[a]



[a] Reagents and conditions: (a) phenol, NaI, 120 °C (1 h), followed by addition of amine, 5 h, 120 °C; (b) EtOH, 5 h, 60 °C (38−88% yield); (c) EtOH, 80 °C (1 h), followed by addition of amine, 5 h, 50 °C (40%). A = 6-chloro-2-methoxyacridine; B = 7-chloroquinoline; C = 1,2,3,4-tetrahydroacridine; D = 6-chloro-1,2,3,4-tetrahydroacridine; E = 6-methoxyquinoline.

two phenylalanine residues displayed remarkable anti-prion activity in a cellular model of prion replication.[23] In this compound, because of a resonance effect of the BQ ring, a hydrophobic and planar system is generated, which might interfere, through hydrophobic interactions, with aromatic residues critical to fibril formation.[23] This hypothesis is corroborated by the key role of planarity as a major determinant for anti-prion activity in a recently synthesized series of bivalent DKP.[17]

Altogether these results allowed us to propose that the planar BQ scaffold may be considered as a privileged motif in modulating protein−protein interactions (PPIs) and as a promising spacer in the search for more effective bivalent anti-prion chemical probes. On this basis, we have here designed a small combinatorial library of bivalent ligands whose general structure is depicted in Figure 2SI. The ligands feature the BQ nucleus as a central core, with two linkers in positions 2 and 5 connecting two terminal moieties. As linkers, we selected three polyamine chains (**24**−**26**, Scheme 1) that would allow exploring different lengths and chemical composition for the different molecules. This is of particular importance, since linker length has been shown by May et al. to be very critical against PrP^{Sc} formation for the bivalent acridines series.[10] As terminal moieties, starting from the consideration that aromatic groups provided the best activity in the previous series of BQ compounds,[23] we selected aromatic prion recognition motifs, such as 6-chloro-2-methoxyacridine (as in **1**−**3**, Scheme 1), 7-chloroquinoline (**4**−**6**), and 1,2,3,4-tetrahydroacridine (THA) (**7**−**9**). Several acridine and quinoline derivatives have been shown to inhibit PrP^{Sc} formation in infected cells and to bind PrP.[14,24,25] Given that the analogous THA-9-amine is active against yeast prion,[26] derivatives **7**−**9** were also designed. As a second step, on the basis of the remarkable profile shown by **5**, three other derivatives (**10**−**12**) were designed with the aim of further optimizing activity in the existing series of compounds. Herein, we present a solution-phase parallel synthesis of a library of bivalent BQ derivatives, which were chosen for their anti-prion activity in ScGT1 cells, together with their capability of inhibiting PrP^{Sc} aggregation and of reducing oxidative stress (OS).

Chemistry and Biology

We have previously reported how a disubstitution reaction of diamines with 2,5-dimethoxy-1,4-benzoquinone provides easy access to a variety of 2,5-diamino-1,4-benzoquinones.[27,28] Encouraged by the good yields and the straightforward work-up associated with this reaction, we decided to synthesize the designed bivalent **1**−**11** using a solution phase parallel synthesis approach. Eleven N-substituted polyamines (**13**−**23**, Scheme 1) were loaded with 2,5-dimethoxy-1,4-benzoquinone into different vessels of a carousel workstation. After heating at 50 °C for 5 h, the desired products formed in moderate to good yields (38−88%). Monovalent **12** was obtained by Michael reaction starting from naftoquinone and amine **17** (40%). The preparation of intermediates **13**−**23** was easily achieved treating in parallel fashion commercially available polyamines **24**−**26** with heteroaryl halides **27**−**31**. Compounds **13**−**23** were obtained in 25−67% yield by reacting a large excess of the polyamine with the corresponding heteroaryl halide (27:1) in phenol and using NaI as a catalyst (Scheme 1). In these conditions we were able to obtain selective monosubstitution at the terminal primary amino group of the polyamine, obviating the need for protection/deprotection of the other amino functionalities. Furthermore, we overcame the low-yield of common S_NAr reactions and the use of costly reagents of Pd-catalyzed amination methodologies.

A cell-screening assay was used to test anti-prion activity across the library of synthesized compounds. Prior, we determined the effects of library compounds on cell viability by calcein-AM assay, measuring viable ScGT1 cells after incubation in the drug-doped medium with various compound concentrations of 10 nM to 10 μM for 5 days (Table 1). Then their ability to reduce PrP^{Sc} in ScGT1 cells was determined by Western blot densitometry of the PK-resistant PrP^{Sc}, in comparison with BiCappa, used as a reference compound. For entries BiCappa, **2**, **5**, **6**, and **10**, we also calculated the EC_{50} values, which represent the effective concentrations for half-maximal inhibition. Cell viability at EC_{50} values were expressed as an average percentage of viable cells versus untreated control. In addition, for BiCappa, **2**, **5**, **6**, and **10**, the capability of inhibiting prion fibril formation was studied in vitro by using a previously reported amyloid seeding assay (Table 1, Figures 1 and 3SI).[24]

Results and Discussion

Preliminarily, the possible toxicity of **1**−**9** was assessed in ScGT1 cells. At 1 μM, the toxicity profiles among the library members varied from 1.5% to 114.8% (Table 1). Treatment with **1** and **3** decreased cell viability to 18.2% and 1.5%,

Table 1. Cell viability and anti-prion activity on ScGT1 cells of library compounds

Cpd	% of viable cells at 1 μM[b]	% of PrP^{Sc} inhibition at 1 μM[c]	EC_{50} (μM)[c]	% of viable cells at EC_{50}[b]	Lag Phase (hours)[d]
BiCappa	75.6 ± 7.1[a]	102.1 ± 2.7[a]	0.32 ± 0.03[a]	92.4 ± 6.2[a]	55 ± 7[*]
1	18.2 ± 1.2	ND			
	80.1 ± 6.3 (at 0.2 μM)	3.1 ± 0.3 (at 0.2 μM)			
2	65.5 ± 5.6	89.7 ± 5.1	0.68 ± 0.05	75.2 ± 8.4	45 ± 10[#]
3	1.5 ± 0.2	ND			
	65.8 ± 4.6 (at 0.2 μM[b])	5.4 ± 0.4 (at 0.2 μM[c])			
4	114.8 ± 7.9	6.2 ± 0.6			
5	100.4 ± 3.6	85.5 ± 3.9	0.73 ± 0.03	99.6 ± 2.7	53 ± 5[#]
6	105.0 ± 7.4	49.1 ± 2.2	1.2 ± 0.1	91.3 ± 4.2	40 ± 10[*]
7	108.0 ± 8.4	7.1 ± 0.9			
8	104.4 ± 5.6	3.6 ± 0.4			
9	95.4 ± 7.4	3.4 ± 0.2			
10	78.6 ± 5.2	105.3 ± 5.5	0.17 ± 0.01	101.5 ± 3.6	57 ± 6[#]
11	87.2 ± 5.8	4.7 ± 0.3			
12	94.3 ± 3.8	2.9 ± 0.1			

[a] Values are the mean of three experiments, standard deviations are given. [b] ScGT1 cells were cultured in DMEM with 10% FBS, plated 25000 cells in each well of 96-well plates. The compounds were dissolved in DMSO (100%) and diluted in PBS 1X before adding various concentrations (10 nM - 10 μM) and incubated for 5 days at 37 °C, 5% CO_2. The results were developed by calcein-AM fluorescence dye and read by microplate reader. [c] The effect of library compounds on inhibition of scrapie prion replication. ScGT1 cells were cultured in DMEM with 10% FBS, split 1:10 into Petri dishes and incubated for 2 days at 37 °C and 5% CO_2. Then, various compound concentrations (10 nM - 2 μM), being non-toxic for the cells, were added to the plates. After a 5-day incubation, proteins of cells were extracted, quantified, digested with proteinase K (PK), and western-blotted. [d] Prion fibril formation inhibitory activity in vitro (Control 45 ± 4 h). Statistical analysis was done by analysis of Student's t-test (n = 4); (*) $p \leq 0.05$, (#) $p \leq 0.01$.

respectively. Because of the toxicity shown, **1** and **3** were studied for their anti-prion activity at a lower concentration (0.2 μM), whereas the other library members were assayed at 1 μM. The synthesized **2**, **4**−**9** were found to cover a broad range of activity against PrP^{Sc} formation, with inhibition spanning from 3.4% to 89.7% (Table 1).

Compounds **1**−**3**, bearing an acridine moiety, displayed a general higher toxicity in the cell viability assay. **2** turned out to be the most active compound, with a submicromolar EC_{50} (0.68 ± 0.05 μM) and a percentage of viable cells at EC_{50} of 75.2%. A different toxicity profile was observed for quinoline derivatives **4**−**6**, which were not toxic to ScGT1 cells (cell viability of > 100% at 1 μM). Intriguingly, **5** and **6** showed also remarkable submicromolar EC_{50} values (0.73 ± 0.03 μM, and 1.2 ± 0.1 μM, respectively; Figure 3SI) comparable to that of BiCappa (0.32 ± 0.03 μM). To note, a series of bisquinolines with a polyamine linker have been already designed and tested in ScN2a cell line but showed a lower activity against prion infection (in the one-digit micromolar range).[25] This might confirm the design rationale, indicating that the presence of a BQ core is critical for activity. The replacement of the 2,6-disubstited acridine ring of **1**−**3** with the unsubstituted THA, as in **7**−**9**, resulted in a complete loss of activity, pointing out to a possible role for the aromatic substituents in the recognition process. Interestingly, these latter compounds did not show toxicity. For all the three series (**1**−**3**, **4**−**6**, and **7**−**9**), data from the cell-screening assay suggest that a linker length of three methylenes is important for optimal activity. Intriguingly, a similar trend was observed by May et al. in their series of analogous bivalent ligands.[10] Altogether, these preliminary results suggest that a specific length of the linker and the presence of a chlorine substituent on the prion recognition motifs might contribute to activity against PrP^{Sc} formation. Regarding toxicity, the presence of the acridine ring seems to be a major determinant, in line with the reported DNA intercalation properties of this heterocycle.[10] Conversely, quinoline and THA moieties do not confer cytotoxicity. Building on these considerations, we decided to synthesize a second set of compounds in which the effect of the substituents on the heteroaromatic ring was investigated by synthesizing

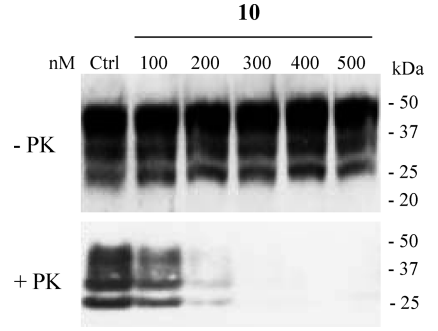


Figure 1. (A) Western blot of protease-digested ScGT1 cell lysates depicting the presence or absence of PrP^{Sc} after treatment with **10** before (up) or after (bottom) PK: Ctrl = control.

the 6-chloro-THA (**10**) and the 6-methoxyquinoline (**11**) derivatives. Furthermore, to probe the bivalent mechanism of action of **5**, its corresponding monomeric derivative **12** was designed.

From the biological studies (Table 1), as expected, quinoline **11**, lacking the chlorine atom, was not toxic against ScGT1 cells while displaying negligible activity against prion replication (inhibition of 4.7%). These results again point out the critical role played by the chlorine substituent of the aromatic ring. This speculation was further confirmed by the outstanding activity shown by **10**. In contrast to **8**, which does not carry the chlorine atom and is devoid of anti-prion activity, **10** showed a remarkable EC_{50} of 0.17 μM, which is the lowest among the present series of derivatives, even better than that of BiCappa. Remarkably, **10** showed a concomitant low toxicity (101.5% of viable cells at EC_{50} value) (Figures 1 and 3SI).

By comparing the dramatically different profiles shown by monovalent **12** and bivalent **5** (2.9% vs 85.5% of inhibition), we were able to provide the definitive proof of principle that two proper substituted aromatic prion recognition motifs connected by a BQ spacer are critical for activity.

To study the mechanism of action of the most active compounds (**2**, **5**, **6**, and **10**) at a molecular level, a fibrillation

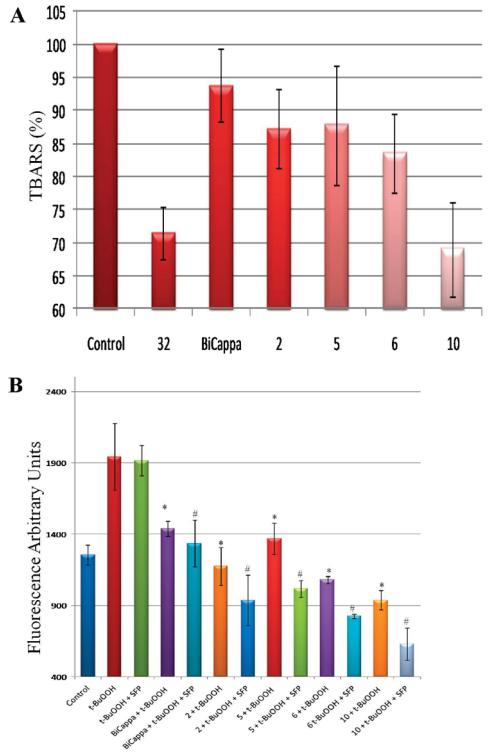


Figure 2. (A) Effect of **32**, BiCappa, **2**, **5**, **6**, and **10** (1 μM) on ScGT1, evaluated by TBARS formation. Values are the mean ± SD (n = 3). (B) Antioxidant activity of BiCappa, **2**, **5**, **6**, and **10** in ScGT1 cells against ROS formation induced by *t*-BuOOH. Experiments were performed with ScGT1 cells treated or not with 2.5 μM SFP: (∗) $p \leq 0.05$ with respect to *t*-BuOOH treated samples; (#) $p \leq 0.05$ with respect to *t*-BuOOH + SFP treated samples.

assay was used. Only **5**, **10**, and BiCappa, at 2 μM, exhibited significant PrP amyloid fibril forming inhibitory activity. In fact, they extended the lag phase to ≥53 h, showing significantly slower kinetics than the control (45 h, Table 1). These results, although preliminary, are in agreement with the starting hypothesis that bivalent ligands might interact directly with the recPrP to prevent its conversion to the misfolded PrP^{Sc} isoform. Furthermore, the idea that hydrophobic and planar molecular features are crucial for perturbing PPIs in the prion fibrillogenesis processes seems confirmed.[23] In addition, a key molecular determinant seems to be the presence of a chlorine substituent on the heteroaromatic terminal moieties.

The PrP^{Sc} infected cells are under OS, mainly caused by mitochondrial dysfunction.[29] In light of this, antioxidants might be beneficial against prion diseases.[30] Indeed, benzoquinones, such as coenzyme Q (CoQ), can scavenge ROS, and CoQ treatment has been proposed for prion and other neurodegenerative diseases.[4,31,32] Thus, we tested the antioxidant potential of the most active BQ derivatives (**2**, **5**, **6**, and **10**) in ScGT1 cell lines by using the thiobarbituric acid reactive substances (TBARS) assay and the a water-soluble derivative of vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, **32**) as a positive control.[5] The assay measures lipid hydroperoxides and aldehydes expressed as an average percent of TBARS of treated cells versus untreated cells. As shown in Figure 2A, **2**, **5**, and **6** displayed low antioxidant activity (83−87%) at 1 μM while **10** behaves similarly to **32** (69% vs 71%, respectively). As expected, BiCappa, which does not carry a BQ scaffold, did not show any antioxidant capacity (93%). We have previously demonstrated that the antioxidant property of related BQ derivatives[27,28] and CoQ itself concerns mainly their reduced hydroquinone forms. NQO1, an inducible enzyme that catalyzes the reduction of quinones to hydroquinones, was shown to be responsible for the production of the CoQ-reduced antioxidant forms, as well as that of BQ derivatives.[27,28] Therefore, since **2**, **5**, **6**, and **10** share the same BQ nucleus, their antioxidant activity was also evaluated in ScGT1, following exposure to *t*-BuOOH, and in the absence or presence of pretreatment with sulforaphane (SFP), an inducer of NQO1. Figure 2B clearly shows that **2**, **5**, **6**, and **10** (at 1 μM) in their oxidized forms show a basal antioxidant activity, but this activity was increased in cells pretreated with SFP, confirming that NQO1 is involved in the activation of BQ derivatives. As expected, the antioxidant activity of BiCappa is not influenced by the overexpression of NQO1.

Conclusion

A library of 11 symmetrical bivalent compounds was synthesized by solution phase parallel synthesis and tested against prion replication. Despite the small number of compounds, four of them (**2**, **5**, **6**, and **10**) were active against prion replication in the submicromolar range, whereas monovalent **12** showed negligible activity. These results confirmed the rationale for the design of bivalent anti-prion ligands. 7-Chloroquinolines (**5** and **6**) and 6-chloro-THA (**10**) derivatives showed a concomitant encouraging low toxicity. Notably, the EC_{50} of **10** was even lower than that displayed by BiCappa, which is a reference compound for prion diseases.[10] Furthermore, **10** showed the largest correlation between the cellular anti-prion activity and the capability of inhibiting PrP fibril formation. Interestingly, for **10** we could also find correspondence between anti-prion and antioxidant activities, in contrast to the results obtained by Miyata et al. in a series of very potent pyrazolone derivatives.[33] Although its mechanism of action is not fully disclosed (see docking studies in Supporting Information), we assume that the bivalent structure of **10** favors the interaction with prion recognition domains, whereas the spacer acts simultaneously as a disrupting element against PPIs and an effective antioxidant moiety. Remarkably, the 6-chloro-THA scaffold emerges as an effective and completely novel prion recognition motif.

In conclusion, the present series of molecules are chemical probes that may facilitate the exploration of the molecular mechanism underlying prion disease. We envisage that a better understanding of the molecular framework of bivalent ligands capable of inhibiting prion aggregation and OS would facilitate the creation of new effective anti-prion agents.

Experimental Section

Chemistry. All starting reagents were of the best grade available from Aldrich. Direct infusion ESI-MS spectra were recorded on a Waters ZQ 4000 apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 MHz (^{1}H) and 50.3 MHz (^{13}C) or at 400 MHz (^{1}H) and 100 MHz (^{13}C). Elemental analysis was used to confirm ≥95% sample purity, and the elemental compositions of the compounds agreed to within ±0.4% of the calculated value.

General Procedure b for the Synthesis of Library Members 1−11. In distinct reactors, 2,5-dimethoxybenzoquinone (1 equiv) was suspended in EtOH (15 mL) and heated to 80 °C until the solid was completely dissolved. After the mixture was cooled to 50 °C, the appropriate amines **13**−**20** (2 equiv) were added to each reaction mixture that became progressively clear and red. Each

mixture was heated at 50 °C for 5 h. After the mixture was cooled, precipitates formed, which were collected by filtration. The solids were dissolved in diethyl ether (7−10 mL), and trifluoroacetic acid (0.2 mL) was added to the solutions to obtain the corresponding trifluoroacetate salts.

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Supporting Information Available: Chemical characterization of **1**−**23** biological methods, and docking results. This material is available free of charge via the Internet at http://pubs.acs.org.

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