Library Design, Synthesis, and Screening: Pyridine Dicarbonitriles as Potential Prion Disease Therapeutics

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Transmissible spongiform encephalopathies (TSEs) or prion diseases are a family of invariably fatal neurodegenerative disorders, and there are no effective therapeutics currently available. In this paper, we report on the design, synthesis, and screening of a series of pyridine dicarbonitriles as potential novel prion disease therapeutics. A virtual reaction-based library of 1050 compounds was constructed. Docking and evaluation using GOLD scores assisted the initial selection of compounds for synthesis. The selection was augmented with further compounds to increase structural diversity. A total of 45 compounds were synthesized via a one-pot three-component coupling reaction. The mechanism of the three-component coupling reaction was investigated, and it was discovered that chemical oxidation is required for the last step, forming the pyridine ring (aromatization). A total of 19 compounds were identified as binders to one or more forms of prion protein by in vitro screening using surface plasmon resonance (SPR). A selection of compounds were investigated for activity in cells, resulting in the discovery of a new inhibitor of PrP^{Sc} formation.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a family of invariably fatal neurodegenerative disorders that are characterized by the appearance of large vacuoles in the cortex and cerebellum. These include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Creutzfeldt−Jacob disease (CJD) in humans.[1] They have attracted intense interest in recent years since a new variant of Creutzfeldt−Jacob disease (vCJD) was discovered in humans, which is thought to have been triggered by the consumption of contaminated beef products.[2−4] Iatrogenic transmission has also been observed via contact with contaminated neurosurgical instruments, tissue grafts, and blood products.[5] As yet, no effective therapy exists for the prevention of either infection or disease progression.[6]

TSEs are associated with a posttranslational conversion of the cell-surface glycosylphosphatidylinositol (GPI)-anchored protein PrP^{C} to its partially protease-resistant isoform PrP^{Sc}.[7] As part of an ongoing medicinal chemistry project, we are looking for compounds that bind to human PrP^{C} ($huPrP^{C}$) and stabilize it against this conversion with the aim of identifying novel prion disease therapeutics.

Pyridine dicarbonitrile **1**, along with three analogues **2**−**4** (Figure 1), were reported to inhibit PrP^{Sc} accumulation in scrapie-infected mouse neuroblastoma cells (ScN2a).[8] However, the mode of action for these compounds is not understood. As part of a larger screening program hunting for affinity binders for cellular prion protein (PrP^{C}), these compounds were analyzed in silico and by SPR for binding to $huPrP^{C}$.

Our modeling studies indicated that all four compounds fitted well into our proposed binding site,[9] and the SPR screening results showed moderate binding of compound **3** and weak binding of compound **2** to PrP^{C}.[10] The pyridine dicarbonitrile substructure was therefore chosen as a basic structural scaffold for the design of a reaction-based library. In this paper we report details on the design, synthesis, and mechanistic investigation of this novel series of pyridine dicarbonitriles and examine their binding to or interaction with $huPrP^{C}$, truncated human PrP^{C} ($t\text{-}huPrP^{C}$), and murine PrP^{C} ($moPrP^{C}$).

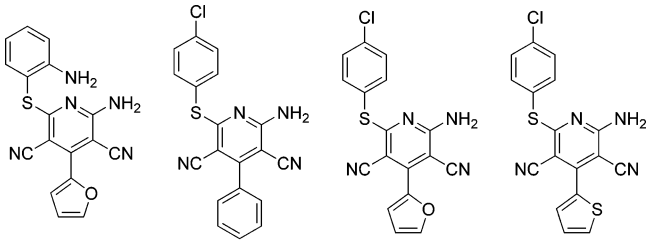

Figure 1. Pyridine dicarbonitriles reported as active in mouse ScN2a cells.[8]

Results and Discussion

Docking. The hypothetical $huPrP^{C}$ binding pocket used in our virtual screening was identified from SYBYL-Molcad surfaces generated from the PrP^{C} NMR structures available from the Brookhaven protein data bank (1QM3, conformer 15).[9] Compounds **1**−**4** were docked and scored using the genetic optimization for ligand docking (GOLD) program, which resulted in docking scores of 62, 72, 69, and 70, respectively. These scores indicate that compounds **1**−**4** fit well in the binding pocket (Figure 2). It can be seen that both nitrile groups are interacting with the protein. Based on these results, a virtual reaction-based library of 1050 compounds from 21 arylthiols and 50 arylaldehydes was generated. They were also docked and scored, allowing compound selection for synthesis.

Library Design. The development of reaction-based library design techniques has allowed access to smaller, more focused libraries and, because the process is based on the actual chemical steps of a synthesis, reduces problems of synthetic accessibility. Using this approach, a virtual library of pyridine dicarbonitriles was prepared.

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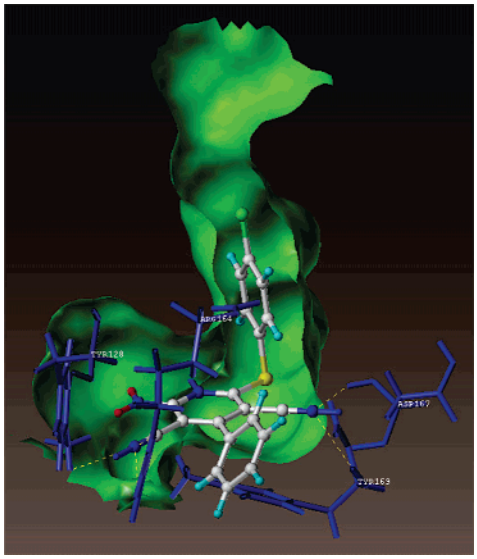
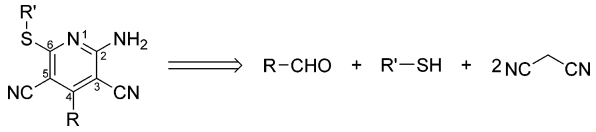


Figure 2. Binding pose of compound **2** (ball-and-stick) as predicted by GOLD within the hypothetical binding pocket (green). The four amino acids involved in hydrogen bonding, tyr-128, agr-164, asp-167 and tyr-169, are shown (blue), and hydrogen bonds are represented by yellow dotted lines.

Scheme 1. Reterosynthesis of Pyridine Dicarbonitriles (core structure numbering shown)



Elghandour et al. have reported a synthesis of pyridine dicarbonitriles from an aldehyde, a thiol, and 2 equiv of malononitrile (Scheme 1).[11] A restricted substructure search of the Available Chemicals Directory (ACD) provided a total of 21 arylthiols and 50 arylaldehydes. In combination, these reagents were used to construct an initial virtual library of 1050 compounds. This library was docked and scored using GOLD as described above, and analysis of the predicted binding pose, along with Lipinski's rule of five,[12] was used in the selection of 45 compounds for synthesis.

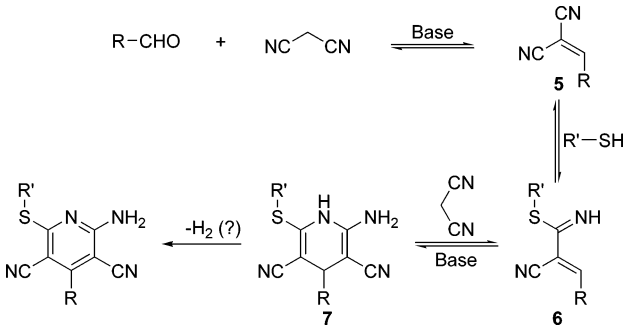
Synthesis. When the one-pot, one-step procedure was applied to our starting materials, the yields were significantly lower than the 75% reported for 2-amino-4-ethyl-6-phenylsulfanylpyridine-3,5-dicarbonitrile (Scheme 1).[11] For this reason, a simple variation on the one-step, one-pot procedure was employed for the investigation of the reaction conditions.

The reported mechanism of pyridine dicarbonitrile formation (Scheme 2) involves the attack of a malononitrile anion upon the aldehyde followed by elimination of water to form adduct **5**, which is followed by addition of thiol to form **6**. Subsequent addition of a second malononitrile anion provides penultimate intermediate **7**, which is followed by (according to Kambe et al.[13]) loss of molecular hydrogen to furnish the product.

Initially, synthesis was therefore carried out by a stepwise addition of reagents, product **8** being prepared in 34% yield. For expediency, this procedure was applied to the parallel synthesis of 36 pyridine dicarbonitriles without further optimization (Tables 2 and 3).

However, we had doubts about the accuracy of the proposed loss of molecular hydrogen as the last stage of the reaction as the mechanism of this process was not clear, and evolution of gas was not observed during the course of the reaction. It was postulated that the required loss of two hydrogen atoms may

Scheme 2. Reported Mechanism for Pyridine Dicarbonitrile Synthesis[5]

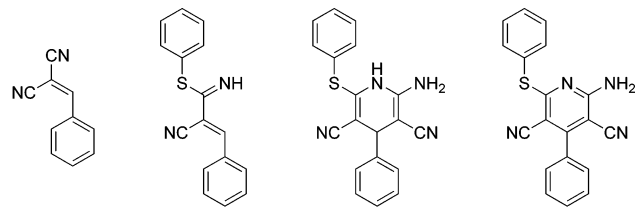


be by aerobic oxidation. To probe the reaction mechanism, a stepwise reaction was carried out under nitrogen. The formation of product as a crystalline solid was normally observed during the final period at reflux. However, in the absence of air, no product formation was observed. On admission of air to the system, product crystallization was observed in the normal way giving **8** in 36% yield. Interestingly, after carrying out the reaction under nitrogen, oxidation could also be achieved using a stoichiometric amount of a 1 M aqueous solution of $KMnO_4$. This resulted in the formation of **8** in 44% yield.

This experiment was repeated with sampling for analysis by mass spectroscopy at each stage of the procedure. Molecular ions from intermediates **9**, **10**, and **11** were observed to be present, but product **8** was only discernible on contact with air (Figure 3). These experiments confirm the presence of the expected intermediates but also show that chemical oxidation is required for the final aromatization of the pyridine ring.

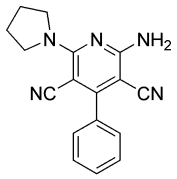
As part of the optimization of the synthesis of pyridine dicarbonitriles, the single-step, one-pot procedure was also investigated further. Heating a solution of benzaldehyde, thiophenol, and 2 equiv of malononitrile in ethanol with a catalytic amount of piperidine at reflux for 3 h resulted in the formation of **8** in 43% isolated yield. The effects of reaction time, overall reaction concentration, ratios of reagents, and reaction temperature were also investigated for their effect on the yield. It was found that the ratios of 1:1:2 for the aldehyde, thiol, and malononitrile, respectively, were indeed optimum. It was also found that both doubling and halving the volume of solvent used had no discernible effect on the yield. Changing the solvent from ethanol to ethylene glycol, to allow access to higher temperatures (130 °C), resulted in a 20% drop in yield. The most significant result came from variation of the reaction time. Reducing the reflux period from 3 h to 1 h gave a yield of 40%, but increasing the reaction to 15 h at reflux raised the yield to 48%. Consequently, subsequent reactions were allowed to reflux overnight.

In an effort to raise the yield further, several alternative bases were evaluated. Twenty-four reactions were carried out in parallel, with ethanol as solvent, and the yields calculated from HPLC analysis of the crude reaction mixture. Piperidine (used



9, Mol. Wt.: 154 **10**, Mol. Wt.: 264 (Plus tautomers) **11**, Mol. Wt.: 330 **8**, Mol. Wt.: 328

Figure 3. Intermediates observed by mass spectroscopy.



12

Figure 4. Observed side product.

Table 1. Investigation of Bases in Both Catalytic and Stoichiometric Amounts

entry	base[a]	no. of equiv	calcd yield [%] of **8**[b]	isol yield [%] of **8**[c]
1	none	–	0	
2	piperidine	0.1	34	48
3	piperidine	1.1	28	
4	morpholine	0.1	29	
5	morpholine	1.1	27	
6	thiomorpholine	0.1	32	
7	pyrrolidine	0.1	29	
8	pyrrolidine	1.1	0	
9	N,N-DIPEA	0.1	41	31
10	N,N-DIPEA	1.1	34	
11	Et_3N	0.1	42	30
12	Et_3N	1.1	31	
13	pyridine	0.1	0	
14	pyridine	1.1	0	
15	2,4,6-collidine	0.1	15	
16	2,4,6-collidine	1.1	24	
17	DMAP	0.1	38	35
18	aniline	0.1	1	
19	*N*-methylaniline	0.1	0	
20	*N*,*N*-dimethylaniline	0.1	0	
21	*N*,*N*-dimethylaniline	1.1	0	
22	*N*,*N*-diethylaniline	0.1	0	
23	K_2CO_3	1.1	0	
24	NaOH	1.1	0	

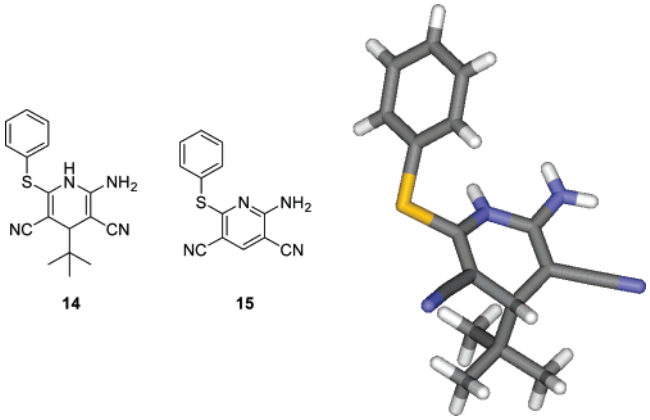
[a] N,N-DIPEA = *N*,*N*-diisopropylethylamine, DMAP = *N*,*N*-dimethylaminopyridine. [b] Calculated from HPLC data. [c] Isolated yield of **8**.

initially), triethylamine, N,N-DIPEA, and DMAP gave the highest yields (Table 1). It can be seen that, with the exception of 2,4,6-collidine, the bases performed better in catalytic amounts. The use of 0.1 equiv of pyrrolidine gave the desired product, whereas the use of 1.1 equiv led to the formation of known product[14] **12** in 52% yield (Figure 4), which may partially explain why the yields of the desired products were, in most cases, lower when stoichiometric amounts of base were used.

To investigate the ease of product recovery, reactions with the four best bases (at 0.1 equiv) were carried out and calculated yields compared to isolated yields. It was found that the use of piperidine gave **8** in the highest isolated yield of 48% (Table 1). The disparity between the calculated and isolated yields may be due to HPLC sample dilution factors. On the basis of these investigations it was decided to continue using piperidine at 0.1 equiv as catalyst.

No further optimization was attempted in the light of recent publications. Chang and co-workers reported the stepwise synthesis of a small library of pyridine dicarbonitriles resulting in yields ranging from 20 to 50%.[15,16] It was therefore presumed unlikely that our yields could be improved significantly. However, although our yields are comparable, the one-step, one-pot procedure offers many advantages.

Library Synthesis. The compounds selected for synthesis from the virtual library were augmented with a view to increasing the structural diversity of the library. Pentanethiol, *N*-acetylcysteamine, and benzylthiol were included to modify position 6. Substitution at position 4 was varied further by the inclusion of 2-thiophenecarboxaldehyde and 2-furaldehyde.



14 **15**

Figure 5. Compounds **14**, **15**, and X-ray crystal structure conformation of **14**.

The pyridine dicarbonitrile library was prepared using a 24-well parallel synthesis apparatus. The stepwise procedure was used initially, as described above. However, once it was found that the one-step, one-pot procedure offered improved yields, subsequent syntheses were approached in this way.

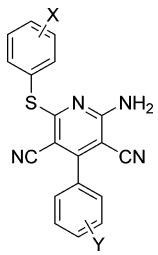
It was found that the outcome of the syntheses was influenced to a greater extent by the aldehyde, rather than the thiol. The use of 3-methoxy- and 4-methoxybenzaldehydes, 4-formylbenzoic acid methyl ester, and 4-methanesulfonylbenzaldehyde consistently gave relatively high yields. The use of both 3-fluoro-4-hydroxy- and 3-chloro-4-hydroxybenzaldehydes furnished product in low yields. All of the syntheses attempted with 3,4-di- and 3,4,5-trihydroxy aldehydes failed to give the desired products. This was also the case with 2-furaldehyde.

Interestingly, in the attempt to synthesize *tert*-butyl compound **13**, although the desired compound was not formed, penultimate intermediate **14** was isolated in 32% yield (Figure 5). This compound was stable in air. Attempts to oxidize **14** under the $KMnO_4$ conditions described above resulted in dealkylation as well as aromatization giving pyridine dicarbonitrile **15** in 77% yield. This was also the case when pyridinium chlorochromate (PCC) was used, giving **15** in 47% yield. However, oxidation was achieved by treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) giving **13** in 25% yield.

Screening (SPR). Screening was carried out against three forms of prion protein: $huPrP^C$, $t\text{-}huPrP^C$, and $moPrP^C$. The interactions between the dicarbonitrile ligands and the prion proteins were measured using SPR technology. The protein was covalently bound to a carboxymethylated dextran surface, and the ligands were dissolved at 40 μM in phosphate buffer containing 6.5% (v/v) DMSO. The ligand was passed over the immobilized protein, and real-time association/dissociation was recorded as sensorgrams. Visual inspection of the sensorgrams was performed to identify compounds which were interacting with the surface rather than the protein. These compounds were removed from the list due to uncertainty over the degree of binding. Responses of less than 2.5 response units (RU) were regarded as not binding, and they have been represented as $0\%RU_{max}$. The observed equilibrium binding was expressed as a percentage of maximum binding for a 1:1 interaction ($\%RU_{max}$). DMSO correction was performed to account for small variations between the DMSO concentration of the running buffer and the sample solution.

Previously a considerable increase in binding has been observed with the age of the chip.[10,17] For this reason all of the compounds reported here were screened in two runs, only allowing one injection per compound, enabling screening of all

Table 2. Synthesis and Screening Results[a]



compd	X	Y	yield [%]	huPrP^C [%RU$_{max}$]	t-huPrP^C [%RU$_{max}$]	moPrP^C [%RU$_{max}$]
8	−	−	43[b]	0	0	0
19	3-Cl	−	18	0	0	0
2	4-Cl	−	−[c]	0	11.4	0
50	3-OH	−	10	70.3	37.6	60.6
51	4-OH	−	16	0	0	0
52	3-NH_2	−	17	8.0	0	8.8
53	4-NH_2	−	11	7.7	0	9.5
48	3-CO_2CH_3	−	3	125.3	26.8	33.2
49	4-CO_2CH_3	−	23	0	0	0
20	−	3-OCH_3	47[b]	0	0	0
21	−	4-OCH_3	34[b]	0	0	0
22	4-OH	3-OH	20	34.0	23.1	43.1
23	4-OH	4-SO_2CH_3	31[b]	0	0	0
24	4-OH	4-CHO	27	−[d]	−[d]	−[d]
25	4-OH	3-F, 4-OH	8	44.2	28.0	53.8
26	4-OH	3-Cl, 4-OH	3	53.4	33.4	64.6
27	4-OH	4-$COCH_3$	6	0	0	0
28	4-OH	4-CO_2CH_3	18	0	0	0
16	4-OH	4-OH	25	−[d]	−[d]	−[d]
29	3-Cl	4-Cl	25[b]	118.7	56.5	52.3
30	3-Cl	3-OCH_3	47[b]	8.6	6.7	0
31	3-Cl	4-OCH_3	40[b]	0	0	0
32	3-Cl	4-CO_2CH_3	46	0	0	0
37	3-CO_2CH_3	4-CO_2CH_3	52	0	0	0
38	4-CO_2CH_3	4-OH	3	0	0	0
39	4-CO_2CH_3	4-CO_2CH_3	14	0	0	0
33	2-F	3-OH	17	17.2	9.2	18.8
17	2-F	3-Cl, 4-OH	3	−[d]	−[d]	−[d]
35	4-CO_2H	4-CO_2CH_3	2	5.9	0	8.5
36	4-$NHCOCH_3$	4-CO_2CH_3	47	9.4	6.4	13.3
34	4-$NHCOCH_3$	2,6-F	3	37.3	20.0	40.1
40	4-$NHCOCH_3$	3,5-F	25	0	0	0

[a] Compounds screened at 40 μM for binding to three forms of PrP^C. [b] Prepared by the one-step, one-pot procedure. [c] Compound purchased from Maybridge, UK. [d] Measurement not possible due to interference with the chip surface.

the compounds within 48 h. Quinacrine was used as a standard at the start and end of each run to quantify the change of binding over time.[18] The standard deviation for quinacrine over the two runs was 11.6%, and this value was assumed to represent the error margin for all the other compounds.

The results are shown in Tables 2 and 3. It was observed that the chip surface interfered with the binding of four compounds (**16**, **17**, **18**, and **24**); therefore, their binding data has been omitted from the tables as it was deemed unreliable. The remaining compounds included 19 binders and 24 non-binders. Most of the binders showed a similar level of interaction with huPrP^C and moPrP^C, whereas the binding toward t-huPrP^C was generally lower.

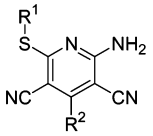
To identify structural motifs important for binding, the compounds were ordered according to their pattern of substitution. The compounds shown in Table 2 were built around the core structure of compound **8**, which was found not to be binding, where the aryl groups of both aldehyde and thiol precursors are unsubstituted. Compounds arising from functionalized aryl thiols and benzaldehyde showed good binding where X = 3-OH and 3-CO_2CH_3, whereas no interaction was detected for X = 4-OH and 4-CO_2CH_3. However, weaker binding was observed where X = 4-Cl, 3-NH_2, and 4-NH_2. In the case of compound **19**, no interaction with the proteins was detected, as was the case for compounds **20** and **21**.

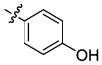
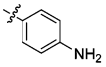
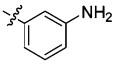
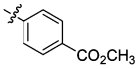
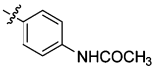
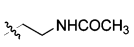
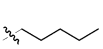
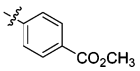
Compounds arising from 4-mercaptophenol where Y = 3-OH, (3-F, 4-OH) and (3-Cl, 4-OH) (**22**, **25**, and **26**) showed good binding, whereas in the cases of **27** and **28** no binding was observed. This indicates that where X = 4-OH substitution at the 3 and/or 4 position of the aryl aldehyde is required for binding; however, no clear commonality could be discerned among the favored functional groups. Compound **29**, derived from 3-chlorothiophenol and 4-chlorobenzaldehyde, bound to huPrP^C strongly, whereas compounds **30**, **31**, and **32** showed weak or no binding.

Of the remaining compounds shown in Table 2, compounds **33** and **34** displayed moderate binding, compounds **35** and **36** bound only weakly, and compounds **37**, **38**, **39**, and **40** showed no binding at all.

The compounds shown in Table 3 depart from the core structure of **8** in that they originate from an aliphatic thiol and/or aldehydes bearing a heterocycle, or in the case of **13**, pivalaldehyde. Of the compounds bearing a thiophene at the 4-position only two, compounds **41** and **42**, showed significant interaction with all three forms of PrP^C. The para- to meta-substitution effect, seen with several compounds in Table 2, is

Table 3. Synthesis and Screening Results



Compd	R^1	R^2	Yield [%]	huPrP^C [%RU$_{max}$]	t-huPrP^C [%RU$_{max}$]	moPrP^C [%RU$_{max}$]
13		-^{t}Bu	25[d]	0	0	0
18			18	-[c]	-[c]	-[c]
4			-[b]	0	16.7	0
54			27	0	0	0
55			9	0	0	0
56			22	0	0	0
41			23	123.6	91.8	72.2
57			17	0	0	0
43			9	0	0	0
42			19	55.5	22.8	52.6
44			19	0	0	0
45			25	96.5	50.8	112.2
58			43	9.0	9.6	0
46			34[a]	0	0	0
47			20[a]	0	0	0

Compounds screened at 40 μM for binding to three forms of PrP^C. [a] Prepared by the one-step, one-pot procedure. [b] Compound purchased from Maybridge, UK. [c] Measurement not possible due to interference with the chip surface. [d] After oxidation of **14** with DDQ.

also observed between compounds **4** and **41**, with the binding raised from 0 to 123.6%RU$_{max}$ respectively. Surprisingly, compounds **4** and **2** (Table 2) showed weak binding to t-huPrP^C but none to huPrP^C. One hypothesis for this anomalous behavior could be that these compounds are interacting with a binding region created by the truncation of the protein.

All of the compounds synthesized with benzylthiol showed binding to huPrP^C with the exception of **44**. The most pronounced binding was seen with **45**, bearing a 3-OH substituent on the aryl aldehyde, followed by **42**. Compounds **46**, **47**, and **13** failed to interact with the protein in all three forms, as did all intermediates and side products isolated (**12**, **14**, **15**, data not shown in tables).

From the data presented above, it may be suggested that the nature of the substituent at the 4-position of the pyridine has less of an influence on binding than that on the thioether at the 6-position. To some extent this was predicted by the binding poses generated in silico, where the thioether resides deep inside the hypothetical pocket and the group at position 4 is placed toward the opening.

A good drug candidate is expected to have a fast and strong association, but a reasonably slow dissociation to maintain a certain drug concentration (Figure 6A). Both fast association and dissociation is not ideal (Figure 6B). The binding comparison above concerned only association, without taking dissociation into consideration. To characterize the interactions between prion protein and the ligands in more detail, the binding data was further analyzed with respect to R1 and R2 and presented in Figure 6C.

Most of the data points are concentrated along the *y* axis, implying a rapid dissociation. However, compounds **25**, **29**, **36**, **41**, **42,** and **48**, where R1 was >10%, showed a slower rate of dissociation. Compounds **25**, **36**, and **42** only showed significant R2 values when binding to one form of PrP^C, whereas compounds **29**, **41**, and **48** exhibited higher R2 values upon

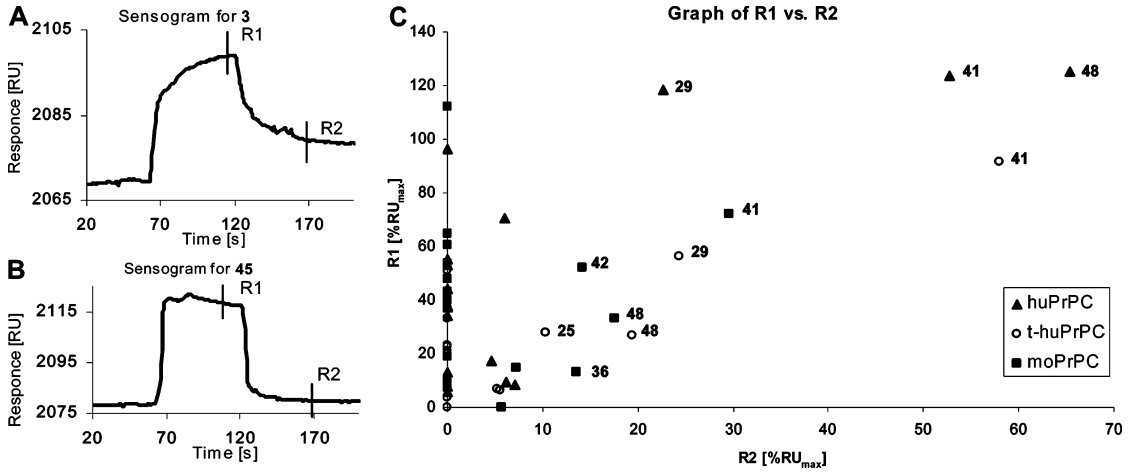


Figure 6. A: Sensorgram from **3** to illustrate association response 1 (R1) and dissociation response 2 (R2) implying slow dissociation. B: Sensorgram from **45** to illustrate R1 and a low R2 implying fast dissociation. C: Graph of R1 vs R2 (Compounds with slow dissociation are labeled).

interaction with both forms of human PrP^C. Interestingly, all of the latter compounds bear meta substitution on the arylthio moiety.

In summary, compounds **25**, **29**, **36**, **41**, **42**, and **48** were deemed to be the most promising candidates for demonstrating biological activity, and as such, were subjected to cell-line assay.

Cell-Line Screening. Biological assays were carried out on SMB cells of mesodermal origin, originally established by the culture of mouse brain clinically affected by Chandler scrapie isolate. The procedure used was based upon that reported by Rudyk et al.[19] Full experimental details can be found in the Supporting Information.

Compounds **25**, **29**, **36**, **41**, **42**, and **48**, together with literature compound **1**, were screened at 24 μM for inhibition of PrP^{Sc} formation. Perrier et al. reported **1** as active in ScN2a cells with an IC_{50} of $\sim$20 μM,[8] so it was noted with interest that, in our SMB cell lines, compound **1**, along with **25**, **29** and **48**, showed no activity. Compounds **36** and **41** were found to be toxic. However, at 24 μM, compound **42** proved positive for PrP^{Sc} inhibition. It is interesting to note that this compound had a favorable R1/R2 ratio as discussed above, and in our assay, demonstrated a higher potency than **1**.

Conclusions

In conclusion, the synthesis of pyridine dicarbonitriles has been thoroughly investigated leading to an improved understanding of the reaction mechanism. Although exploration of the reaction conditions did not lead to very high yields, a library of 45 pyridine dicarbonitriles was synthesized and screened for binding to three forms of PrP^C. A total of 19 compounds were found to bind to $huPrP^C$, and 6 displayed the slow dissociation characteristics expected in drug candidates. One compound, **42**, showed a higher activity than literature compound **1**. The pyridine dicarbonitriles therefore represent a class of compounds worthy of further investigation as potential prion disease therapeutics.

Experimental Section

Modeling. All the ligands were prepared for GOLD docking using SYBYL 6.9 and used as MOL2 files. Energy minimization was achieved by using steepest descent and conjugate gradient methods prior to docking. Molecular docking studies were performed using GOLD 2.1 in 10 independent genetic algorithm (GA) runs, and for each of these a maximum number of 100000 GA operations were performed on a single population of 100 individuals. Crossover, mutation, migration (95, 95 and 10 respectively), hydrogen bonding (4.0 Å), and van der Waals (2.5 Å) parameters were used as default. A docking sphere was placed within the hypothetical binding pocket of the PrP^C [1QM3 (15)] with a user-defined origin and a radius of 15 Å.

Commercially available structures for the virtual library design were retrieved from the ACD. SMARTS (SMiles ARbitrary Target Specification) strings were written consisting of the following features: any thiophenol or benzaldehyde with any of the following substituents at either or both the meta- and para-positions, or any single substituent at either position (Cl, F, OH, $NHCH_3$, $COCH_3$, $NHCOCH_3$, CO_2CH_3, CO_2H). To avoid steric clashes with the rest of the molecule, only fluorine was allowed at the ortho-position. This resulted in a total of 21 arylthiols and 50 arylaldehydes within chosen cost parameters. In combination, these reagents were used to construct an initial library of 1050 compounds using Legion 6.8 (SYBYL module). 2-Amino-6-(phenylthio)-4-phenylpyridine-3,5-dicarbonitrile was defined as a product core structure. Benzaldehyde and thiophenol were defined as reactant cores for their respective analogues. Groups that were not part of the core were labeled as R_1, R_2, R_3, R_4, R_5. These R groups were extracted as fragment SLNs. Mapping of the reactants to the product was carried out, and the library was generated.

SPR Screening. Screening was performed using a BIAcore 3000 (BIAcore, Uppsala, Sweden) equipped with a CM5 sensor chip.[10] *N*-Hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide hydrochloride (EDC), 1 M ethanolamine, HBS-EP buffer, surfactant P20, and regeneration solution (10 mM glycine-HCl, pH 3.0) were purchased from BIAcore. Sodium phosphate, ethylenediamine tetraacetic acid (EDTA), sodium chloride, sodium hydroxide, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. $HuPrP^C$, $t\text{-}huPrP^C$, and $moPrP^C$ were kindly provided by the Institute for Animal Health (Compton, UK).

HBS-EP buffer was used for immobilization of the protein. The CM-dextran on the sensor chip was activated by mixing equal volumes of 100 mM NHS and 400 mM EDC followed by injection of the mixture over the chip surfaces for 7 min at a flow rate of 5 μL/min. The $huPrP^C$, $t\text{-}huPrP^C$, and $moPrP^C$, each at a concentration of 2 μg/mL, was injected through flow cells 2, 3, and 4 for 7 min. Flow cell 1 was used as a control channel. The surfaces were washed thoroughly immediately after the ethanolamine blocking step with three consecutive injections of a mixture of 25 mM NaOH and 1 M NaCl at of 8 s intervals. The surface was then equilibrated with the running buffer for 30 min prior to the injection of sample solutions. On average, an immobilization response of 3000−4000 RU was achieved for each protein.

Screening was run at 25 °C with a flow rate of 30 μL/min using phosphate buffer as a running buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20) containing 6.5% DMSO. DMSO calibration using buffer

samples containing 5.5−7.5% DMSO was carried out to correct for solvent effects.

Each analytical cycle consisted of an injection of a 40 μM solution of sample in running buffer for 1 min (association phase) followed by running buffer for 3 min (dissociation phase). Two subsequent surface regenerations were carried out at a flow rate of 35 μL/min. First, a 30 s injection of 25 mM NaOH/1 M NaCl with 0.0005% SDS (pH 8.5), followed by a 35 s injection of 10 mM glycine-HCl (pH 3.0). A typical run consisted of 10 buffer injections, followed by a DMSO calibration block in triplicate. A single injection of quinacrine was followed by 19 samples, each a single analytical cycle. A second DMSO calibration block was followed by a further 19 analytical cycles, with another quinacrine injection and a third DMSO calibration block to finish. The binding was expressed as $\%RU_{max}$ to rank the binding affinity of compounds, which can be calculated by the following equation:

$$\%RU_{max} = \frac{\text{RU of compound} \times \text{MW protein}}{\text{RU immoblised protein} \times \text{MW compound}} \times 100$$

Synthesis. All reagents were purchased directly from commercial sources and used as supplied. Melting points were measured using a Bibby-Sterilin SMP10 melting point apparatus and are uncorrected. Accurate mass and nominal mass measurements were measured using a Waters-Micromass LCT electrospray mass spectrometer. TLC was performed using aluminum backed silica gel 60 plates (0.20 mm layer). Flash column chromatography was carried out using Fluorochem silica gel 60 Å. HPLC was carried out using a Phenomonex Luna ODS (150 mm × 4.6 mm, 5 μm particle size) column using acetonitrile/water (5% to 100% organic over 20 min at 1 mL/min). Detection was at 254 nm. All compounds were isolated in >95% purity unless otherwise stated (see Supporting Information).

Stepwise One-Pot Procedure: 2-Amino-4-phenyl-6-phenylsulfanylpyridine-3,5-dicarbonitrile (8). To a stirred solution of benzaldehyde (0.51 mL, 5.00 mmol) in ethanol (10 mL) was added a solution of malononitrile (0.33 g in 2.0 mL of ethanol, 5.00 mmol) and 3 drops of piperidine. The pale yellow solution was then refluxed for 75 min. To this was then added thiophenol (0.51 mL, 5.00 mmol), and the mixture was refluxed for a further 45 min. Another 1 equiv of malononitrile (0.33 g in 2.0 mL of ethanol, 5.00 mmol) was added, and the reaction mixture was refluxed for a further 60 min. The reaction mixture was then exposed to air overnight. The yellow crystalline precipitate formed was collected by suction filtration, washed with *n*-hexane/ethanol (9:1), followed by *n*-hexane, and then dried in vacuo. Product **8** was obtained as a yellow crystalline solid (34% yield). Mp 227−228 °C (Lit.,[13] 223−224 °C); *m*/*z* (ES); 329 ($[M + H]^+$); found 329.0845 ($C_{19}H_{13}N_4S$ $[M + H]^+$ requires 329.0861).

2-Amino-4-*tert*-butyl-6-phenylsulfanyl-1,4-dihydropyridine-3,5-dicarbonitrile (14). As precipitate was not observed on standing, and the reaction mixture was poured onto crushed ice (50 mL), neutralized with a few drops of concentrated HCl, and allowed to stand for 2 h. No solids were observed. Extraction of the aqueous phase with $CHCl_3$ followed by flash column chromatography (EtOAc/*n*-hexane, 6:4, $R_f = 0.3$) gave product **14** as a yellow powder (32% yield). Mp 198−199 °C; *m*/*z* (ES), 311 ($[M + H]^+$); found 311.1323 ($C_{17}H_{19}N_4S$ $[M + H]^+$, requires 311.1330).

2-Amino-4-(4-hydroxyphenyl)-6-(4-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (16). After ice treatment followed by flash column chromatography (EtOAc, $R_f = 0.7$), product **16** was isolated as a yellow powder (25% yield). Mp >300 °C; *m*/*z* (ES), 359 ($[M - H]^-$); found 359.0604 ($C_{19}H_{11}N_4O_2S$ $[M - H]^-$ requires 359.0603).

2-Amino-4-(3-chloro-4-hydroxyphenyl)-6-(2-fluorophenylsulfanyl)pyridine-3,5-dicarbonitrile (17). After ice treatment followed by flash column chromatography (petroleum ether/EtOAc, 2:1, $R_f = 0.6$) followed by recrystallization from EtOAc/*n*-hexane, product **17** was isolated as a yellow powder (3% yield). Mp 261−263 °C; *m*/*z* (ES), 397 ($[M + H]^+$); found 397.0313 ($C_{19}H_{11}ClFN_4OS$ $[M + H]^+$, requires 397.0326).

2-Amino-6-phenylsulfanyl-4-thiophen-2-yl-pyridine-3,5-dicarbonitrile (18). Product **18** was isolated as a dark yellow powder (18% yield). Mp 207−208 °C; *m*/*z* (ES), 335 ($[M + H]^+$); found 335.0412 ($C_{17}H_{11}N_4S_2$ $[M + H]^+$, requires 335.0425).

2-Amino-6-(3-chlorophenylsulfanyl)-4-phenylpyridine-3,5-dicarbonitrile (19). Product **19** was isolated as a light brown powder (18% yield). Mp 212−213 °C; *m*/*z* (ES), 363 ($[M + H]^+$); found 363.0489 ($C_{19}H_{12}ClN_4S$ $[M + H]^+$, requires 363.0471).

2-Amino-4-(3-hydroxyphenyl)-6-(4-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (22). After ice treatment, no precipitate was observed. The resultant mixture was extracted thoroughly with chloroform. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Purification of the crude product by flash column chromatography (THF, $R_f = 0.73$) gave **22** as a brown powder (17% yield). Mp 292−293 °C decomp; *m*/*z* (ES); 361 ($[M + H]^+$); found 361.0742 ($C_{19}H_{13}N_4O_2S$ $[M + H]^+$ requires 361.0759).

2-Amino-4-(4-formylphenyl)-6-(4-hydroxyphenylsulfanyl)pyridine-3,5-dicarbonitrile (24). After ice treatment, followed by flash column chromatography by gradient elusion (petroleum ether/EtOAc, 2:1 to 1:1, $R_f = 0.2$) product **24** was isolated, as a reddish-yellow powder (27% yield). Mp 275−276 °C decomp; *m*/*z* (ES), 371 ($[M - H]^-$); found 371.0599 ($C_{20}H_{11}N_4O_2S$ $[M - H]^-$ requires 371.0603).

2-Amino-4-(3-fluoro-4-hydroxyphenyl)-6-(4-hydroxyphenylsulfanyl)pyridine-3,5-dicarbonitrile (25). After ice treatment, followed by flash column chromatography (EtOAc, $R_f = 0.6$), product **25** was isolated as a yellow powder (8% yield). Mp > 300 °C; *m*/*z* (ES), 379 ($[M + H]^+$); found 379.0663 ($C_{19}H_{12}FN_4O_2S$ $[M + H]^+$, requires 379.0665).

2-Amino-4-(3-chloro-4-hydroxyphenyl)-6-(4-hydroxyphenylsulfanyl)pyridine-3,5-dicarbonitrile (26). After ice treatment followed by flash column chromatography (dichloromethane/methanol, 95:5, $R_f = 0.1$), product **26** was isolated as a yellow powder (3% yield). Mp 274−275 °C decomp; *m*/*z* (ES), 395 ($[M + H]^+$); found 395.0367 ($C_{19}H_{12}N_4ClO_2S$ $[M + H]^+$ requires 395.0370).

4-(4-Acetylphenyl)-2-amino-6-(4-hydroxyphenylsulfanyl)pyridine-3,5-dicarbonitrile (27). Isolated by flash column chromatography (petroleum ether/EtOAc, 1:1, $R_f = 0.3$) gave product **27** as a white powder (6% yield). Mp 261−263 °C decomp; *m*/*z* (ES), 387 ($[M + H]^+$); found 387.0900 ($C_{21}H_{15}N_4O_2S$ $[M + H]^+$, requires 387.0916).

4-[2-Amino-3,5-dicyano-6-(4-hydroxyphenylsulfanyl)pyridin-4-yl]benzoic acid methyl ester (28). Product **28** was isolated as a white powder (18% yield). Mp 299 °C decomp; *m*/*z* (ES), 425 ($[M + Na]^+$); found 425.0676 ($C_{21}H_{14}N_4NaO_3S$ $[M + Na]^+$, requires 425.0684).

4-[2-Amino-6-(3-chlorophenylsulfanyl)-3,5-dicyanopyridin-4-yl]benzoic acid methyl ester (32). Product **32** was isolated as a dark yellow powder (48% yield). Mp 247−248 °C; *m*/*z* (ES), 421 ($[M + H]^+$); found 421.0526 ($C_{21}H_{14}ClN_4S$ $[M + H]^+$, requires 421.0543).

2-Amino-6-(2-fluorophenylsulfanyl)-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile (33). After ice treatment followed by flash column chromatography (EtOAc, $R_f = 0.6$), product **33** was isolated as a white powder (17% yield). Mp 232−233 °C; *m*/*z* (ES), 363 ($[M + H]^+$); found 363.0732 ($C_{19}H_{12}FN_4OS$ $[M + H]^+$, requires 363.0716).

***N*-{4-[6-Amino-3,5-dicyano-4-(2,6-difluorophenyl)pyridin-2-ylsulfanyl]phenyl}-acetamide (34).** Isolation by flash column chromatography (petroleum ether/EtOAc, 1:5, $R_f = 0.3$) gave product **34** as a yellow powder (3% yield). Mp 261−263 °C; *m*/*z* (ES), 444 ($[M + Na]^+$); found 444.0720 ($C_{21}H_{13}F_2N_5NaOS$ $[M + Na]^+$, requires 444.0707).

2-Amino-6-(4-carboxyphenylsulfanyl)-4-(4-methylesterphenyl)-pyridine-3,5-dicarbonitrile (35). The product was isolated by filtration and recrystallization from methanol/dichloromethane followed by flash column chromatography (petroleum ether/THF,

1:3, $R_f = 0.3$) gave product **35** as a brown powder (2% yield). Mp 288−290 °C decomp; *m/z* (ES); 431 ($[M + H]^+$); found 431.0830 ($C_{22}H_{15}N_4O_4S$ $[M + H]^+$ requires 431.0814).

4-[2-(4-Acetylaminophenylsulfanyl)-6-amino-3,5-dicyanopyridin-4-yl]benzoic acid methyl ester (36). Product **36** was isolated as a pale yellow powder (47% yield). Mp 271−273 °C; *m/z* (ES); 444 ($[M + H]^+$); found 444.1145 ($C_{23}H_{18}N_5O_3S$ $[M + H]^+$ requires 444.1130).

4-[6-Amino-3,5-dicyano-4-(3-methyl ester)pyridin-2-ylsulfanyl]benzoic acid methyl ester (37). Product **37** was isolated as a dark brown powder (52% yield). Mp 260−261 °C; *m/z* (ES), 467 ($[M + Na]^+$); found 467.0806 ($C_{23}H_{16}N_4NaO_4S$ $[M + Na]^+$, requires 467.0790).

4-[6-Amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-ylsulfanyl]benzoic acid methyl ester (38). Product **38** was isolated as a white powder (3% yield). Mp > 300 °C; *m/z* (ES), 403 ($[M + H]^+$); found 403.0884 ($C_{21}H_{15}N_4O_3S$ $[M + H]^+$, requires 403.0865).

2-Amino-6-(4-methylesterphenylsulfanyl)-4-(4-methylesterphenyl)pyridine-3,5-dicarbonitrile (39). Product **39** was isolated as a light yellow powder (14% yield). Mp 294−295 °C; *m/z* (ES), 445 ($[M + H]^+$); found 445.0956 ($C_{23}H_{17}N_4O_4S$ $[M + H]^+$, requires 445.0971).

N-{**4-[6-Amino-3,5-dicyano-4-(3,5-difluorophenyl)pyridin-2-ylsulfanyl]phenyl**}**acetamide (40).** Product **40** was isolated as a yellow powder (25% yield). *m/z* (ES), 444 ($[M + Na]^+$); found 444.0699 ($C_{21}H_{13}F_2N_5NaOS$ $[M + Na]^+$, requires 444.0707).

2-Amino-6-(3-chlorophenylsulfanyl)-4-thiophen-2-ylpyridine-3,5-dicarbonitrile (41). Product **41** was isolated as a brown powder (23% yield). Mp 204−205 °C; *m/z* (ES), 369 ($[M + H]^+$); found 369.0050 ($C_{17}H_{10}ClN_4S_2$ $[M + H]^+$, requires 369.0035).

2-Amino-6-benzylsulfanyl-4-thiophen-2-ylpyridine-3,5-dicarbonitrile (42). Product **42** was isolated as a dark brown crystalline solid (19% yield). Mp 202−204 °C; *m/z* (ES), 347 ($[M + H]^+$); found 347.0410 ($C_{18}H_{13}N_4S_2$ $[M + H]^+$, requires 347.0425).

N-**[4-(6-Amino-3,5-dicyano-4-thiophen-2-ylpyridin-2-ylsulfanyl)phenyl]acetamide (43).** Isolated by flash column chromatography (petroleum ether/EtOAc, 1:3, $R_f = 0.2$) gave product **43** as a yellow powder (9% yield). Mp 261−263 °C decomp; *m/z* (ES), 390 ($[M - H]^-$); found 390.0497 ($C_{19}H_{12}N_5OS_2$ $[M - H]^-$, requires 390.0483).

2-Amino-6-benzylsulfanyl-4-phenylpyridine-3,5-dicarbonitrile (44). Product **44** was isolated as yellow crystalline solid (19% yield). Mp 212−214 °C; *m/z* (ES), 341 ($[M - H]^-$); found 341.0864 ($C_{20}H_{13}N_4S$ $[M - H]^-$, requires 341.0861).

2-Amino-6-benzylsulfanyl-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile (45). After ice treatment followed by flash column chromatography (EtOAc, $R_f = 0.6$), product **45** was isolated as a yellow powder (25% yield). Mp 190−192 °C; *m/z* (ES), 359 ($[M + H]^+$); found 359.0981 ($C_{20}H_{15}N_4OS$ $[M + H]^+$, requires 359.0967).

3-(6-Amino-3,5-dicyano-4-phenylpyridin-2-ylsulfanyl)-benzoic acid methyl ester (48). After ice treatment, recrystallization from methanol followed by washing with dichloromethane gave product **48** as a white powder (3% yield). Mp 247−248 °C; *m/z* (ES), 387 ($[M + H]^+$); found 387.0919 ($C_{21}H_{15}N_4O_2S$ $[M + H]^+$, requires 387.0916).

4-(6-Amino-3,5-dicyano-4-phenylpyridin-2-ylsulfanyl)-benzoic acid methyl ester (49). Product **49** was isolated as a white powder (23% yield). Mp 297−298 °C; *m/z* (ES), 387 ($[M + H]^+$); found 387.0912 ($C_{21}H_{15}N_4O_2S$ $[M + H]^+$, requires 387.0916).

2-Amino-6-(4-hydroxyphenylsulfanyl)-4-thiophen-2-ylpyridine-3,5-dicarbonitrile (50). After ice treatment product **50** was isolated as a yellow powder (27% yield). Mp 236−238 °C; *m/z* (ES), 351 ($[M + H]^+$); found 351.0391 ($C_{17}H_{11}N_4OS_2$ $[M + H]^+$, requires 351.0374).

2-Amino-6-(3-hydroxyphenylsulfanyl)-4-phenylpyridine-3,5-dicarbonitrile (51). After ice treatment product **51** was isolated as a yellow powder (10% yield, 93% purity). Mp 228−229 °C; *m/z* (ES), 345 ($[M + H]^+$); found 345.0808 ($C_{19}H_{13}N_4OS$ $[M + H]^+$, requires 345.0810).

2-Amino-6-(4-hydroxyphenylsulfanyl)-4-phenylpyridine-3,5-dicarbonitrile (52). After ice treatment followed by dichloromethane wash gave product **52** as a white powder (16% yield). Mp 254−256 °C; *m/z* (ES), 345 ($[M + H]^+$); found 345.0803 ($C_{19}H_{13}N_4OS$ $[M + H]^+$, requires 345.0810).

2-Amino-6-(3-aminophenylsulfanyl)-4-phenylpyridine-3,5-dicarbonitrile (53). Isolated by flash column chromatography (petroleum ether/EtOAc, 1:1, $R_f = 0.1$) gave product **53** as a whitish-yellow powder (11% yield). Mp 230−232 °C; *m/z* (ES), 344 ($[M + H]^+$); found 344.0966 ($C_{19}H_{14}N_5S$ $[M + H]^+$, requires 344.0970).

2-Amino-6-(4-aminophenylsulfanyl)-4-phenylpyridine-3,5-dicarbonitrile (54). After ice treatment, recrystallization from methanol, gave product **54** as a yellow powder (17% yield). Mp 162−164 °C; *m/z* (ES), 344 ($[M + H]^+$); found 344.0956 ($C_{19}H_{14}N_5S$ $[M + H]^+$, requires 344.0970).

2-Amino-6-(4-aminophenylsulfanyl)-4-thiophen-2-ylpyridine-3,5-dicarbonitrile (55). After ice treatment, product **55** was isolated as a dark brown powder (9% yield). Mp 220−221 °C decomp; *m/z* (ES), 350 ($[M + H]^+$); found 350.0538 ($C_{17}H_{12}N_5S_2$ $[M + H]^+$, requires 350.0534).

2-Amino-6-(3-aminophenylsulfanyl)-4-thiophen-2-ylpyridine-3,5-dicarbonitrile (56). Product **56** was isolated as a dark reddish brown powder (22% yield). Mp 237−238 °C; *m/z* (ES), 350 ($[M + H]^+$); found 350.0522 ($C_{17}H_{12}N_5S_2$ $[M + H]^+$, requires 350.0534).

4-(6-Amino-3,5-dicyano-4-thiophen-2-ylpyridin-2-ylsulfanyl)-benzoic acid methyl ester (57). Product **57** was isolated as a yellow powder (17% yield). Mp 260−261 °C; *m/z* (ES), 393 ($[M + H]^+$); found 393.0492 ($C_{19}H_{13}N_4O_2S_2$ $[M + H]^+$, requires 393.0480).

2-Amino-6-benzylsulfanyl-4-(4-fluorophenyl)pyridine-3,5-dicarbonitrile (58). Product **58** was isolated as a yellow powder (43% yield). Mp 188−189 °C; *m/z* (ES), 361 ($[M + H]^+$); found 361.0939 ($C_{20}H_{14}FN_4S$ $[M + H]^+$, requires 361.0923).

One Step, One-Pot Procedure: 2-Amino-4-phenyl-6-phenylsulfanylpyridine-3,5-dicarbonitrile (8). To a stirred solution of benzaldehyde (0.51 mL, 5.00 mmol) in ethanol (10 mL) containing 3 drops of piperidine was added a solution of malononitrile (0.66 g in 4.0 mL of ethanol, 10.00 mmol) and thiophenol (0.51 mL, 5.00 mmol). The reaction mixture was heated at reflux for 18 h, after which the reaction mixture was exposed to air for 3 h. The yellow crystalline precipitate formed was collected by suction filtration, washed with *n*-hexane/ethanol (9:1) and with *n*-hexane, and then dried in vacuo. Product **8** was obtained as a yellow crystalline solid (705 mg, 43% yield).

2-Amino-4-(3-methoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (20). Product **20** was isolated as a white powder (47% yield). Mp 278−279 °C; *m/z* (ES), 359 ($[M + H]^+$); found 359.0982 ($C_{20}H_{15}N_4OS$ $[M + H]^+$, requires 359.0967).

2-Amino-4-(4-methoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (21). Product **21** was isolated as a pale yellow powder (34% yield). Mp 251−253 °C; *m/z* (ES), 359 ($[M + H]^+$); found 359.0981 ($C_{20}H_{15}N_4OS$ $[M + H]^+$, requires 359.0967).

2-Amino-6-(4-hydroxyphenylsulfanyl)-4-(4-methanesulfonylphenyl)pyridine-3,5-dicarbonitrile (23). Product **23** was isolated as a yellow powder (31% yield). Mp 203−204 °C; *m/z* (ES), 423 ($[M + H]^+$); found 423.0591 ($C_{20}H_{15}N_4O_3S_2$ $[M + H]^+$ requires 423.0586).

2-Amino-4-(4-chlorophenyl)-6-(3-chlorophenylsulfanyl)pyridine-3,5-dicarbonitrile (29). Product **29** was isolated as a white powder (25% yield). Mp 217−220 °C; *m/z* (ES), 395 ($[M - H]^-$); found 394.9937 ($C_{19}H_9Cl_2N_4S$ $[M - H]^-$, requires 394.9925).

2-Amino-6-(3-chlorophenylsulfanyl)-4-(3-methoxyphenyl)pyridine-3,5-dicarbonitrile (30). Product **30** was isolated as a white powder (47% yield). Mp 211−212 °C; *m/z* (ES), 393 ($[M + H]^+$); found 393.0592 ($C_{20}H_{14}ClN_4OS$ $[M + H]^+$, requires 393.0577).

2-Amino-6-(3-chlorophenylsulfanyl)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (31). Product **31** was isolated as a pale yellow powder (40% yield). Mp 192−194 °C; *m/z* (ES), 393 ($[M + H]^+$); found 393.0592 ($C_{20}H_{14}ClN_4OS$ $[M + H]^+$, requires 393.0577).

***N*-[2-(6-Amino-3,5-dicyano-4-phenylpyridin-2-ylsulfanyl)-ethyl]acetamide (46).** Product **46** was isolated as a white powder (34% yield). Mp 224−226 °C; *m*/*z* (ES), 338 ($[M + H]^+$); found 360.0895 ($C_{17}H_{15}N_5NaOS$ $[M + Na]^+$ requires 360.0895).

4-(2-Amino-3,5-dicyano-6-pentylsulfanylpyridin-4-yl)-benzoic acid methyl ester (47). Product **47** was isolated as a yellow solid (20% yield). Mp 197−199 °C; *m*/*z* (ES), 381 ($[M + H]^+$); found 381.1395 ($C_{20}H_{21}N_4O_2S$ $[M + H]^+$ requires 381.1385).

2-Amino-6-phenylsulfanylpyridine-3,5-dicarbonitrile (15) ($KMnO_4$ procedure). To a stirred solution of **14** (50.0 mg, 0.15 mmol) in ethanol (5.0 mL) was added a solution of $KMnO_4$ (23.7 mg, 0.15 mmol) in ethanol (15 mL) dropwise, and the reaction was carried out overnight at room temperature. TLC showed the presence of starting material, so the reaction was therefore heated at reflux for 3 h, after which full conversion was achieved. The reaction mixture was allowed to cool to room temperature and poured into 50 mL of ice−water, and product **15** was isolated as a white powder (77% yield). Mp 202−204 °C, *m*/*z* (ES), 251 ($[M - H]^-$); found 251.0380 ($C_{13}H_7N_4S$ $[M - H]^-$, requires 251.0391).

2-Amino-6-phenylsulfanylpyridine-3,5-dicarbonitrile (15) (PCC procedure[20]). Silica gel 60 Å (2.88 g) was added to a solution of pyridinium chlorochromate (PCC) (206.9 mg, 0.96 mmol) in acetone (4.0 mL). The solvent was evaporated under reduced pressure, and the resulting solid was dried at 75 °C overnight. Compound **14** (100 mg, 0.32 mmol) was added to a stirred suspension of silica gel supported PCC (206.9 mg, 0.96 mmol) in dichloromethane at room temperature. After 25 min, the reaction was found to be complete by TLC. The solid was filtered, and the solution was concentrated under reduced pressure to give product **15** as a white powder (47% yield).

2-Amino-4-*tert*-butyl-6-phenylsulfanylpyridine-3,5-dicarbonitrile (13). To a stirred solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (109.0 mg, 0.48 mmol) in dichloromethane (13 mL) was added **14** (100.0 mg, 0.32 mmol), and the reaction was stirred overnight at room temperature. The reaction mixture was evaporated to dryness, and the resultant solids were extracted with 20 mL of *n*-hexane/EtOAc (6:4). After the removal of all volatiles under reduced pressure, the crude product was purified by flash column chromatography (*n*-hexane/EtOAc, 6:4, $R_f = 0.6$). Product **13** was obtained as a brown powder (25% yield). Mp 157−159 °C, *m*/*z* (ES), 307 ($[M - H]^-$); found 307.1005 ($C_{17}H_{15}N_4S$ $[M - H]^-$, requires 307.1017).

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Supporting Information Available: Experimental details of the cell-line assays, IR and ^1H and ^{13}C NMR spectroscopic data, X-ray crystallographic data for compounds **8** and **14**, and assessment of compound purity by HPLC. This material is available free of charge via the Internet at http://pubs.acs.org.

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