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Original article

Discovery of 6-substituted indole-3-glyoxylamides as lead antiprion agents with enhanced cell line activity, improved microsomal stability and low toxicity

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ABSTRACT

A series of highly potent indole-3-glyoxylamide based antiprion agents was previously characterized, focusing on optimization of structure–activity relationship (SAR) at positions 1–3 of the indole system. New libraries interrogating the SAR at indole C-4 to C-7 now demonstrate that introducing electron-withdrawing substituents at C-6 may improve biological activity by up to an order of magnitude, and additionally confer higher metabolic stability. For the present screening libraries, both the degree of potency and trends in SAR were consistent across two cell line models of prion disease, and the large majority of compounds showed no evidence of toxic effects in zebrafish. The foregoing observations thus make the indole-3-glyoxylamides an attractive lead series for continuing development as potential therapeutic agents against prion disease.

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1. Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of rare but invariably fatal neurodegenerative disorders afflicting both humans and animals. The most common such condition affecting humans is Creutzfeldt–Jakob Disease (CJD), and other examples include familial fatal insomnia (FFI) and Gerstmann–Sträussler–Scheinker syndrome (GSS). Animal prion diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer, elk and moose. In recent years, the latter of these has been recognized as a significant epidemic amongst wildlife populations across certain regions of North America [1].

The fundamental molecular event common to all TSEs is post-translational refolding of normal cellular prion protein, PrP^{C}, into its disease-associated isoform, PrP^{Sc}. This β-sheet rich form of the protein readily forms insoluble aggregates, deposition of which is thought to be central to pathogenesis and disease progression. Despite in vitro characterization of a large number of antiprion compounds capable of clearing PrP^{Sc} from infected cell lines, little therapeutic efficacy in vivo has been achieved to date, even in the face of considerable research effort [2]. An effective treatment to arrest or reverse progression of clinical prion disease therefore remains an important goal.

We recently characterized a series of indole-3-glyoxylamides **1** as potent antiprion agents (Fig. 1) [3], many of which demonstrated low nanomolar EC_{50} values against a prion-infected cell line (SMB.s15) [4]. This cellular system is one of several models [5] for prion disease that persistently produce PrP^{Sc} and is therefore used in screening for potential therapeutics. Previous efforts were focused around developing the structure–activity relationship

Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; CWD, chronic wasting disease; FFI, familial fatal insomnia; GSS, Gerstmann–Sträussler–Scheinker syndrome; hpf, hours post fertilization; MAPK, mitogen-activated protein kinase; PrP^{C}, normal cellular prion protein (or PrP-sen); PrP^{Sc}, disease-causing isoform (or PrP-res); SAR, structure–activity relationship; ScN2a, scrapie-infected neuroblastoma cells; SMB, scrapie-infected mouse brain; TBST, tris-buffered saline (pH 7.6) containing 0.05% Tween 20; TSE, transmissible spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease.

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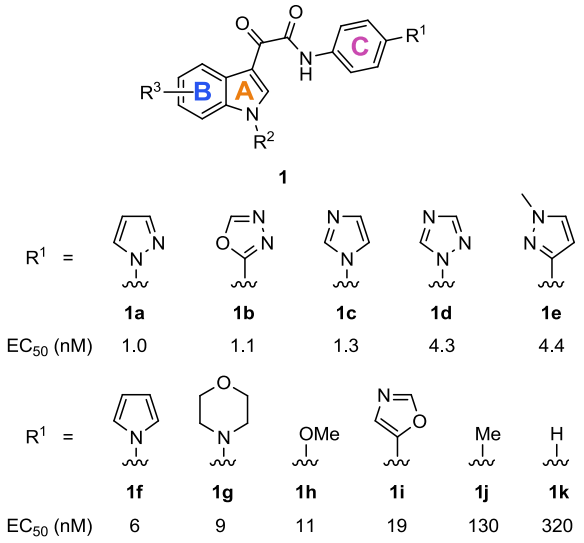


Fig. 1. Representative members of the indole-3-glyoxylamide lead series previously identified as potent inhibitors of PrP^{Sc} accumulation. In all of the examples **1a**–**1k**, $R^2 = R^3 = H$.

(SAR) at positions 1–3 of the indole ring (ring A, Fig. 1), including investigation of the most suitable glyoxylamide substituent, which was found optimally to consist of a *para*-substituted aniline moiety (ring C). The effect of substitution at indole C-4 to C-7 (ring B) is one important aspect of the SAR which was not considered in this earlier work, and assessment of such modifications is therefore a focus of the current study.

In addition to their highly potent in vitro antiprion activity, compounds of general structure **1** constitute an encouraging lead series given the wide variety of drug candidates containing the indole-3-glyoxylamide moiety (Fig. 2). Indeed, this substructure could be thought of as a 'privileged scaffold' [6] for drug discovery, considering the significant number of such compounds in various stages of clinical or preclinical development, across a range of biological activities (Table 1). Some attrition has been inevitable, but generally seems not to be due to adverse events in the case of this compound class. Thus, given the range of related examples which have already progressed to clinical studies for other disease indications (Table 1), we were encouraged that the antiprion indole-3-glyoxylamides are certainly amenable to tuning toward acceptable physical and pharmacokinetic properties. The structures in lead series **1** may thus be considered a promising starting point, with good potential for the development of clinically useful antiprion agents.

We therefore undertook to establish the effect of introducing substituents around 'ring B' (Fig. 1) upon cell line activity, in order to complete a basic understanding of the antiprion SAR across the whole of the central indole-3-glyoxylamide framework. In addition to this extension to the known SAR, it was also considered important to identify compounds with the best preclinical potential by consideration of other relevant properties. The criteria chosen were in vitro metabolic stability, and in vivo evaluation in zebrafish as an initial toxicity screen.

2. Chemistry

Synthesis of the new screening compounds substituted in 'ring B' was carried out using a protocol similar to that reported previously [3] (Scheme 1). In the presence of electron-withdrawing substituents (R^3 = F, Cl), it was found necessary to extend the duration of the first step (reaction with oxalyl chloride) to 24 h to achieve complete conversion to the intermediate glyoxylyl chorides. In terms of final yield, this approach actually proved advantageous for all of the substituted indoles, and was therefore adopted as a general method during the preparation of screening libraries. Initially, compounds derived from either methyl- or chloro-substituted indoles were synthesized. All such products were obtained successfully, with the exception of those derived from 4-chloroindole. In this case, it appeared that the extent of reaction with oxalyl chloride had only been very small.

3. Results and discussion

3.1. Investigation of antiprion structure–activity relationship

Methyl- and chloro-substituted series derived from parent structures **1h**, **1j**, **1g** and **1k** all displayed varying antiprion potency in the SMB cell line (Table 2). This range of R^1 groups (OMe, Me, morpholine and H, respectively, for the parent compounds listed) was chosen firstly because it represents a range of activities within the existing lead series (EC_{50} values between 11 and 320 nM), and secondly for ease of synthesis. Whereas compounds containing a five-membered aromatic heterocycle in the R^1 position are the

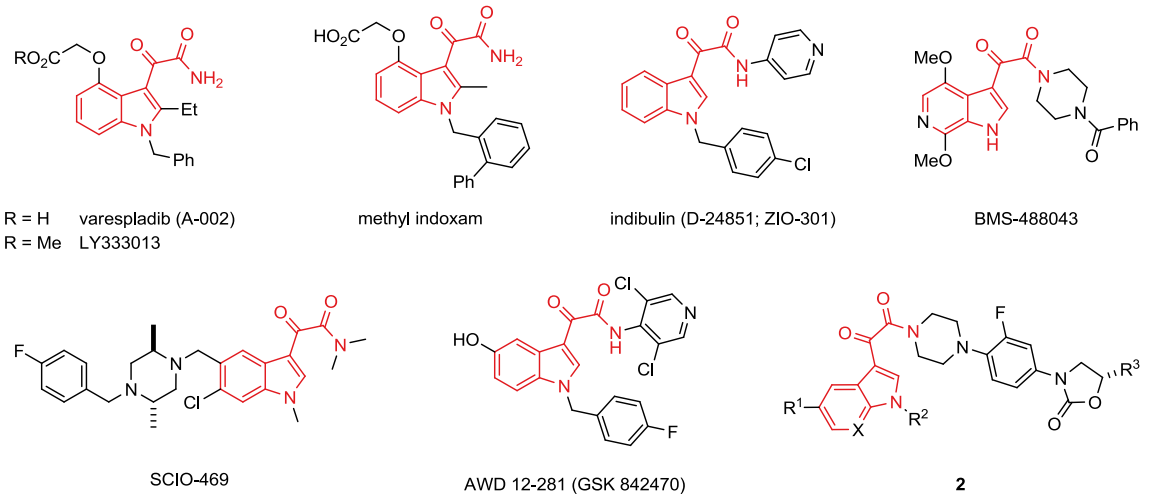


Fig. 2. Several compounds containing an indole-3-glyoxylamide (or closely related) substructure have progressed to preclinical or clinical evaluation.

Table 1
Indole-3-glyoxylamides and related compounds undergoing past or present clinical evaluation.

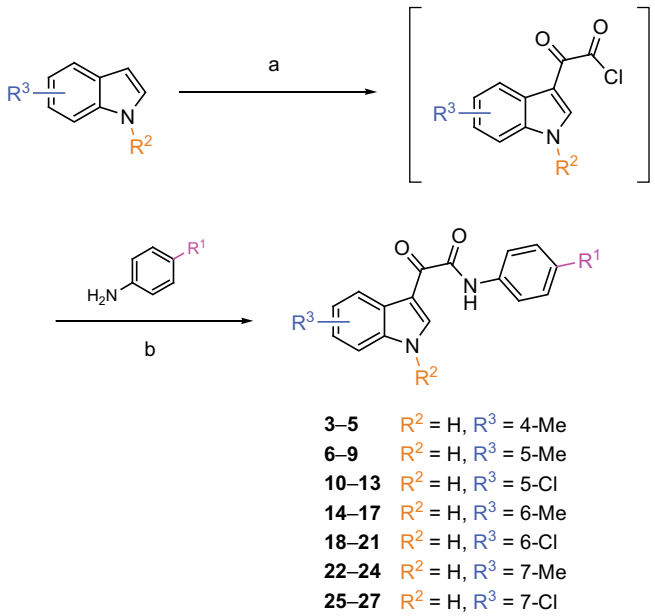
Compound	Action	Indication	Status	Reference(s)
Varespladib (A-002)	Secretory phospholipase (sPLA2) inhibitor	Acute coronary syndrome	Phase II completed Phase III 2010–2011	[7] [8]
SCIO-469	Selective p38α-MAPK inhibitor	Post-surgical dental pain	Phase II	[9]
		Multiple myeloma	Phase II	[10]
		Rheumatoid arthritis	Discontinued	[11]
		Fracture healing (as analgesic)	Preclinical	[12]
		Wound healing (in diabetes)	Preclinical	[13]
Indibulin (D-24851; ZIO-301)	Tubulin polymerization inhibitor	Solid tumors [14]; Breast cancer [15]	Phase I/II	[14,15]
BMS-488043	HIV-1 attachment inhibitor	HIV infection	Phase I	[16]
Methyl indoxam	General phospholipase (PLA_2) inhibitor	Obesity	Preclinical	[17]
2 (several examples)	Antibacterial	Systemic infection	Preclinical	[18]
LY333013	Secretory phospholipase (sPLA2) inhibitor	Rheumatoid arthritis	Discontinued	[19]
AWD 12-281 (GSK 842470)	Phosphodiesterase-4 (PDE4) inhibitor	Inflammatory disorders	Discontinued	[20]

most effective leads, they possess poor solubility, making their efficient synthesis and isolation in pure form somewhat problematic. Therefore, more readily accessible analogs were chosen in the first instance, in order to establish some general trends in the SAR of derivatives substituted in 'ring B'.

Clear effects were indeed observed on introduction of substituents in these positions (Table 2). Derivatization at C-4 was especially detrimental to activity (**3**–**5**), with EC_{50} values raised by at least two orders of magnitude. The 5-substituted analogs (**6**–**13**) similarly showed a considerable worsening of antiprion effect, albeit not to the same extent, with most EC_{50} values about one order of magnitude higher relative to the parent structures. Compounds modified at C-7 (**22**–**27**) were also less potent than their unsubstituted counterparts, though in most cases the deterioration in biological activity was not as pronounced. However, introduction of either a chloro-, or in particular a methyl, group at C-6 produced the desired improvement in EC_{50} values, indicating this is the only site on 'ring B' which may be usefully modified to enhance the antiprion activity of lead series **1**.

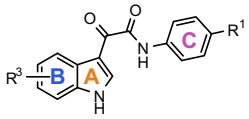
The above trends in potency are obvious from inspection of a graphical presentation of the data (Fig. 3). Within each series for a given R^3 substituent, EC_{50} values hold to the established order of R^1 = H (least active) > Me ≥ OMe > morpholine (most active). Similarly, where R^1 is held constant, there is a consistent trend following the general order 4-Me (not shown) >>5-Me > 5-Cl > 7-Me > 7-Cl > H ≥ 6-Cl > 6-Me. In the light of these results, additional compounds were prepared for cell line evaluation, focusing on further variations to substitution at indole C-6.

This second series of screening compounds was prepared using the same synthetic approach as detailed above (Scheme 1),



Scheme 1. Preparation of indole-3-glyoxylamide screening compounds. Reagents and conditions: (a) oxalyl chloride, THF, room temp, 24 h; (b) $^{i}Pr_2NEt$ or 2,6-lutidine, 45 °C, 18 h.

Table 2
Antiprion activity of indole-3-glyoxylamides substituted with either methyl- or chloro-groups at positions C-4 to C-7.[a]



Ref.	R^3	R^2	SMB.s15 EC_{50}, μM[b]
1h [3a]	–	OMe	0.011 ± 0.006
1j [3a]	–	Me	0.13 ± 0.02
1g [3a]	–	morpholine	0.009 ± 0.002
3	4-Me	OMe	2.36 ± 1.21
4	4-Me	Me	>20
5	4-Me	morpholine	0.68 ± 0.10
6	5-Me	OMe	0.88 ± 0.31
7	5-Me	Me	0.90 ± 0.30
8	5-Me	morpholine	0.083 ± 0.005
9	5-Me	H	11.5 ± 3.3
10	5-Cl	OMe	0.47 ± 0.04
11	5-Cl	Me	0.56 ± 0.12
12	5-Cl	morpholine	0.069 ± 0.004
13	5-Cl	H	1.63 ± 0.81
14	6-Me	OMe	0.012 ± 0.003
15	6-Me	Me	**0.051 ± 0.008**
16	6-Me	morpholine	**0.0031 ± 0.0006**
17	6-Me	H	**0.044 ± 0.028**
18	6-Cl	OMe	0.0099 ± 0.0005
19	6-Cl	Me	0.10 ± 0.03
20	6-Cl	morpholine	0.0073 ± 0.0001
21	6-Cl	H	**0.074 ± 0.010**
22	7-Me	OMe	0.15 ± 0.04
23	7-Me	Me	1.40 ± 0.13
24	7-Me	morpholine	0.016 ± 0.003
25	7-Cl	OMe	0.024 ± 0.017
26	7-Cl	Me	0.47 ± 0.12
27	7-Cl	morpholine	0.0072 ± 0.0054

[a] Synthetic yields for each compound are listed in the Supplementary Information.
[b] EC_{50} values in bold type represent an improvement of at least a factor of two over the unsubstituted analog.

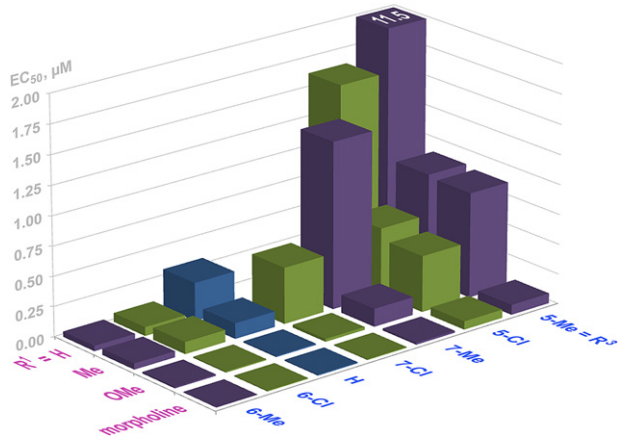


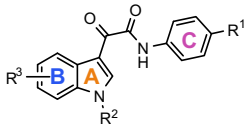
Fig. 3. Graphical representation of EC_{50} values presented in Table 2, showing clear trends in activity with variation of R^1 and R^3. Bar heights correspond to observed EC_{50} concentrations. An enlarged version of this Figure is included within the Supplementary Information for improved clarity.

beginning with the appropriate substituted indole. We also prepared 6-Cl analogs of some 1-methylindole derived structures, to ascertain whether introduction of the C-6 substituent would improve the activity of these compounds where R^2 = Me. Derivatives substituted with small alkyl groups at R^2 are less active than when R^2 = H [3], but are more soluble. This increased solubility likely originates from a substitution which can disrupt intramolecular hydrogen-bonding in the solid state, and also reduces the marked planarity of the molecules; both of these effects have been suggested or adopted elsewhere as strategies to improve aqueous solubility of drug candidates [21]. It was therefore instructive to compare whether 6-Cl substitution could recover some of the potency lost by introduction of a methyl group at R^2. Surprisingly, the 6-chloro-1-methyl entities (**30**–**31**) performed more poorly than both the equivalent 1-Me (**32**–**33**) and 6-Cl (**28** and **18**) compounds, suggesting that derivatization at C-6 cannot be used to compensate for the loss of activity encountered on introducing an alkyl group at N-1. In contrast to the small but noticeable improvement seen for earlier examples (**18**–**21**, Table 2), 6-Cl derivatives of **1f** and **1a** (**28** and **29**, respectively) actually showed a small drop in activity compared with their parent compounds, although both new analogs still proved very potent as antiprion agents (EC_{50} < 20 nM; Table 3).

A small set of other groups at C-6 was also investigated (Table 3). Fluoro-compounds **34**–**36** showed a little improvement over their related chloro- (**28**, **29**, **18**) analogs, and comparable activities to the C-6 unsubstituted (**1f**, **1a**, **1h**) parent compounds. In contrast, 6-^{i}Pr derivatives **37**–**38** were greatly compromised in antiprion potency compared to their unsubstituted counterparts. The most effective modifications to 'ring B' proved to be introduction of a 6-cyano (**39**–**40**) or 6-nitro (**41**–**42**) group, with the former producing a reduction in EC_{50} by essentially an order of magnitude. The preceding trends (summarized graphically in the Supplementary Information; Fig. S1) suggest that strongly electron-withdrawing groups at C-6 offer the best route to an optimal antiprion effect, although the effectiveness of the 6-Me compounds (**14**–**15**) does provide an intriguing exception to this general trend.

Thus, SAR focused upon positions C-4 to C-7 of the indole ring had established that substitution at positions 4, 5 and 7 was detrimental to activity, whereas certain analogs with groups introduced at C-6 showed much improved activity. The 6-Me, 6-CN and 6-NO_2 series were identified as the most valuable, in terms of offering improved antiprion potency over that of the unsubstituted parent structures.

Table 3
Second-generation library of substituted indole-3-glyoxylamide antiprion compounds, focusing on varation of substitution at C-6.[a]



Ref.	R^3	R^1	R^2	SMB.s15 EC_{50}, μM[b]
1f [3a]	–	H	1*H*-pyrrol-1-yl	0.006 ± 0.003
1a [3a]	–	H	1*H*-pyrazol-1-yl	0.0010 ± 0.0004
28	6-Cl	H	1*H*-pyrrol-1-yl	0.017 ± 0.007
29	6-Cl	H	1*H*-pyrazol-1-yl	0.0061 ± 0.0010
30	6-Cl	Me	1*H*-pyrrol-1-yl	0.27 ± 0.10
31	6-Cl	Me	OMe	1.63 ± 0.83
32	–	Me	1*H*-pyrrol-1-yl	0.17 ± 0.05
33 [3a]	–	Me	OMe	0.068 ± 0.018
34	6-F	H	1*H*-pyrrol-1-yl	0.0077 ± 0.0016
35	6-F	H	1*H*-pyrazol-1-yl	0.0012 ± 0.0009
36	6-F	H	OMe	0.013 ± 0.004
37	6-^{i}Pr	H	OMe	0.33 ± 0.06
38	6-^{i}Pr	H	Me	0.61 ± 0.01
39	6-CN	H	OMe	**0.0022 ± 0.0004**
40	6-CN	H	Me	**0.0109 ± 0.0014**
41	6-NO_2	H	OMe	0.0105 ± 0.0012
42	6-NO_2	H	Me	**0.0350 ± 0.0028**

[a] Synthetic yields for each compound are listed in the Supplementary Information.
[b] EC_{50} values in bold type represent an improvement of at least a factor of two over the unsubstituted analog.

3.2. Comparison of activity in SMB and ScN2a cells

To further appropriate the significance of the present indole-based compounds as an important class of antiprion compounds, a selection of screening library members, representative of a range of activities, was also evaluated in scrapie-infected neuroblastoma cells (ScN2a). This cell line has been widely employed as a common model of prion disease, including screening for potential therapeutics. The present results allowed comparison of PrP^{Sc} clearance in both neuronal (ScN2a) and non-neuronal (SMB) cells of murine origin, and demonstrated good correlation between the two host cell types (Table 4). Statistical analysis suggested there is a significant correlation of antiprion activity between the two cell models (P < 0.0001, Pearson r = 0.9731; see Supplementary Information,

Table 4
Observed EC_{50} values of selected screening compounds in two scrapie-infected cell lines.[a]

Ref.	SMB.s15 EC_{50}, μM	ScN2a EC_{50}, μM
1a	0.0010 ± 0.0004	0.014 ± 0.006
1c	0.0013 ± 0.0002	0.016 ± 0.006
1g	0.009 ± 0.002	0.065 ± 0.054
1h	0.011 ± 0.006	0.091 ± 0.040
1j	0.13 ± 0.02	0.206 ± 0.046
25	0.024 ± 0.017	0.247 ± 0.099
26	0.47 ± 0.12	1.26 ± 0.24
29	0.0061 ± 0.0010	0.175 ± 0.038
39	0.0022 ± 0.0004	0.011 ± 0.000
40	0.0109 ± 0.0014	0.026 ± 0.010

[a] An expanded version of this Table is included in the Supplementary Information (Table S2), which includes structures to aid clear comparison of results.

Fig. S2), although this result is not conclusive given the relatively small number of data pairs ($n = 11$).

Although actual EC_{50} values were consistently higher in the ScN2a model, the compounds still retained excellent antiprion potency, with the most active analogs maintaining nanomolar level activities. For example, compounds **1a**, **1c** and **39** displayed EC_{50} values of 14 nM, 16 nM and 11 nM respectively, compared with 1.0 nM, 1.3 nM and 2.2 nM in the SMB cell line. The most important aspect of these results is that structure–activity relationships proved essentially identical between the two cell lines. Amongst the compounds unsubstituted on 'ring B', the general trend in EC_{50} with respect to R^1 was consistent, i.e. R^1 = Me (**1j**) >> OMe (**1h**) > morpholine (**1g**) >> 1*H*-pyrazol-1-yl (**1a**). Introduction of a 7-Cl substituent remained detrimental to activity in both cell models (**25** > **1h** and **26** > **1j**), whereas 6-Cl substitution raised EC_{50} markedly in the ScN2a model (**29** >> **1a**) in contrast to the more marginal differences seen in SMB cells. In both cases, 6-cyano compounds **39** and **40** proved at least an order of magnitude more potent than their unsubstituted analogs (**1h** and **1j** respectively), reinforcing the positive result of this modification outlined above (Fig. 4). Thus, the SAR previously established by SMB cell line screening was maintained across the ScN2a experiments, strengthening the significance of the indole-3-glyoxylamides as a lead series, and suggesting a common mechanism of action in both cell models. Although the mode of action of these indole-based compounds is not yet known, it has previously been established that they do not display a discernible interaction with PrP^C itself [3].

3.3. Preliminary evaluation of toxicity and metabolic stability

In addition to potent activity at the screening stage, other factors are important in selecting candidate compounds for progression into animal studies. To further inform selection of lead compounds for in vivo evaluation, additional assays were performed to assess stability and toxicity of the present indole libraries. The importance of early-stage assessment of potential drug safety is increasingly becoming recognized in discovery programs, as a contributing factor to reduce late-stage attrition. Over recent years, zebrafish assays for early screening of safety pharmacology have emerged as an important tool in this respect, due to their relatively low cost and generally good predictivity of mammalian adverse drug effects [22]. In order to further evaluate the antiprion-active indole-3-glyoxylamides as a source of potential prion disease therapeutics, the survival rate of early zebrafish larvae upon exposure to each compound was determined, to search for any evidence of acute toxicity. Animals were dosed at 48 h post fertilization (hpf) with compounds at 5 μM, and three days later the number of live fish remaining was recorded and compared to untreated controls (for a full summary of results see the Supplementary Information section, Fig. S3). Toxicity screening routinely employs zebrafish larvae, as opposed to mature fish, since the early larvae are transparent and direct observation of developing organs and structures is therefore possible.

It was pleasing to find that under the assay conditions, over half of the test compounds showed no effect on zebrafish survival, including the 6-Me and 6-CN series (**14**–**17** and **39**–**40**) displaying some of the most potent antiprion activity. Structures where R^1 = Me or morpholine were rather often associated with a mortality rate of at least 20%, suggesting these substitutions at the *p*-position of the aniline moiety should be avoided; indeed, compound **1j** (R^1 = Me) proved the most toxic of all those evaluated.

Animals treated with three of the most potent antiprion agents—**1a** (SMB EC_{50} 1.0 nM), **16** (3.1 nM) and **39** (2.2 nM)—were examined in detail after three days' treatment and staining with acridine orange (Fig. 4), and were developmentally normal with no evidence for apoptosis or obvious deformities. This general absence of acute toxic effects was encouraging, whilst at the same time, the zebrafish screen had identified some R^1 substitutions (Me, morpholine) which should preferably be avoided. In addition, substitution at indole C-5 correlated with evidence of toxic effects (mortality ≥ 20%), although given the considerable drop in antiprion potency also associated with such modifications, this observation was not of major concern.

It was also considered important to identify any 6-substituted library members which would be unsuitable leads because of susceptibility to first pass metabolism. As such, their stability toward mouse liver microsome preparation was assessed and compared to that of 6-unsubstitued analogs **1h** and **1j**. Through this work it became clear that all of the 6-substituted analogs showed improved microsomal stability (Fig. 5), suggesting that indole C-6

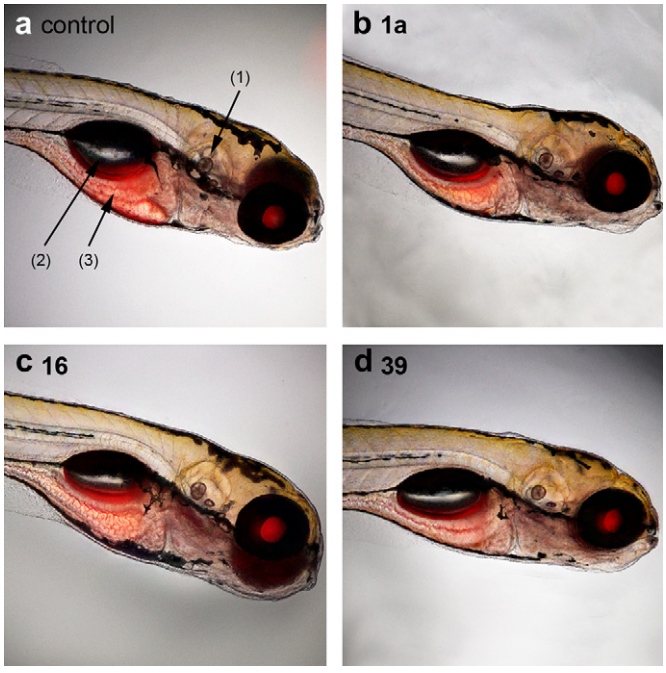


Fig. 4. Early zebrafish larvae (48 hpf) exposed to three potent lead compounds (at 5 μM for 3 d) proved developmentally normal. Images presented here were obtained after acridine orange staining, and some key anatomical features are labeled for reference: (1) otic capsule; (2) swim bladder; (3) yolk.

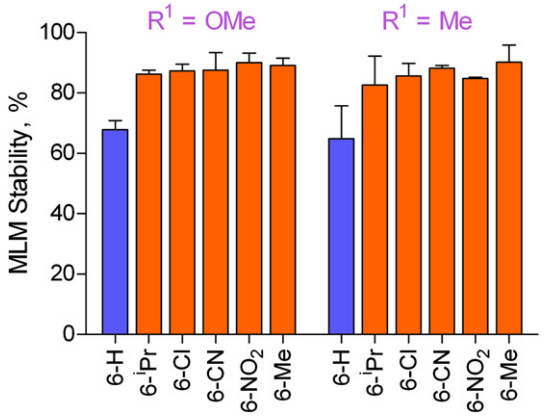


Fig. 5. Introduction of a variety of substituents at indole C-6 imparts improved microsomal stability. These data are tabulated in full in the Supplementary Information (Table S3).

might be a locus of metabolism in the unsubstituted compounds. It was pleasing to note that for the substituents found to enhance antiprion activity (6-Me, 6-CN, 6-NO_2), these compounds also displayed a high degree of stability over the incubation period(≥85% remaining after 30 min), implying an in vitro half-life in the order of several hours.

The foregoing results point toward the 6-Me and 6-CN series as a source of lead compounds showing the best balance between antiprion activity, toxicological safety and metabolic stability. In addition, the introduction of these substitutions is also beneficial when considering solubility. Compounds such as **1a**–**f**, possessing a heteroaromatic group at the R^1 position, show the best antiprion potency but suffer from the drawbacks of very low solubility and a strong tendency to form aggregates in aqueous solution—problems which were documented previously [3b]. It was therefore pleasing to discover that introduction of a 6-Me or 6-CN group into more soluble parent compounds (e.g. **1g**, **1h** or **1j**) lowered the antiprion EC_{50} into the same range as that for **1a**–**f**, whilst still retaining reasonable solubility (up to at least 10 μM, by observation in the antiprion screening assays). In contrast, the 6-CN and 6-NO_2 derivatives of **1a** could not be isolated from an attempted synthesis, presumably due to a further step down in solubility for these analogs.

4. Conclusions

The indole-3-glyoxylamide series previously identified as promising antiprion agents [3] has been extended by considering modifications at positions C-4 to C-7 of the indole system. Analogs bearing various substituents at C-6 (R^3 = 6-Me, 6-CN or 6-NO_2) were found to display EC_{50} values up to an order of magnitude lower than their unsubstituted parents, together with improved stability toward microsomal metabolism and low toxicity. In particular, the 6-Me and 6-CN compounds were identified as those with the best balance between the properties investigated, making them a priority for further progression toward animal studies. Significantly, the high degree of antiprion potency observed for the indole-based compounds in general has been confirmed in a second prion-infected cell line, coupled with observation of a common SAR across the two disease models. No evidence of acute toxicity in zebrafish was seen for the large majority of the screening set. The present results thus confirm the antiprion-active indole-3-glyoxylamides as an important lead series in addressing the urgent need for clinically effective agents against prion disease.

5. Experimental section

5.1. Biology

5.1.1. Assessment of antiprion activity in SMB cells

Compounds were screened for inhibition of PrP^{Sc} formation in SMB cells of mesodermal origin (SMB.s15 cell line) as described previously [3], using a procedure based upon that reported by Rudyk et al. [23]. Cells were grown in tissue culture-treated plastic dishes in Medium 199, supplemented with 10% newborn calf serum (heat inactivated), 5% fetal calf serum (heat inactivated), and penicillin–streptomycin at 10 mg L^{-1} at 37 °C in an atmosphere of 5% CO_2 in air at 95% relative humidity. Medium was changed every third or fourth day, and every 7 days confluent cells were passaged using 0.05% trypsin and 0.002% EDTA at a split ratio of 4. To assess the effects of compounds, cells were distributed into 96-well cluster plates at 12,000 cells per well and incubated for 24 h to allow for cell attachment. The compounds were prepared at 200 times the required concentration in DMSO as stock solutions then transferred, at a 10-fold dilution, into Hank's balanced salt solution. This solution was then transferred at a further 20-fold dilution into the cell medium. The cells were incubated with the compound-containing medium for 5 days. After this time, cell viability was assessed by the MTT assay following the standard protocol supplied with the reagent (Sigma), which indicated that none of the test compounds were toxic to the cells up to a concentration of 20 μM. For dot blot (cell blot) analyses, cells were extracted using lysis buffer (10 mM Tris–HCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 0.5% v/v NP40, and 0.5% w/v sodium deoxycholate), and the content of the well was loaded onto a nitrocellulose membrane (0.45 μm) under gentle vacuum at a total cellular protein concentration of approximately 30–40 μg/well (determined by the Bradford assay following the protocol supplied with the reagent; Sigma). The membrane was air-dried and subjected to 75 μg mL^{-1} proteinase K digestion for 1 h at 37 °C. The reaction was stopped with 1 mM phenylmethylsulfonyl fluoride (PMSF) in 20 mM Tris–HCl-buffered saline (TBS) and the membrane washed extensively with TBS and immersed in 1.8 M guanidine thiocyanate in TBS for 10 min at room temperature. After further washing with TBS, the membrane was blocked using 5% fat-free milk powder in phosphate-buffered saline (PBS), processed with 0.2 μg mL^{-1} mouse mAb anti-PrP 6H4 (Prionics), and developed using an ECL kit (Amersham Pharmacia Biotech). Every experiment was carried out in triplicate and an average value for PrP^{Sc} concentration calculated, relative to an untreated control (DMSO only), together with a standard deviation. Curcumin was employed as a positive control, and effected essentially complete clearance of PrP^{Sc} at the concentration used (10 μM). Test compounds were initially screened at 1, 10 and 20 μM and were considered to be active if PrP^{Sc} levels were reduced to less than 70% of that of the untreated control after 5 days' exposure. Compounds showing activity were re-screened over a range of concentrations to determine an EC_{50} value, such experiments being repeated at least twice (in triplicate) to validate the results so obtained. Screening in the SMB.s15 line was previously validated by investigating two well documented antiprion agents as positive controls [3a]. EC_{50} values of 0.95 μM and 0.42 μM were observed for curcumin and quinacrine, respectively, compared to reported values of 0.01 μM for curcumin (in ScNB cells) [24], and 0.3–0.5 μM for quinacrine (in ScN2a cells) [25].

5.1.2. Assessment of antiprion activity in ScN2a cells

The methods employed were similar to previously published protocols [26], with the following modifications. ScN2a cells (N2a cells infected with the Rocky Mountain Laboratory prion strain) were seeded into black wall, clear bottom, tissue culture-treated plates (Greiner) at 4×10^5 cells per well in 100 μL of assay medium: Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, GlutaMax and 500 μg mL^{-1} geneticin. Test compounds were dissolved in 100% DMSO and diluted in assay medium at 200× final concentration before addition to the assay plates (0.5% final DMSO concentration). Compound addition occurred 4 h after cell seeding. After 5 days' incubation at 37 °C in a humidified and 5% CO_2-enriched environment, lysates were generated as previously described [26] and transferred to high-binding ELISA plates (Greiner) coated with anti-PrP Fab antibody D18 [27], for overnight incubation at 4 °C. The plates were then washed three times with TBST before addition of horseradish peroxidase-conjugated D13 antibody [27] (1:1000 dilution in 1% BSA/PBS, 100 μL), and incubation at rt for 1 h. Following this treatment, the plates were washed seven times with TBST, then 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; 100 μL) was added to each well. After 10 min, absorbance at 405 nm was recorded using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Dose–response curves were generated over a concentration range from three independent experiments. Calcein cell viability assays were run in separately seeded 96-well black wall plates as previously described [27], with all screening

compounds showing no discernible cytotoxicity up to the maximum concentration tested (32 μM).

5.1.3. Animal husbandry

Zebrafish (*Danio rerio*) of the AB wild-type strain were reared under standard conditions on a 14:10 light:dark cycle [28]. Embryos were collected using breeding traps in aquarium tanks, and sorted and staged accordingly. Fish were kept in E3 medium [29] (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.3 mM $MgSO_4$, 0.00001% methylene blue) at 28 °C in dark conditions. All procedures were performed in accordance with the UK Home Office Animals Scientific Procedures Act (1986), and other relevant legistlation.

5.1.4. Drug administration (zebrafish toxicity assay)

Prior to addition of the drugs, 48 hpf embryos were dechorionated using pronase (protease from *Streptomyces griseus*, Sigma; 3 mg mL^{-1}) for 15 min, then rinsed in methylene blue-free E3 medium three times, and dispensed into 96-well plates at 3 fish per well in a fixed volume of 200 μL. Compounds were dosed at 5 μM, from 10 mM DMSO stocks at a final DMSO concentration of 1% v/v. Plates were maintained at 28 °C for a further 72 h, with survivability of larvae assessed at this point (day 5) and compared to that of vehicle-treated control groups. No food was provided during the test. All experiments were conducted twice, each time in triplicate, and full results are presented in the Supplementary Information (Fig. S2).

5.1.5. Acridine orange staining

In order to determine any extent of apoptotic cellular death, acridine orange was applied to treated sample fish at 1 μg mL^{-1} in E3 medium for 30 min. Excess dye was removed by successive washes with the same medium (3 × 10 min). The animals were anesthetized using 160 μM tricaine, then mounted in 1% low melting point agarose (Seakem) containing 160 μM tricaine and analyzed using a Nikon AZ100M microscope. Images were captured using a DS-Ri1 digital camera with NIS-Elements software.

5.1.6. Microsomal stability assay

A mixture of test compound (5.0 mM solution in DMSO, 0.8 μL), isocitrate dehydrogenase (31.4 μL, 2 units), 0.2 M potassium phosphate buffer (pH 7.0, containing 10 mM DL-isocitric acid trisodium salt and 10 mm $MgCl_2$; 228 μL), and microsome preparation from mouse liver (0.5 mg mL^{-1} in the same buffer; 100 μL) was incubated in an Eppendorf tube for 5 min at 37 °C. NADPH (10 mM solution; 40 μL) was then added to initiate the reaction. Contents of the tube were mixed using aspiration/dispensation five times, then a $t = 0$ aliquot (150 μL) was drawn immediately and quenched with ice-cold acetonitrile (300 μL), containing 8 μM 1-(2-methyl-1*H*-indol-3-yl)-2-morpholinoethane-1,2-dione [3a] as internal reference standard. The remaining reaction mixture was maintained at 37 °C for 30 min, then a second aliquot removed and diluted as above. The quenched solutions were each vortexed for 30 s and centrifuged at 4000 rpm (45° fixed-angle rotor F-45-12-11) for 10 min, then the supernatant analyzed by HPLC: Genesis 4 μm C18 column, 4.6 × 150 mm; 5–95% MeCN/H_2O over 4 min, hold 6 min at 95% MeCN; flow rate 1 mL min^{-1}; UV detection at 267 nm. Each analysis was performed in triplicate and metabolism results after 30 min compared with those at $t = 0$. The percentage of drug remaining, together with the standard deviation, is reported for each test sample.

5.2. Chemistry

5.2.1. General procedures

Anhydrous grade THF was obtained from an in-house 'Grubbs' solvent purification system; all other solvents and reagents were purchased from commercial sources and used as supplied. Reactions were performed under an inert atmosphere of N_2, and parallel synthesis was carried out on a Radleys 12-position carousel. Parallel extractions were carried out with liquid–liquid extraction columns, 20 mL sample loading capacity, in conjunction with a carousel work-up station. ^1H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a Bruker AV-1400 spectrometer. Accurate mass and nominal mass measurements were acquired using a Micromass LCT Premier XE mass spectrometer. Purity of screening compounds was assessed by HPLC: Genesis 4 μm C18 column (150 × 4.6 mm) or Ace 3 μm C18 column (125 × 4.6 mm); 30–100% MeCN in water over 12 min, hold at 100% MeCN for 10 min; 15 μL injection; UV detection at 254 nm; flow rate 1.0 mL min^{-1}. For compounds **3**, **4** and **38**–**42**, further purification by preparative HPLC was necessary, beginning with a solution of the crude product in MeCN/DMSO (1:1) and employing an Alltech Alltima HP C18 HL 5 μm column at flow rate of 20 mL min^{-1}, using the eluent specified in each individual case. The vast majority of compounds were isolated in >95% purity; a complete list of HPLC purities and analytical chromatograms, along with full characterization data for each screening compound, is provided in the Supplementary Information (Table S1).

*5.2.2. Indole-3-glyoxylamides **3**–**32**, **34**–**42**; general procedure*

A dry 50 mL capacity carousel reaction tube was charged with the relevant substituted indole (1.5 mmol), and this starting material dissolved in dry THF (12 mL). Oxalyl chloride (144 μL, 209 mg, 1.65 mmol) was added and the mixture stirred at room temp. After 24 h, either *N*,*N*-diisopropylethylamine (590 μL, 438 mg, 3.4 mmol) or 2,6-lutidine(396 μL, 364 mg, 3.4 mmol) was introduced to the reaction, followed by the relevant amine (1.8 mmol). The temperature was raised to 45 °C, and heating continued for 18 h. The solvent was evaporated, then the residue resuspended in a mixture of ethyl acetate (20 mL) and brine (10 mL) and stirred vigorously for 30 min. After passing through a liquid–liquid extraction column, ensuring washing through of the column with additional ethyl acetate, a second extraction was carried out with sat. NH_4Cl (10 mL) in the same manner. Product solutions were then evaporated to dryness giving the crude indole-3-glyoxylamide derivatives, which were purified as necessary (see Supplementary Information for individual compound details). Typically, two sequential recrystallizations, from ethyl acetate–hexane followed by 2-propanol–water, afforded final products of good purity. For products derived from poorly nucleophilic anilines (*p*-1*H*-pyrazol-1-ylaniline,*p*-pyrrol-1-ylaniline and *p*-morpholinoaniline), a further modification was used. Immediately following introduction of the arylamine to the reaction mixture, DMAP (18 mg, 0.15 mmol, 10 mol%) was added to assist the final step, as its use was found to enhance the extent of conversion in the final step in such cases.

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Appendix. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.06.013.

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