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Isatin derivatives, a novel class of transthyretin fibrillogenesis inhibitors

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ABSTRACT

The isatin core structure was found to be a novel chemical scaffold in transthyretin (TTR) fibrillogenesis inhibitor design. Among the series of isatin analogues prepared and tested, the nitro compound 1,3-dihydro-3-[(4-nitrophenyl)imino]-2*H*-indol-2-one (**2r**) is as potent as triiodophenol, which is one of the most active known TTR inhibitors. The *E*/*Z* stereochemistry of these molecules in solution, elucidated by ^{1}H NMR, does not influence their biological activity. The compounds do not bind to the native tetrameric TTR suggesting that their inhibitory action is independent of the protein binding and stabilization.

Isatin (1*H*-indole-2,3-dione), an endogenous compound identified in many organisms, shows a wide range of biological activities.[1–3] Owing to these properties, derivatives of isatin have been developed for therapeutic applications. A recent example is the compound SU11248 (Sutent), a 5-fluoro-3-substituted-2-oxoindole that has received FDA approval for the treatment of gastrointestinal stromal tumors[4] and advanced renal cell carcinoma.[5] Other halogenated derivatives of isatin, including the 5,6,7-tribromoisatin,[6] a number of 5,7- dibromoisatins,[7,8] and a series of 5-bromo-(2-oxo-3- indolinyl)thiazolidine-2,4-diones,[9] have also been reported to exhibit potential as anticancer drugs when assayed in various cancer cell lines. Examples of the diverse biological activities of isatin derivatives are the 3-arylimino-2-indolones, which are potent and selective galanin GAL_3 receptor antagonists[10] and DNA gyrase inhibitors.[11]

Among the already known families of isatin analogs, the isatin-3-arylimines show a basic structure of two bridged aromatic rings that can readily accommodate different functional groups. This simple structural feature is present in many pharmacophores. Thus, several families of transthyretin (TTR) fibrillogenesis inhibitors[12] with structural patterns as diverse as tetrahydroquinolines, dihydropyridines, benzodiazepines, phenoxazines, stilbenes and benzoxazoles have in common this two bridged aromatic system.[13] Moreover, TTR small molecule inhibitors usually bear halogen substituents in one ring and hydrophilic functions in the other. To the best of our knowledge, in spite of these apparent structural analogies, isatin derivatives have not yet been tested as potential TTR fibrillogenesis inhibitors.

TTR is a plasma protein involved in the transport of thyroid hormones and, by interacting with retinol binding protein, of vitamin A. This protein self-assembles as a homotetramer of 55 kDa leaving a central hydrophobic channel with two symmetrical binding sites. Being an amyloid prone protein, TTR is linked to a distinctive group of amyloid diseases. Deposits of wild type TTR in the heart and peripheral nerves appear to cause senile systemic amyloidosis, whereas most of the one hundred already identified TTR mutants result in two groups of diseases known as familial amyloidotic polyneuropathy and familial amyloidotic cardiomyopathy.[14] The aggregation pathway of TTR into amyloid fibrils is not yet well characterized but most fibrillogenesis models postulate several steps, including dissociation of the tetramer, changes in monomer conformation, aggregation of conformationally modified monomers into non-fibrillar oligomers that later form protofibrils and further elongate into mature fibrils.[15]

This mechanism, along with the fact that binding of thyroid hormones to TTR results in tetramer stabilization, suggests that amyloid fibril formation can be inhibited by small molecule compounds structurally similar to thyroid hormones. Indeed, this hypothesis has been confirmed by the identification of several families of compounds that bind to TTR and stabilize the ground state of the protein proportionally to the dissociation constants.[16] Such a

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stabilizing effect of the tetrameric TTR is sufficient to arrest or retard the cascade of events leading to the formation of aggregates and protein precipitation that can be observed in vitro when the soluble TTR is challenged by an acidic pH. Thus, in vitro experiments of TTR aggregation in the presence of inhibitors that measure the rate and amount of protein precipitation by turbidimetry constitute the standard test for the identification and characterization of novel TTR fibrillogenesis inhibitors.[17,18]

Binding of inhibitors to TTR has also been demonstrated to occur ex vivo in human and transgenic animal blood plasma samples and in vivo using cell cultures.[19,20] Thus, TTR tetramer stabilization is considered to be one of the most promising approaches for the discovery of the first effective pharmacological intervention for TTR amyloid diseases. However, in spite of this evidence, a compound that prevents in vivo deposition of TTR aggregates after being administered to an animal model of these diseases has still not been identified.

The isatin-3-arylimines here studied were prepared in a straightforward manner using already known methods[21] following the reaction depicted in Scheme 1. Commercially available isatin, *N*-methylisatin or 5-substituted isatins were reacted with different commercial 3-substituted anilines in refluxing ethanol. Direct crystallization of reaction mixtures by spontaneous solvent evaporation afforded the isatin imines of Table 1 in good yields.

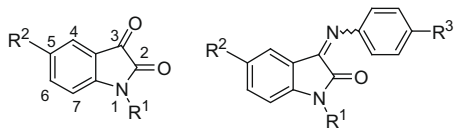
The potential fibrillogenesis inhibitory properties of these compounds were assessed using a high throughput screening in vitro assay previously validated in our labs.[17] The method measures the ability of each individual compound to inhibit the turbidimetry induced by acidification of transparent solutions of the TTR variant Y78F, which is one of the most amyloidogenic mutants of TTR. Because protein aggregation rates are proportional to turbidimetry rates, different kinetic aggregation parameters can be deduced after testing a series of concentrations of each inhibitor. Most meaningful are IC_{50} and RA (%). Values of these parameters for the prepared isatin analogs and for thyroid hormone T_4 (thyroxine) and triiodophenol (TIP) as reference compounds are reported in Table 1.

Reduction of amyloidosis, RA (%), is defined as the percent of reduction of the fibril formation rate at a high inhibitor concentration relative to the rate at zero concentration of the tested compound. RA (%) values of 100% indicate that the inhibitor is able to fully prevent fibril formation. On the other hand, IC_{50} is the inhibitor concentration at which the initial rate of fibril formation is half the rate in the absence of the inhibitor. Since the TTR tetramer can hold up to two molecules of inhibitor, the maximum binding stoichiometry is 2:1. However, an optimal inhibitor is expected to require the lowest ratio of just 1:1. Accordingly and given that the TTR concentration in these assays was 7.2 μM, a good inhibitor would display a maximum response at half of the protein concentration, this is, 3.6 μM. This concentration would be the maximum value of IC_{50} for a given TTR aggregation inhibitor that acts exclusively through a tetramer stabilization mechanism.

Isatin (**1a**) did not show significant inhibitory activity when assayed at concentrations within the range 0–40 μM. No activity was also recorded for the corresponding isatin-3-imine derivatives of aniline and 4-methyl and 4-iodo substituted anilines (**2a**, **2d** and **2l**). However, good to excellent values of inhibitory potency were observed for both the methoxy (**2f**, IC_{50} = 8.1 μM) and the nitro (**2r**, IC_{50} = 2.8 μM) derivatives. The excellent potency of the nitro derivative was even superior to that of triiodophenol (TIP, IC_{50} = 3.2 μM), which is one of the most potent TTR fibrillogenesis inhibitors known to date.

In addition, when further examining the influence of a second substitution at the C-5 position in one of these already active isatin-3-imines, namely compound **2f**, it was found that all of them (compounds **2h** to **2k**, R^2 = CH_3, Br, I and NO_2, respectively) deplete rather than potentiate the activity of the parent compound, suggesting that this 5-position is crucial and should be unoccupied for effective TTR inhibition. When the influence of a second substitution at C-5 was examined in the inactive compound isatin-3-

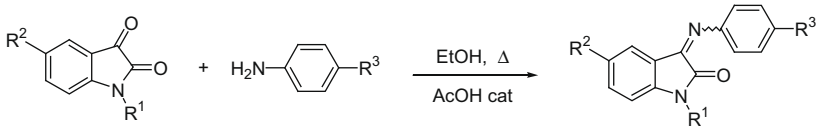
Table 1
TTR fibrillogenesis inhibition properties of isatin-imines measured in vitro by using the kinetic turbidimetric assay[a]



1a-b **2a-s**

Compound	R^1	R^2	R^3	IC_{50}[b] (μM)	RA[c] (%)
1a	H	H	H	>50	n.a. (24)[d]
1b	CH_3	H	H	6.3	100
2a	H	H	H	>50	31
2b	CH_3	H	H	11.0	77
2c	H	I	H	>50	1
2d	H	H	CH_3	>50	n.a. (14)[d]
2e	CH_3	H	CH_3	6.3	8.6
2f	H	H	OCH_3	8.1	100
2g	CH_3	H	OCH_3	7.7	81
2h	H	CH_3	OCH_3	>50	32
2i	H	Br	OCH_3	>50	48
2j	H	I	OCH_3	>50	42
2k	H	NO_2	OCH_3	>50	24
2l	H	H	I	>50	0
2m	CH_3	H	I	>50	0
2n	H	CH_3	I	>50	n.a. (7)[d]
2°	H	Br	I	>50	16
2p	H	I	I	>50	23
2q	H	NO_2	I	>50	n.a. (7)[d]
2r	H	H	NO_2	2.8	90
2s	CH_3	H	NO_2	6.3	86
TIP				3.2	80
T_4				10.5	95

[a] Parameters obtained from fitting the data of 'initial rates of fibril formation (v_0)' versus 'inhibitor concentration ([*I*])' to Eq. 1 (Supplementary data), except when indicated as (d). IC_{50} and RA (%) values are the mean of three experiments.
[b] Concentration of inhibitor at which the initial rate of fibril formation is half the rate at [*I*] = 0.
[c] Percentage of reduction of the fibril formation rate at a high (infinite) inhibitor concentration relative to the rate at [*I*] = 0.
[d] Inhibitor for which v_0 versus [*I*] follows a linear dependence. RA (%) is not applicable (indicated by n.a.). In brackets, RA (%) at 40 mM: percentage of reduction of fibril formation rate at 40 mM inhibitor concentration.



Scheme 1. Synthesis of 3-arylimino-2-indolones. Reagents and conditions: isatin (0.5 mmol), aniline (0.5 mmol), EtOH, cat. CH_3COOH, reflux, 45 min.

imine **2l**, inactive compounds (**2n**–**q**, $R^2 = CH_3$, Br, I and NO_2, respectively) were also obtained. The same occurred when the inactive isatin-3-imine **2a** was converted into the 5-iodo derivative **2c**.

Surprisingly, *N*-methylisatin (**1b**) was a good inhibitor, as were its isatin-3-imine derivatives (**2b**, **2e**, **2g** and **2s**, R^2 = H, CH_3, OCH_3, NO_2 respectively), with the exception of the totally inactive 5-iodo-aniline derivative (**2m**). The potencies of these analogues were in the same range as the natural thyroid hormone T_4. This is in sharp contrast with the aforementioned total inactivity of isatin (**1a**) and the isatin-3-imine derivatives of aniline (**2a**) and 4-methyl substituted aniline (**2d**).

This rough SAR analysis corroborates that the substitution pattern of the isatin and isatin-3-arylimine derivatives here tested is crucial for activity. The most potent compounds identified were those exhibiting polar substituents at position 4 of the aniline moiety, namely compounds **2r** and **2f**, while compounds with less polar groups or lipophilic substituents, namely **2a**, **2d** and **2l** were not active. This may suggest that an electrostatic interaction (dipolar, hydrogen bond, etc.) critical for activity may be at play. On the other hand, given that compounds of considerable potency were obtained when isatin (**1a**) and the isatinimine derivatives of aniline (**2a**) and 4-methyl substituted aniline (**2d**) were transformed into the corresponding *N*-methyl derivatives (**1b**, **2b**, and **2e**, respectively), it could be hypothesized that the lipophilicity of the isatin moiety at the 1 position is a positive contributing factor for activity.

It is well known that 3-arylimino-2-indolones exist as *E* and *Z* mixtures of isomers that can be analyzed by ^{1}H NMR, but as they readily interconvert in solution they cannot be separated by chromatography.[4,10] To investigate if the differences in activity are correlated to the stereochemistry of these compounds in solution, an NMR study of the isatin-3-arylimines **2f**, **2g**, **2l**, and **2m** was conducted (see Supplementary data). Within this group, the two methoxyphenyl-3-imino derivatives of isatin and *N*-methylisatin (compounds **2f** and **2g**) were very potent fibrillogenesis inhibitors while the iodinated analogues (**2l** and **2m**) were totally inactive.

The stereochemistry of the imines was established unequivocally by NMR techniques using 2D homonuclear (COSY and NOESY) and heteronuclear (HSQC and HMBC) experiments. The *E* stereochemistry was assigned to the major isomer in DMSO-d_6 solutions because the NOESY experiments show a neat correlation between H_4 and $H_{2'}$ and also a weak correlation between H_4 and $H_{3'}$ for these major isomers. In addition, the signal for H_4 of the *E*-isomer was considerably shifted upfield (approx. 1 ppm) relative to the H_4 signal of the parent isatin and the minor Z-isomers. Moreover, the chemical shifts of H_4, H_5, H_6 and H_7 of the *Z*-isomers showed little difference in chemical shift to those for the parent isatin (see Supplementary data). All the ^{1}H spectra of these two pairs of isatin-3-arylimines show the same 8:2 ratio of *E*/*Z* isomers so the differences of activity within these compounds can not be explained in terms of varying isomer compositions.

To determine if the mechanism of the inhibitory activity of these compounds proceeds by tetramer stabilization, isatin binding to TTR was investigated. A labeled thyroxine (^{125}I-T_4) displacement test that measures the ability of each inhibitor to displace labeled T_4 from its binding site on TTR was used.[22] The method gives affinity values relative to T_4. To our surprise, regardless of their fibrillogenesis inhibition activity, none of the isatin derivatives was able to displace ^{125}I-labeled thyroxine from the complex with tetrameric TTR, indicating that none of them are TTR ligands. More direct proof of the lack of binding affinity was obtained by isothermal titration calorimety experiments on the most active isatin derivative (**2r**). In spite of being very effective at inhibiting TTR aggregation, the compound completely failed to bind to soluble TTR even at concentrations as high as 40 μM. These results are a strong indication that, in contrast with the most common TTR amyloidosis inhibitors, the isatin analogs do not act by inducing kinetic stabilization of the tetramer by ligand binding. In turn, this suggests that other possible interactions between isatins and TTR soluble aggregates inhibit TTR amyloidogenic processes. Although much work is required to elucidate the molecular phenomena underlying the inhibitory activity of these compounds, the high potency of compounds such as **2r** make isatin a very promising new scaffold for the design and development of potential drugs for TTR-related amyloid diseases. In addition, since isatins act by a different mechanism from that of the known families of tetramer stabilizing inhibitors, which up to now have failed to show their effectivity in animal models, the isatins here reported offer a new possibility for achieving this goal.

In conclusion, a new class of TTR fibrillogenesis inhibitors based on isatin is reported. This family comprises simple derivatives such as *N*-methylisatine (**1b**) as well as 3-arylimino-2-indolones such as 1,3-dihydro-3-[(4-nitrophenyl)imino]-2*H*-indol-2-one (**2r**), with a potency matching that of triiodophenol (TIP) but lacking TTR binding affinity properties. Furthermore, no correlation between the biological activity and the stereochemistry of such compounds in solution was found. By acting with a novel yet unknown mechanism, the isatins may open new avenues for the discovery of inhibitors that are active in animal models. The study of this mechanism may also provide new insights for the complete characterization of TTR amyloid processes.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.004.

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