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# Discovery of novel non-peptide inhibitors of BACE-1 using virtual high-throughput screening

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ABSTRACT

A novel series of isatin-based inhibitors of β-secretase (BACE-1) have been identified using a virtual high-throughput screening approach. Structure–activity relationship studies revealed structural features important for inhibition. Docking studies suggest these inhibitors may bind within the BACE-1 active site through H-bonding interactions involving the catalytic aspartate residues.



Alzheimer's disease (AD) accounts for the majority of dementia diagnosed in patients after the age of 60.[1] The formation of insoluble extracellular amyloid plaques by the accumulation of amyloid β-peptide (Aβ) is one of the key pathological features in the brains of AD sufferers.[2] As outlined by the amyloid cascade hypothesis,[3] Aβ is generated by the proteolytic cleavage of the β-amyloid precursor protein (APP). β-Secretase (β-site APP cleaving enzyme-1, or BACE-1) is the first of two enzymes responsible for the sequential processing of APP, and is considered rate-limiting in the proteolytic cleavage process.[4] Since BACE-1 knockout mice have been shown to be viable with only mild phenotypic changes affecting peripheral nerve development, the inhibition of BACE-1 is considered a promising therapeutic approach for the treatment of AD.[5]

BACE-1 belongs to the family of pepsin-like human aspartyl proteases, characterized by a single transmembrane domain. With the catalytic site located on the lumenal side of the membrane, BACE-1 processes the substrate APP at Met671-Asp672 ($APP_{770}$ numbering) to release soluble sAPPβ. The membrane-bound fragment C99 is then cleaved by γ-secretase to generate the amyloidogenic Aβ.

A number of inhibitors of BACE-1 have been reported.[6] In the case of the earliest reports, the majority of these involved peptide-based structures with relatively high molecular weights and unfavorable properties in terms of penetration of the blood-brain barrier (BBB). More recently, however, a range of potent non-peptidic inhibitors of moderate size have been described.[6] In light of the wealth of X-ray crystallographic data available for BACE-1, we were interested in designing small molecule non-peptide inhibitors using structure-based methods.[7] Herein, we report the discovery of a novel series of non-peptide inhibitors of BACE-1 using the virtual high-throughput screening software eHiTS.[8]

eHiTS is an exhaustive flexible docking algorithm with a scoring function which incorporates both empirical and knowledge-based features.[9,10] Input ligands are divided into rigid fragments and flexible connecting chains.

The rigid fragments are then docked independently within the active site of BACE-1 and assessed for the best pose combination before re-connection with the connecting chains to generate the final docked conformation. Using the high-throughput screening mode within the program, three compound libraries containing a total of 250,000 commercially available compounds[11] were screened against the BACE-1 X-ray crystal structure co-crystallized with a peptide-based inhibitor (PDB code 1M4H).[12] From the top-ranked structures, six molecules were selected for purchase, based upon predicted binding affinity and synthetic tractability (see Supplementary data). These compounds were tested in an in vitro activity assay using recombinant human BACE-1 and a quenched

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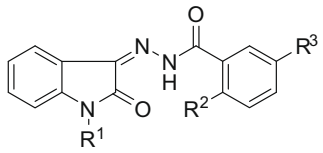
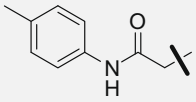
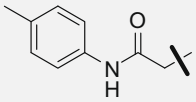
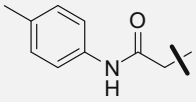
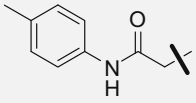
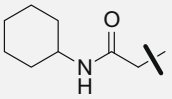
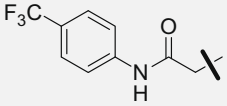
**Table 1**
Compound library for SAR studies of a small molecule BACE-1 inhibitor



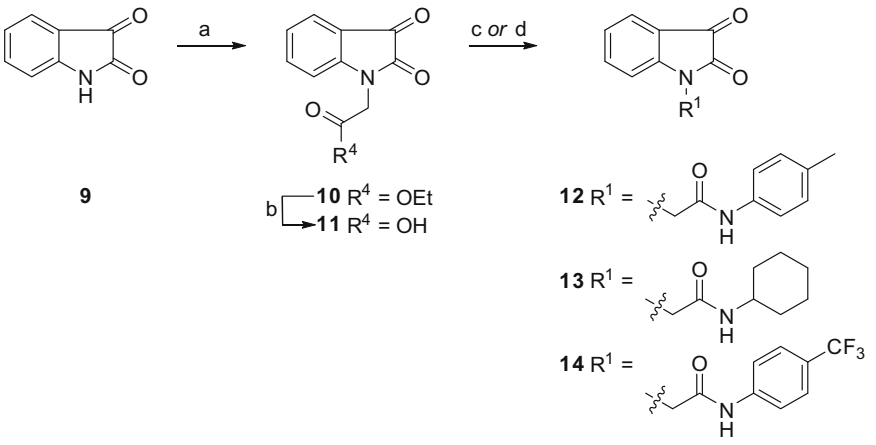
**1-8**

| Compound | $R^1$ | $R^2$ | $R^3$ | BACE-1 inhibition at 100 μM[a], % ($IC_{50}$ in brackets, μM)[b] |
|---|---|---|---|---|
| **1** |  | OH | $NO_2$ | 84.5 ± 2.1 (2.4 ± 0.3) |
| **2** |  | H | $NO_2$ | 28.8 ± 3.6 |
| **3** |  | OMe | $NO_2$ | 20.2 ± 0.1 |
| **4** |  | OH | H | 71.1 ± 2.4 |
| **5** | H | OH | $NO_2$ | 35.2 ± 4.9 |
| **6** | Me | OH | $NO_2$ | 14.3 ± 4.9 |
| **7** |  | OH | $NO_2$ | 92.0 ± 2.5 (4.8 ± 0.1) |
| **8** |  | OH | $NO_2$ | 74.6 ± 3.4[c] |

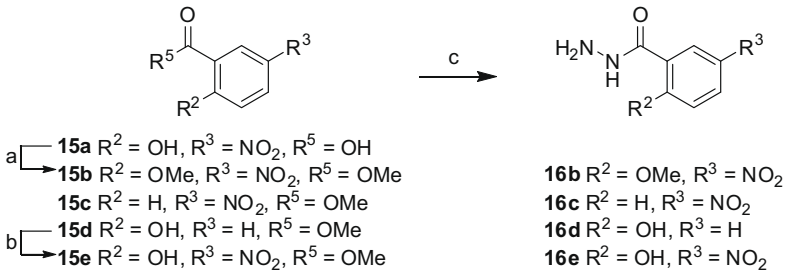
[a] Inhibition of BACE-1 activity measured as a percentage of the control activity in an in vitro BACE-1 activity assay (see text). Values shown are mean ± SEM.
[b] $IC_{50}$ only determined for compounds attaining over 80% BACE-1 inhibition at 100 μM.
[c] Precipitation of assayed compound observed during incubation.

fluorescent peptide substrate based on the Swedish mutant APP sequence (SEVNLDAEFK).[13] Of the six compounds assayed, compound **1** was identified as a BACE-1 inhibitor with an $IC_{50}$ value of 2.4 μM (Table 1).

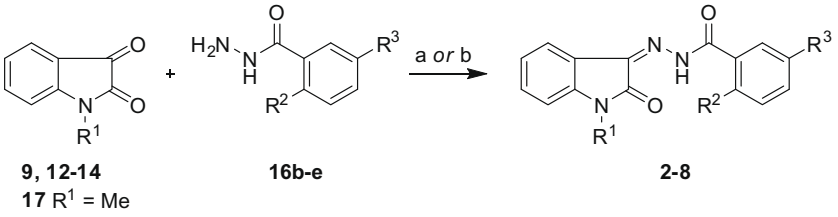
A series of structural analogs (**2**–**8**, Table 1) were synthesized to probe the structural features required for biological activity. These molecules were prepared using standard methods involving coupling of various isatins with hydrazides.[14] A range of N-substituted




**Scheme 1.** Synthesis of isatin-*N*-acetamides **12**–**14**. Reagents and conditions: (a) ethyl bromoacetate, $K_2CO_3$, DMF, 0 °C, 16 h, 52%; (b) 10% NaOH/EtOH, rt, 10 min, quantitative; (c) (i) $SOCl_2$, 60 °C, 3 h; (ii) R–$NH_2$, $CH_2Cl_2$, 0 °C, 20 h; (d) R–$NH_2$, HOBt, EDAC, $CH_2Cl_2$, 0 °C, 20 h, 55–62%.




**Scheme 2.** Synthesis of hydrazides **16b–e**. Reagents and conditions: (a) MeI, KOH, DMSO, rt, 16 h, 65%; (b) fuming $HNO_3$, AcOH, heat, 20 min, 40%; (c) hydrazine monohydrate, MeOH, reflux, 3 h, 36–85%.




**Scheme 3.** Synthesis of analogs **2–8**. Reagents and conditions: (a) AcOH, reflux, 3 h; (b) AcOH, microwave, 300 W, 150 °C, 30–60 min, 38–88%.

isatins were prepared by reacting isatin **9** with ethyl bromoacetate to give ester **10**, which was subsequently hydrolyzed to give isatin-*N*-acetic acid **11**.[15]

Isatin-*N*-acetamides **12–14** were prepared by either forming the acid chloride intermediate (method c), or direct coupling with the appropriate amine using HOBt and EDAC (method d) (Scheme 1).

The hydrazides were prepared by reaction of appropriately substituted methyl benzoates **15b–e** with hydrazine to give hydrazides **16b–e** (Scheme 2).[16,17] Condensation of the isatins with the hydrazides using either conventional reflux in acetic acid or under microwave irradiation delivered the desired structures in the compound library (Scheme 3).

Firstly, we investigated the effects of altering the phenolic functionality of inhibitor **1** on BACE-1 inhibition. Removal of the phenolic OH (Table 1, as in compound **2**) resulted in a significant reduction in BACE-1 inhibition at 100 μM, indicating that the presence of the phenolic moiety is important for BACE-1 inhibition. The methoxy analog compound **3** showed only 20% BACE-1 inhibition at 100 μM, further supporting the importance of the phenolic functionality in the inhibition of BACE-1.

The role of the nitro group in compound **1** was also explored. The des-nitro version (compound **4**) showed a BACE-1 inhibition of 71% at 100 μM, indicating that the nitro function appears not to be important for binding to BACE-1.

Next, we introduced different substituents onto the isatin portion of the inhibitor in order to investigate the importance of the acetamide portion in **1**. BACE-1 inhibition dropped considerably when $R^1$ was replaced by H or Me (Table 1, compounds **5** and **6**, respectively). The detrimental effects observed indicated an essential role for the acetamide portion of compound **1** in BACE-1 inhibition. By retaining all the structural features of the original hit compound **1**, compounds **7** (*p*-tolyl moiety of **1** replaced with a cyclohexyl ring) and **8** (*p*-trifluoromethyl in place of the *p*-tolyl moiety in **1**) show comparable levels of BACE-1 inhibition (92% and 75%, respectively, at 100 μM) to that of compound **1** (Table 1). The selectivity of these inhibitors for BACE-1 was established following our observation that they do not appear to inhibit the activity of the closely related enzyme BACE-2 (data not shown). Additionally, in order to rule out aggregation-related non-specific inhibition, we observed that the presence or absence of detergent had no effect upon the inhibitory activities. Furthermore, control experiments involving fluorescence measurements of the substrate in the presence of the inhibitors, but in the absence of enzyme, established that the inhibitors had no effect on the fluorescence behavior of the substrate.

Having established the important molecular features within these isatin-derived inhibitors required for BACE-1 inhibition, we wished to probe further the possible binding pose of compound **1** within the BACE-1 active site. It has been previously reported that isatin 3-benzoylhydrazone **18** (Fig. 1), a substructure of **1**, adopts a near planar structure in solution due to the delocalization of the π electrons along the –N–NH–C(=O)– scaffold in addition to an intramolecular H-bond between the isatin carbonyl and the hydrazone N–H.[18] For compound **1**, modeling indicated that the tendency for planarity of the molecule is reinforced by additional intramolecular H-bonding involving the phenolic O–H to yield either of the two conformations **19** and **20** (Fig. 2).

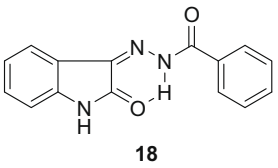



**Figure 1.** Structure of isatin 3-benzoylhydrazone **18**, appears to adopt a planar conformation.

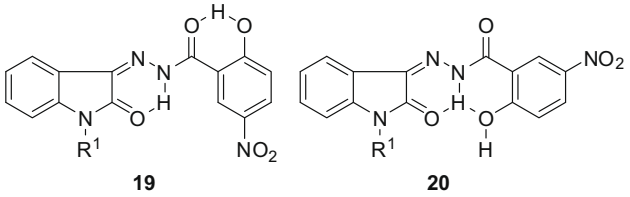



**Figure 2.** The two important conformations of compound **1** involving the formation of intramolecular H-bonding with the phenolic O–H.

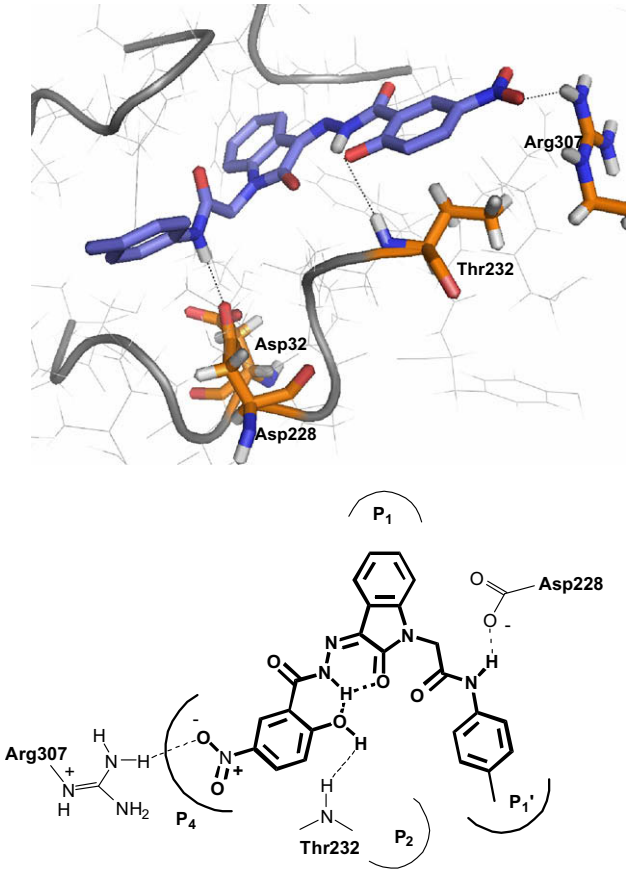



**Figure 3.** (top), binding pose of **1** (corresponding to conformer **20**) in the BACE-1 active site generated using AutoDock; (bottom), schematic showing predicted H-bond contacts and positions of BACE-1 substrate binding pockets.

AutoDock[19] was used to separately dock the two proposed favored conformations **19** and **20** of compound **1** into the active site of BACE-1 (PDB code 1M4H). While the docking of conformer **19** did not give any solutions consistent with the observed biological activity (data not shown), docking of conformer **20** revealed a binding pose which was consistent with the observed activities of compounds **1**–**8**.

Figure 3 (top) shows the lowest-energy binding pose identified for conformer **20** within BACE-1. It is noteworthy that an analogous binding pose was also identified using eHiTS. The acetamide moiety is predicted to occupy the catalytic site, with the acetamide N–H acting as an H-bond donor to the catalytic residue Asp228 (H-bond length = 1.86 Å). This is consistent with the observed biological activity as the structural analogs lacking the acetamide portion (compounds **5** and **6**) displayed very poor activity towards BACE-1. The phenol unit is predicted to make an H-bond contact with the backbone nitrogen of Thr232 (H-bond length = 2.20 Å) and to partly occupy the $P_2$ substrate pocket. This feature implies the phenol might be involved in both the formation of the intramolecular H-bonding network, and also in intermolecular H-bonding interactions with the enzyme. The nitro group in **1** is predicted to extend into the $P_4$ pocket (Fig. 3, bottom, and see also the Supplementary data), possibly participating in weak H-bonding with the side chain of Arg307 (H-bond length = 2.13 Å), consistent with the slight decrease in the binding affinity exhibited by compound **4**.

In summary, using the virtual high-throughput screening software eHiTS, we have discovered a novel non-peptidic inhibitor of BACE-1 based on an isatin motif. Studies of the biological activity of structural variants in combination with in silico docking suggest the inhibitor adopts a planar conformation, which is stabilized by intramolecular H-bonding from the phenolic moiety in **1**. Additionally, binding to BACE-1 appears to involve H-bonding interactions between the *p*-tolylamide of **1** and the catalytic residue Asp228. A recent report[20] detailing the discovery of a series of potent small molecule BACE-1 inhibitors compares the ligand efficiency (LE)[21] of a range of reported inhibitors of BACE-1. In this study, the authors noted that despite the high potency of the previously reported peptide-based BACE-1 inhibitors such as OM99-2 ($K_i$ = 1.6 nM),[22] the relatively high molecular weights of these systems (e.g., OM99-2 has a molecular weight of 893) often result in them having relatively poor ligand efficiency (e.g., LE = 0.19 for OM99-2). For the present study, although still somewhat below the preferred minimum value of LE = 0.3,[21] compound **1** (molecular weight = 461) has LE = 0.22 and therefore, is closer to the preferred value than the potent but considerably larger peptidic inhibitors reported previously. Although the relatively poor solubility of the present series of compounds (e.g., *c*log *P* values for compounds **1** and **7** are 3.97 and 3.66, respectively), coupled with the presence of the nitro and phenolic moieties render them challenging candidates for further development, we have demonstrated that eHiTS is a powerful screening tool to identify biologically active compounds quickly and efficiently.

## Acknowledgments

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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.2009.09.103.

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14. *Typical procedure*: To a solution of isatin **9** (3.4 mmol) in DMF (10 mL) was added potassium carbonate (5.1 mmol). Ethyl bromoacetate (4.1 mmol) was added dropwise at 0 °C, and the reaction was stirred at room temperature for 16 h. The precipitate was poured into an ice-water mixture, and the orange

precipitate formed filtered, washed with water, and recrystallized in water to give ester **10**. 10% NaOH was added to a stirring ethanolic solution of **10**, and the solution stirred at room temperature until the color changed to yellow. Conc. HCl was added to obtain an orange precipitate, which was filtered and recrystallized in water to give isatin-*N*-acetic acid **11**. To a solution of **11** (4.9 mmol) and cyclohexylamine (12.25 mmol) in anhydrous DCM was added HOBt (5.9 mmol) and EDAC (5.9 mmol) at 0 °C, and the reaction was stirred at room temperature for 20 h. The organic layer was diluted, washed with 2 M aq HCl (3×25 mL), satd $NaHCO_3$ (3 × 25 mL), brine (30 mL), dried with $MgSO_4$, and the solvent removed in vacuo to give the crude product **13**, which was recrystallized in MeOH/$H_2O$ (Scheme 1). Methyl salicylate **15d** (10.0 mmol) was reacted with fuming nitric acid (10.0 mmol, 10% v/v) premixed with glacial AcOH, and the reaction heated until the color changed to brown. The mixture was cooled and water was added to give a brown precipitate, which was filtered and purified using flash chromatography on silica gel with 4:1 petroleum ether (40–60 °C)/EtOAc as eluent to afford methyl 5-nitrosalicylate **15e**. Hydrazine monohydrate (30.0 mmol) was added to a methanolic solution of **15e** (7.5 mmol), and the reaction heated under reflux for 3 h. The mixture was cooled to 0 °C, the yellow precipitate was then filtered and recrystallized from MeOH to give **16e** (Scheme 2). Amide **13** (0.35 mmol) was mixed with hydrazide **16e** (0.35 mmol) in glacial AcOH, and the reaction was heated to 150 °C in a CEM microwave reactor at 300 W for 30 min. The precipitate was filtered and washed with EtOAc to give pure product **7**. Mp >250 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 14.30 (br s, 1H), 8.83 (d, 1H), 8.31 (s, 1H), 7.89 (br s, 1H), 7.68 (d, 1H), 7.45 (t, 1H), 7.21–7.16 (m, 2H), 7.00 (d, 1H), 4.39 (s, 2H), 3.59 (m, 1H), 1.77–1.69 (m, 4H), 1.58–1.53 (m, 1H), 1.30–1.17 (m, 5H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 164.1, 161.7, 159.6, 142.9, 139.7, 136.6, 130.9, 128.4, 127.1, 122.5, 120.1, 119.2, 117.9, 117.5, 109.5, 109.3, 47.5, 41.9, 31.7, 24.6, 23.9; HRMS (ES-) $m/z$ 464.1578 $[M-H]^-$; $C_{23}H_{22}N_5O_6^-$ requires 464.1576; HPLC (5–95% MeCN/$H_2O$) $t_R$ 23.91 min (peak area 100%).

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