In Vitro Assay Development and HTS of Small Molecule Human ABAD/17β-HSD10 Inhibitors as Therapeutics in Alzheimer's Disease

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Abstract

A major hallmark of Alzheimer's disease (AD) is the formation of neurotoxic aggregates composed of the amyloid-β peptide (Aβ). Aβ has been recognized to interact with numerous proteins resulting in pathological changes to the metabolism of patients with Alzheimer's disease (AD). One such mitochondrial metabolic enzyme is amyloid-binding alcohol dehydrogenase (ABAD), where altered enzyme function caused by the Aβ-ABAD interaction, is known to cause mitochondrial distress and cytotoxic effects, providing a feasible therapeutic target for AD drug development. Here we have established a high-throughput-screening (HTS) platform for the identification of modulators to the ABAD enzyme. A pilot screen with a total of 6759 compounds from the NIH Clinical Collections (NCC) and SelleckChem libraries and a selection of compounds from the BioAscent diversity collection, has allowed validation and robustness to be optimised. The pilot screen revealed 16 potential inhibitors in the low □M range against ABAD with favourable physicochemical properties for blood-brain barrier penetration.

Abbreviations

17β-HSD10	=	17β-hydroxysteroid dehydrogenase type 10
ABAD	=	amyloid-binding alcohol dehydrogenase
AD	=	Alzheimer's disease
AICD	=	amyloid precursor protein intracellular domain
APP	=	amyloid precursor protein
Aβ	=	amyloid-β peptide
BACE	=	β-secretase
BBB	=	blood–brain barrier
BSA	=	bovine serum albumin
DMSO	=	dimethyl sulfoxide
DTT	=	dithiothreitol
ER	=	endoplasmic reticulum
ETC	=	electron transport chain
FAC	=	final assay concentration
HTS	=	high throughput screen
RCCs	=	redox cycling compounds
ROS	=	reactive oxygen species
TEM	=	transmission electron microscopy
S/B	=	signal to background
SDR	=	short chain dehydrogenase reductase
ThT	=	thioflavin T
STD NMR	=	saturated transfer difference NMR

Introduction

Alzheimer's disease (AD) is the most common form of dementia, and with current therapies at best palliative the development of a drug that can halt or even reverse the progression of AD is an essential goal in order to manage this debilitating disease. Despite being identified over 100 years ago the underlying cause of AD is a contentious issue with proponents for a number of theories. However, the amyloid cascade hypothesis which implicates amyloid-β peptide (Aβ) as the main causative agent, has been generally accepted.[1] Amyloid binding alcohol dehydrogenase (ABAD, or 17β-hydroxysteroid dehydrogenase type 10) was first identified as an Aβ binding protein in 1997 using a yeast two hybrid system.[2] A finding which was subsequently confirmed using a number of techniques.[2–4] ABAD is known to interact with the two major plaque forming isoforms of Aβ, namely Aβ(1-40) and Aβ(1-42), leading to distortion of the enzyme structure and inhibition of its normal function as an energy provider for cells.[5,6] *In vitro* experiments have shown that the interaction between ABAD and Aβ is cytotoxic and ABAD's function is altered with a build-up of ROS and toxins leading to mitochondrial dysfunction.[7] Using site directed mutagenesis and surface plasmon resonance (SPR) Lustbader *et al*.[3] identified the L_D loop of the ABAD protein as the binding site for Aβ, and subsequently synthesised a 28 amino acid peptide encompassing this region (amino acids 92-120) that was termed the ABAD-decoy peptide (ABAD-DP). Again using SPR it was shown that this ABAD-DP could inhibit the binding of ABAD to Aβ(1-40) and Aβ(1-42). Significantly, the inhibition of the interaction between ABAD and Aβ by ABAD-DP was shown to translate into a cytoprotective effect in cell culture

experiments.[8] Cultured wild type cortical neurons exposed to Aβ(1-42) showed a significant increase in cell death, as measured by cytochrome-c release, whilst those pre-incubated with the ABAD-DP did not.[8] Critically for this work demonstrated that inhibition of the ABAD-Aβ interaction may offer a novel therapeutic avenue for the treatment of AD.

Other than the disruption of the ABAD-Aβ interaction, there is a second approach which may hold merit in treating AD; the direct modulation of ABAD activity. *In vitro* experiments with SHSY-5Y cells administered with the ABAD inhibitor, AG18051, show a reduction in mitochondrial dysfunction and oxidative stress associated with the interaction between ABAD and Aβ, and are protected from Aβ mediated cytotoxicity.[9] Suggesting that inhibition of ABAD may also be a viable therapeutic approach in the treatment of complexities that exist within AD. In order to screen and identify new efficacious drug-like inhibitors we have already developed an ABAD activity assay,[10] utilising the loss of absorbance of the enzyme's cofactor NADH (at 340 nM) as ABAD converts it to NAD^+ as its readout. However, as very few modulators of ABAD activity have been reported and with limited compound diversity as starting points for those that have, we optimised this assay further to achieve an improved, robust assay amenable to high-throughput screening (HTS) on a 384-well plate scale.

Materials and Methods

All aqueous solutions were prepared with deionized water (Millipore, UK) and all chemicals purchased from Sigma Aldrich, UK unless stated. ABAD protein

was expressed and purified as described in Aitken *et al.* 2016.[11] The initial 96-well plate screening assay conditions are described in Hroch *et al.* 2016.[10] Assay buffer used contains: 10 mM Tris.HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.005 % Tween and 0.01 % BSA. As indicated for some experiments the assay buffer was supplemented with 100 units/ml bovine liver catalase. All experiments were conducted in clear bottom 384-well low volume microplates (Corning) with a final assay volume of 20 µl and the reaction progress was monitored by oxidation of NADH as determined by reduction in absorbance at 340 nm read on the EnVision plate reader (PerkinElmer).

Acetoacetyl-CoA Enzyme Kinetics

To determine the kinetics for acetoacetyl-CoA and the effect of ABAD concentration upon reaction rate, a matrix titration experiment was set up. Previously determined conditions[10] were used with a fixed concentration of 700 µM NADH and a starting concentration of up to 200 µM acetoacetyl-CoA. Doubling dilutions of acetoacetyl-CoA were then coupled with doubling dilutions of ABAD, which started with a maximum concentration of 40 nM. Data collected from the experiment was analysed using a standard template from which reaction progress curves were analysed to calculate initial velocities and obtain Km values using the Michaelis-Menten equation (XLFit, ID Business Solutions).

NADH Enzyme Kinetics

To determine the kinetics for NADH and the effect of ABAD concentration upon reaction rate a matrix titration experiment was set up. Previously

determined conditions[10] were used, 120 µM acetoacetate and a starting concentration of up to 1000 µM NADH. Doubling dilutions of NADH were then coupled with doubling dilutions of ABAD, which started with a maximum concentration of 40 nM. Data were analysed as described previously.

Assay Development

An optimal ABAD concentration was established using a range of concentrations from 0.625, 1.25, 2.5 and 5 nM with measurements taken up to 60 minutes after the reactions were started to capture the full extent of the reaction linearity. Assay DMSO tolerance was tested from 20 % using a 10 point serial dilution with reads being taken up to 60 minutes as before. Reagent stability experiments were carried out whereby reactions were started at times of 0, 1, 2, 4 and 8 hours after reagent preparation. All reagents were maintained at room temperature throughout the course of the experiment.

Compound Screening

For primary screening an endpoint assay format was adopted in which an Echo acoustic liquid dispenser (Labcyte) was used to transfer 20 nl of either reference standard, DMSO control (0.5 % final assay concentration (FAC)) or test compounds (10 µM FAC) to the assay plate. 10 µL of ABAD enzyme in assay buffer (2.5 nM FAC) was then added to the plates using the Preddator liquid handling robot (Redd and Whyte) and plates were incubated at room temperature for 15 minutes. 10 µL of substrate mixture (100 µM acetoacetyl-CoA and 100 µM NADH FAC) was added with the Preddator. To start the

reaction and the plates were left to incubate for 35 minutes at room temperature before the absorbance at 340 nm was read on the EnVision plate reader (PerkinElmer). For kinetic assay format follow up experiments the same protocol was adopted but the absorbance at 340 nm was monitored constantly for 45 minutes from immediately after the reaction was started.

Physiochemical predictions

Published values were obtained for the NCC/Selleckchem hits (where available) from DrugBank (http://www.drugbank.com) and where there were no published values, predictions were made using ChemAxon Marvin suite (http://www.chemaxon.com).

Results and Discussion

To miniaturise our 96-well plate enzyme activity assay into one suitable for HTS, i.e. 384-well plate, we have taken a number of considerations into account. Firstly, our assay needed to be sensitive to inhibitors with a relevant and developmentally tractable mechanism of action. This required characterisation of the ABAD enzyme kinetics using the buffering conditions and 384-well microplates that were intended for use in the screen. Substrate concentrations for screening were then selected based upon which substrate binding site was deemed most tractable for discovering selective and potent inhibitors. Typically the substrate would be screened at or close to its K_m, as this provides a balanced chance of finding competitive, non-competitive or uncompetitive inhibitors, and with regards to ABAD, acetoacetyl-CoA was considered to be the best substrate to target, because NADH is a ubiquitous

cofactor for numerous proteins. As such a concentration of NADH in excess of its experimentally determined K_m was deemed the best approach to de-sensitise the assay to NADH competitive molecules. In addition to the concentration of substrates the concentration of enzyme is also vitally important in a screening assay. It must be high enough so that the enzyme is stable in solution over the course of the assay and can produce enough substrate turnover to reliably monitor reaction progress. However, it should not be too high that the reaction runs too quickly, thus allowing effective monitoring of the linear steady-state of the reaction. A low protein concentration should also avoid additional issues such as excessive protein requirement for large scale screening purposes or encountering limitations in the determination of IC_{50} for high affinity inhibitors too early in a development programme.[11] Finally, the screening assay needed to provide a robust reliable signal that allowed a significant compound effect to be identified from non-effect. This was determined by the Z'-value, a screening statistic that takes into consideration the signal window and the standard deviation with an ideal value of between 0.5 and 1 for a robust assay.[12]

It was hypothesised that a larger signal to background ratio would be seen if the assay could be adapted to be measured as a fluorometric assay. Additionally, moving to a measurement at longer wavelengths would help reduce the potential for compound interference of the assay signal. Several methods were explored to utilise a fluorescence based screening method, including direct measurement of NADH fluorescence; however this resulted in very poor Z' values and signal. An alternative approach was to try all three commercially available Amplite fluorimetric kits (AAT Bioquest) but these

resulted in signal saturation at all concentrations of NADH/NAD^+ tested (despite kit guidelines) or proved to be cumbersome for HTS purposes with many addition steps whilst still resulting in poor Z' values. As our absorbance assay produced very high Z' values indicating the assay was very robust, it was decided to reject this hypothesis and accept the lower signal to background ratio and continue with an absorbance HTS assay.

Enzyme Kinetics

To determine the kinetics for acetoacetyl-CoA and NADH and to investigate the reaction rate dependence of ABAD concentration a matrix titration of acetoacetyl-CoA vs. ABAD and NADH vs. ABAD was conducted using our previously determined buffering conditions.[10] Fixed concentrations of either NADH (700 µM) or acetoacetyl-CoA (200 µM) were used, while the second substrate was titrated respectively. Doubling dilutions of substrate were then coupled with doubling dilutions of ABAD starting with a maximum concentration of 40 nM. Activity time courses and initial velocities were obtained and after optimisation it was decided to use a lower concentration of NADH (100 µM), which is still in excess of Km and a higher starting concentration of acetoacetyl-CoA (800 µM) in order to obtain a full response. **Figure 1A and 1B** demonstrate the final kinetic data, where the Km for acetoacetyl-CoA was 117 ± 28 µM and a Km of 32.96 ± 4.47 µM for NADH was achieved.

Previous findings[10] suggested that NADH is required in excess of its Km in order to minimise the sensitivity of the assay to NADH competitive inhibitors. However, a concentration of NADH that was too high resulted in a

very high level of NADH background signal, this decreased the signal change relative to background that was observed by the activity of ABAD. The concentration of ABAD was also assessed; an optimal concentration produced a suitable change in absorbance, whilst still remaining in the initial velocity phase for an appropriate amount of time. This resulted in an increased signal to background and improved Z-prime. Four concentrations of ABAD were investigated (0.625, 1.25, 2.5 and 5 nM), where 0.625 nM and 1.25 nM ABAD produced very little signal window and the reaction at 5 nM was extremely fast. 2.5 nM ABAD produced a functional signal whilst it remained in the initial velocity phase for approximately 30-45 minutes. The NADH concentration had little effect on this experiment, especially at the higher concentrations indicating that the reaction was more dependent on acetoacetyl-CoA. It was determined that 62.5 µM and 125 µM NADH produced consistent results, therefore the excess concentration of NADH used in the final assay was 100 µM.

DMSO Tolerance

As most screening compounds are solubilised in DMSO, the tolerance of the enzyme assay to this solvent was assessed. Industry standard assays require a tolerance of at least 0.5% DMSO.[13] Using the established kinetic conditions DMSO tolerance was evaluated up to 20% of DMSO. Our ABAD activity assay was found to be tolerant up to approximately 2.5% DMSO, although less than 1% DMSO is more favourable **(Figure 2A)**.

Reagent Stability

For logistical HTS purposes it is important to understand for how long and under what conditions the various assay reagents are stable. Firstly, enzyme and substrate solutions were prepared in assay buffer and maintained at room temperature. Reactions were then started at time 0, 1, 2, 4 and 8 hour intervals after preparation with absorbance values **(Figure 2B)** used to compare Z' values and signal to background values for each time point **(Figure 2C and 2D)**. In addition to this, the effect of freeze/thawing ABAD was also assessed. These experiments confirmed that while the reagents used for this assay are stable for a prolonged length of time when stored appropriately, when prepared and kept at room temperature they are stable for up to two hours and that ABAD is not stable when freeze/thawed. Furthermore, the addition of bovine serum albumin (BSA) appeared to stabilise the enzyme and produced Z' values of 0.7-0.75 and the reaction still appeared in the initial velocity phase during the 45 minute incubation period so it was decided to also include 0.01% BSA in the assay buffer.

Collectively, these assay variations allowed an optimised 384-well plate scale (~20 □L) assay to be established, with more than suitable Z' values. The key criteria are shown in Table 1.

Robustness Set Testing

In order to evaluate what type of undesirable screening compounds our assay was susceptible to pick up as false positives, 118 small molecules with known HTS liabilities[14,15] were screened at two concentrations (10 and 1 µM). There was a percentage hit rate of 27 % of compounds at 10 µM and 9 % of

compounds at 1 µM, the full breakdown of the classification of compounds can be found in **Table 2.**

A very high percentage (81 % at 10 µM and 38 % at 1 µM) of the redox cycling compounds (RCCs) appeared to inhibit the effect of ABAD. The buffer for this assay contains the reducing agent DTT. RCCs generate H_2O_2 in the presence of strong reducing agents and H_2O_2 can indirectly inhibit the catalytic activity of proteins.[16] This is often a major source of false positives when performing a HTS. Identifying that a target is sensitive to inhibition by RCCs during assay development allows steps to be taken to either minimise the sensitivity to RCCs through optimisation of the assay buffer or to design a screening triage process that identifies and eliminates RCCs.[17] Unfortunately, despite alternative agents being available it was not possible to remove or replace DTT from the assay buffer as this reagent was found to be essential for protein stability; however, addition of catalase which degrades H_2O_2, abolished RCC-mediated inhibition of ABAD **(Table 2).** The addition of catalase to the assay buffer did negatively impact upon the S/B and Z' scores so its inclusion was not considered ideal for primary screening however it was used in lower throughput follow up assays with a higher replication level to identify and eliminate RCCs.

Primary Validation Screen

To further assess the performance of the ABAD assay, a small HTS of 1564 known bioactive compounds from the NIH Clinical Collections (NCC) and Selleckchem, FDA-approved Drug libraries, combined with 5195 diverse compounds from the BioAscent Compound Cloud

(http://www.bioascent.com/compoundcloud/) were screened producing 262 and 508 potential hits respectively, where hit compounds were classed as those producing a >20 % effect on ABAD inhibition. The screen proved very robust, with Z' values between 0.75 and 0.77 and CV% across the plates varying between 0.4 and 2.6 %. Signal to background varied between 1.40 and 1.49. These 770 potential hit compounds were then screened kinetically against ABAD to identify compounds, which genuinely inhibit product formation rather than simply absorb light at 340 nM and are therefore false positives when screened in the endpoint assay. De-selection of these hits using the catalase assay to remove any redox cycling compounds left 9 confirmed hits from the BioAscent collection and 8 hits from the NCC/ SelleckChem libraries **(Table 3),** final hit rates of 0.17% and 0.5% respectively. Best hit compound pIC_{50} curves for ABAD ± catalase can be found in supplementary information (**Figure S1 and Table S1**). A future step will be to obtain freshly prepared samples of these hit compounds and test them in orthogonal ABAD assays to validate target engagement.

A key aspect of any potential ABAD inhibitor is that in order to be of therapeutic value in AD they should be CNS penetrant. As the NCC and Selleckchem libraries are composed of a focussed set of FDA-approved drugs and NIH clinical candidates, these properties have already been published.[18] Physiochemical properties were calculated for the BioAscent compounds using ChemAxon software, where calculator Plugins were used for structure property prediction and calculations,[19] to give a direct comparison to those published for the NCC/Selleckchem hits **(Table 3).** Notably all of these hit compounds appear to be in good agreement with the optimal values for CNS

bioavailability[20,21], with the predicted aqueous solubility values also being satisfactory for many of the compounds. The optimal values for CNS penetrance were taken as MW ≤500, logP ≤5, HBA ≤7, HBD ≤3, TPSA ≤90 Å, $logS_{7.4}$ ≤-4, $logD_{7.4}$ = 0-3.[20,21]

The BioAscent compounds were selected principally on the basis of diversity, and so structure-activity relationships are limited in the resulting data. However, future work may include purchasing structurally similar scaffolds from their larger 100,000 library and once the hit compounds have been further validated, these could be acquired to develop SAR further.

The NCC/Selleckchem hits revealed several interesting molecules that have already been used and characterised as drugs for CNS diseases. For example, Liothyronine (a synthetic form of the thyroid hormone) has previously been used to treat hyperthyroidism and myxoedema, as well as in an augmentation strategy in treating major depressive disorder in combination with antidepressants.[22] However, there are links to thyroid replacement therapy and the advancement of AD symptoms, therefore this drug may not be the most promising starting point for a potential therapeutic for AD.[23]

Another former FDA approved drug identified in our screen was Alosetron.HCl (Lotronex, GSK). This compound blocks serotonin by targeting the $5\text{-}HT_3$ receptor. This drug was shown to be effective in treating irritable bowel syndrome, but was officially withdrawn from the market in 2000 due to adverse side effects before being re-introduced in 2002 for patients who did not respond to conventional treatments.[24] Compounds which act against serotonin have already been implicated as a potential therapeutic strategy for Alzheimer's disease due to the role of

serotonin-receptors in cognition and memory,[25] and an increase in serotonin signalling was associated with less Aβ accumulation in cognitively normal individuals.[26] With 5-HT_3 antagonists capable of freely passing the blood–brain barrier, and already implicated in other neurological conditions (Schizophrenia and anxiety)[24] analogues based on this structure may prove to be more promising as a therapeutic agent against ABAD.

Ellagic acid, a natural phenol antioxidant and taxifolin or silymarin, a member of the flavonoid family are two of the more commercially available hits from this screen. Using TEM and Thioflavin T, a β-sheet dye that fluoresces in the presence of Aβ aggregates, Taxifolin has been shown to inhibit Aβ aggregation, although the mechanism of action is still to be determined.[27]

Although not the most promising compound within the 16 hit compounds, Raloxifene.HCl, appeared as a hit in both the NCC and Selleck libraries, however it was also the least active. Raloxifene has been licenced since 1997 (Evista, Eli Lilly) and used to treat osteoporosis in post-menopausal women.[28] Raloxifene has already participated in a small clinical trial for AD where it was shown that a large dose of the drug resulted in the reduced risk of cognitive impairment in postmenopausal women.[29] It is already known that the inhibition of ABAD restores the amyloid-β-mediated deregulation of estradiol,[9] and with Raloxifene classed as a selective estrogen receptor modulator this compound proves to be very interesting and its analogues require further investigation as possible therapeutics against ABAD.

Overall we have optimised and evaluated our ABAD enzyme activity assay into a robust 384- well plate HTS, and used it to identify several novel molecules that modulate ABAD activity and have physicochemical properties suggesting CNS penetration. These could be developed further into lead like candidates against ABAD, a therapeutic target in AD. We have also identified several FDA approved drugs that with further investigation may be classed as re-purposed drugs if they prove to have a role in AD prevention/therapy.

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Conflict of Interest

The authors declare no competing financial interest.

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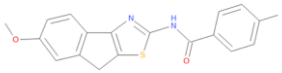
Table 1: Assay development summary table. This table highlights key HTS criteria met and established for our ABAD enzyme activity assay.

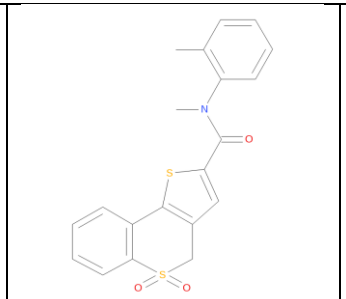
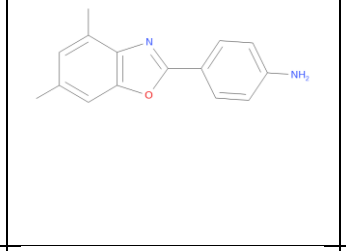
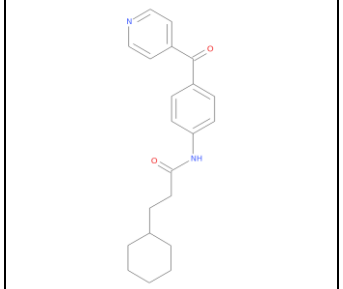
HTS Criteria	Experimentally Established Final Assay Values
Assay format	384-well-plate (clear)
Homogeneous assay	No wash steps
Reaction volume	20 µL
Selected Substrate Concentrations	100 µM acetoacetyl-CoA, 100 µM NADH
Full Plate Signal/Background	1.5
Full Plate Z' value	>0.7
Tolerance to DMSO	Tolerant up to ~2.5% DMSO
Minimal signal pattern on plates CV<10%	<3 %
Incubation times up to 4 hours	Incubate for 35-40 minutes before reading
Buffer composition	10 mM Tris.HCl, 150 mM NaCl, pH7.4, 1 mM DTT, 0.005 % Tween, 0.01% BSA
Substrate K_m	100 µM acetoacetyl-CoA, 20 µM NADH
Reagent Stability	Over 8 hours when reagents are stored appropriately, but 2 hours stability at room temperature

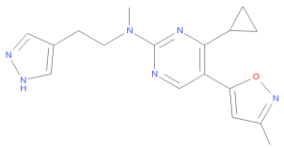
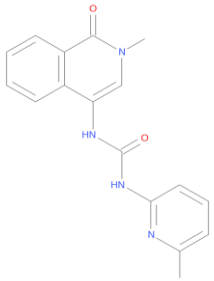
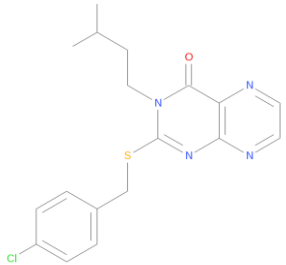
Table 2: Robustness screening set

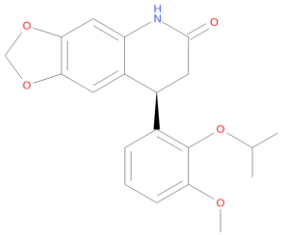
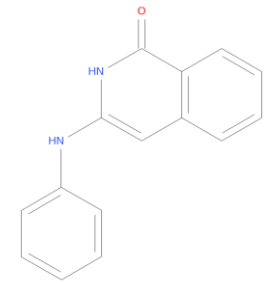
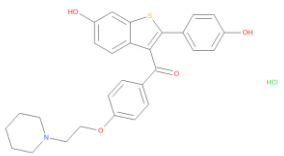
Unwanted physical property	Number of Compounds	Number active at 10 µM	Number active at 1 µM	Number active at 10 µM + Catalase (100 units/well)	Number active at 1 µM + Catalase (100 units/well)
Aggregators	35	11	1	4	1
Chelating	10	1	0	0	0
Coloured	10	0	0	0	0
Fluorescent	17	1	2	0	0
Redox cycling	21	17	8	0	0
Luciferase	15	2	0	1	0
Reactive	10	0	0	0	0
Total	118	32	11	5	1

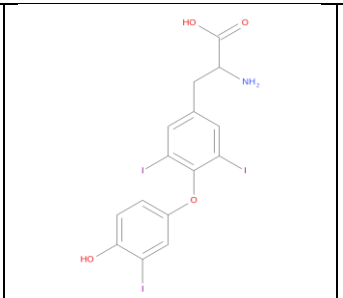
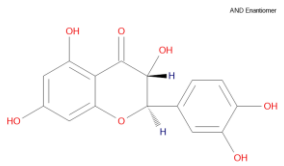
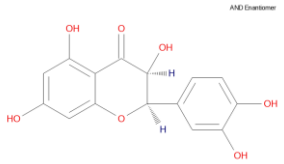
Table 3: Physiochemical properties of 16 best hit compounds

Compound Number	Compound ID and Drug Name where applicable	Structure	MW	pIC_{50} no catalase	pIC_{50} with catalase	logP	HBA/HBD	TPSA (Å)	$logS_{7.4}$	$logD_{7.4}$
Optimal Properties [20,21]	-	-	*≤500*	-	-	*≤5*	*≤7/≤3*	*≤90*	*≤ -4*	*0-3*
1	BCC0060072		318.14	4.5 ± 0.03	4.4 ± 0.04	4.88	3/1	50.69	-4.38	2.25

2	BCC0081684		393.14	5.0 ± 0.07	4.9 ± 0.05	3.97	4/3	63.93	-4.47	2.13
3	BCC0087550		237.12	5.0 ± 0.03	4.9 ± 0.05	4.88	1/0	26.03	-5.05	5.02
4	BCC0090417		328.25	4.3 ± 0.02	4.3 ± 0.07	4.39	1/2	41.99	-5.35	3.86

5	BCC0100281		324.17	4.6 ± 0.03	4.4 ± 0.05	2.13	5/1	83.73	-2.27	2.19
6	BCC0083458		322.14	4.1 ± 0.03	4.0 ± 0.07	2.06	5/2	74.33	-3.17	0.60
7	BCC0082593		393.14	4.2 ± 0.03	4.1 ± 0.07	3.75	4/0	58.45	-4.99	3.36

8	BCC0066281		355.14	4.3 ± 0.04	4.2 ± 0.05	3.05	5/1	66.02	-3.87	3.05
9	BCC0011022		236.09	4.5 ± 0.05	-	3.05	2/2	41.13	-5.05	3.05
10	SAM002548975 (Raloxifene HCl)		473.16	2.7	-	5.69	5/2	70.00	-6.00	5.34

11	SAM003107539 (Liothyronine)		350.79	4.5	-	2.80	4/3	92.78	-4.5	2.76
12	SAM001246778 (Taxifolin)		304.96	4.3	-	1.82	7/5	127.45	-2.18	1.65
13	SAM001246760 (Taxifolin)		304.96	4.0	-	1.82	7/5	127.45	-2.18	1.65

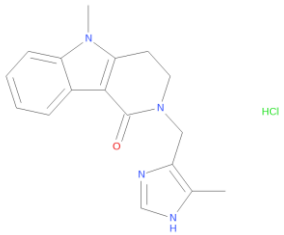
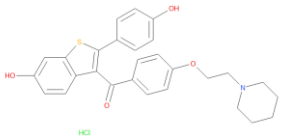
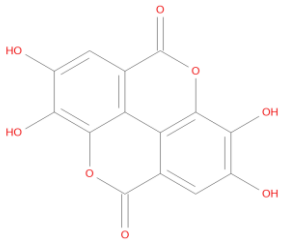
14	SAM001246782 (Alosetron HCl)		292.17	4.1	-	0.98	2/1	53.92	-2.80	1.19
15	S1227 (Raloxifene HCl)		473.16	5.0 ± 0.05	4.9 ± 0.07	5.69	5/2	70.00	-6.00	5.34
16	S1327 (Ellagic Acid)		302.01	4.8 ± 0.05	4.6 ± 0.04	2.32	6/4	133.52	-2.60	-1.83

Figure Legends:

Figure 1: Initial velocity curves for ABAD (2.5 nM) under increasing concentrations of NADH **(A)** and acetoacetyl-CoA **(B),** where Km values= 32.96 ± 4.47 µM and 117 ± 28 µM for NADH and acetoacetyl-CoA were calculated (n=3).

Figure 2: DMSO tolerance and reagent stability testing. (A) Starting concentration of 20% DMSO with a 10 point 1:2 dilution. The DMSO optimal assay concentration was found to be 1% DMSO or lower. **(B-D)** Comparison of reagent stability, where samples in the **left panel** are left at room temperature and **right panel** where samples are at 4°C over an 8 hour sample preparation time course. Reliability was measured by monitoring changes to the maximum and minimum absorbance values **(B)**, Z-primes **(C)** and S/B **(D)**.