Identifying Novel Candidates for Re-Purposing as Potential Therapeutic Agents for Alzheimer's disease

Ballard C¹, Creese B¹, Gatt A², Doherty P², Francis PT^{1,2}, Whitfield D², Corbett A¹, Corcoran J², Hanger D², Thuret S², Killick R^{1,2}, Williams G²

¹ - University of Exeter Medical School

² - King's College London

For correspondence –

Professor Clive Ballard
Pro-Vice-Chancellor and Executive Dean, Professor of Age-related Diseases
College of Medicine and Health
Main Medical School Building,
College of Medicine and Health, St Luke's Campus, Magdalen Road, Exeter, EX1 2LU
c.ballard@exeter.ac.uk
+44(0)1392722911

Abstract

The current paper describes the identification of novel candidate compounds for repositioning as treatments for Alzheimer's disease (AD) from the CMAP library. Candidate compounds were identified based on inverse correlation with transcriptome signatures developed from meta-analyses of Alzheimer RNA expression studies using the SPIED platform. The 78 compounds with a significant inverse correlation were taken forward into an in vitro programme using 6 well validated screening assays relevant to potential treatment targets in AD. Nineteen of the compounds were hits in at least 2 of these assays. A description of each of these compounds is presented.

Background

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disease affecting more than 45 million people worldwide (Livingston et al 2017). Two classes of symptomatic pharmacological treatments, memantine and cholinesterase inhibitors, confer modest but important short term benefits (Birks and Harvey 2018, Howard et al 2012) but treatments with a more sustained impact are urgently needed. Despite considerable efforts, there are still no effective disease modifying treatments for AD, and there have been no new licensed therapeutics for 20 years. A number of candidate disease-modifying compounds for AD, mainly focussed on single specific disease targets, have failed in clinical trials over the last five years, with some recent high profile failures (Egan et al 2019, Honig et al 2018). Robust innovative approaches are urgently needed to re-invigorate drug discovery for AD and identify compounds with multiple impacts on key pathways that will modify disease course and make a real difference to the lives of millions of people worldwide.

Drug repositioning offers a novel, attractive and cost-effective alternative (Corbett et al 2012). It has been the basis of successful therapies in many clinical areas including diverse areas such as cancer (Heckman-Stoddard et al 2017) smoking cessation (Evins et al 2019) and Parkinson's disease (Hubsher et al 2012). The established safety of candidate compounds is a huge advantage, and the

time and cost associated with bringing these compounds to clinical trials is massively reduced (Corbett et al 2012).

The overarching aim this work is to identify novel potential candidates for re-purposing as treatments for AD based on mRNA expression profiles.

Method

Overview

The work was undertaken in 3 key stages: (1) Developing a transcript signature for early and mild Alzheimer's disease based on published mRNA expression studies, (2) using this signature to identify the most promising novel compounds for repurposing as potential new treatments for Alzheimer's disease based on the signatures compounds within the connectivity map (CMAP library) and related databases (3), to evaluate the "hits" in a broad range of Alzheimer related in-vitro assays examining amyloid and non-amyloid related potential treatment targets.

AD associated transcriptional profiles

Transcriptional profiles associated with AD were obtained from the NCBI GEO database [Barrett, T. et al. Nucleic Acids Res. 2007, 35, D760-5.] by performing queries for series containing samples derived from post-mortem AD patient brains for various stages of the disease, together with mouse models of the disease. Profiles were defined as $f = \frac{\langle d \rangle - \langle c \rangle}{\langle d \rangle + \langle c \rangle}$, where the brackets indicate averages of the control (c) and disease (d) samples. The statistical significance is measured by a student's t-test and those folds falling below the 95% confidence interval were dropped. The human disease versus control AD set comprises 21 profiles derived from 14 series (NCBI GEO accession: GSE84422, GSE37263, GSE36980, GSE39420, GSE36980, GSE1297, GSE29378, GSE48350, GSE15222, GSE26972, GSE37264, GSE28146, GSE5281, GSE13214). Cognitive decline was based on decline in MMSE represented by two profiles from two independent series and CDR profiles from one series. Murine model profiles were gathered from 5xFAD and 3xTG datasets giving seven profiles from three series (NCBI GEO accession: GSE50521, GSE119756, GSE101144, GSE77574) for the 5xFAD set and nine profiles from eight series (NCBI GEO accession: GSE31624, GSE15128, GSE36237, GSE92926, GSE60460, GSE60911, GSE36981, GSE35210) for the 3xTG set. BRAAK stage progression profiles (NCBI GEO accession: GSE1297, GSE84422, GSE48350, GSE106241) were generated with a linear mixed model analysis, by fitting the gene expression level across the samples in the series to a linear function of the BRAAK stage with categorical calls on cell type and gender as covariates. The resulting residual correlation Z score for gene expression against BRAAK stage constituted the BRAAK profile. 12 profiles were extracted from four series, with six following full BRAAK progression from level 0 to level 6 and six profiles corresponding to mild BRAAK pathology, level 0 to level 3. Similar profiles were generated for psychiatric measures MMSE and CDR (NCBI GEO accession: GSE48350, GSE1297, GSE84422). In the case of the MMSE profile the regression signs were reversed as MMSE scores decrease with disease progression.

Compound candidate list

Each of the AD associated profiles was queried against CMAP and LINCS drug transcriptional profile databases. Each compound is assigned a rank based on the top anti-correlation across the profiles in each category: AD, BRAAK, BRAAK/mild AD, COG, 5FAD, 3TG. Table 1 lists the candidate compounds together with their top ranks in the AD profile sets. There is a good agreement between CMAP and LINCS.

CMAP and LINCS databases

CMAP data was downloaded from the Broad connectivity map site (www.broadinstitute.org/connectivity-map-cmap). This consisted of probe sets for each sample ranked according to expression level relative to batch control. The data consists of 6,100 samples covering 1,260 drugs and four cell types. The relative probe expression ranks, defined as $1 - \frac{R - R_{min}}{R_{max} - R_{min}}$, where R is the rank of a given gene's expression change (R_{max} being the highest and R_{min} being the lowest ranks), were averaged over replicates ignoring cell type and filtered based on significance using a one sample student's t-test. For genes with multiple probes the probe with the largest significant change was mapped to the gene. This resulted in a unique profile for each drug in CMAP. The compound data can be queried through SPIED (Williams 2013).

LINCS data was based on the GEO depositories GSE92742 and GSE70138, which provide Z-scores for compound versus control treatment data. Replicates were combined and statistically filtered giving a dataset unique for compound/cell type pairings.

Evaluation of the neuroprotective efficacy of the best candidate drugs in vitro

The selected assays included:

Mouse cortical neuron survival in presence of a-beta: This is an established assay for analysis of neuronal cell death following insult by a-beta and followed well established protocols (Goncalves et al 2013). In brief, mouse cortical neurons were treated with oligomeric amyloid and candidate compound in 96-well plate format for three days. The outcome measure was cell survival using Tunel measurement. Compounds conferring a significant improvement in cell survival ($p < 0.05$) were considered to be "hits".

Dendritic Spine Loss: This assay evaluated the ability of compounds to block A β -driven spine loss will maintain neuronal viability and potentially protect cognition in vivo. Primary rat neurons cultured for 24 days and transfected with EGFP.30 minutes pre-treatment with compound, followed by four-hour incubation with 20uM A β_{25-35} oligomers. Fixation was undertaken for immunocytochemistry. Antibodies against PSD-95 and synapsin were imaged through confocal microscopy. Dendritic spine morphology scores were rated for: (1) Identified valid cell bodies, (2) Average length neurites / neuron; (3) No. branch points / neuron; (4) No. PSD-95 puncta / neurite; (5) No. Synapsin-1 puncta / neurite; (6) No. synapses / neuron calculated as overlap of PSD-95 and synapsin-1. Compounds conferring significant benefits in the combined spine loss score ($p < 0.05$) were considered to be "hits" (Killick et al 2018)

APP-mediated wnt restoration: The wnt pathway is known to be disrupted in AD pathology, providing a potential target for AD treatments. Luciferase-based Wnt reporter gene assay was performed in Human HEK293T neuron-like cells in a 96-well format. TOPflash and FOPflash genes were used as reporters. Cells were treated overnight with the reporter, wnt3a and Dkk1. Each replicate was $n = 8$. Benefit was evaluated based on restoration of canonical wnt signalling activity as measured by luciferase output. Compounds conferring significant benefit with respect to wnt restoration ($p < 0.05$ and Effect Size of 20%) were considered to be "hits" (Killick et al 2018)

DKK: Blocking A β induction of Dkk1 blocks many important neurotoxic effects of A β , and therefore forms an important potential treatment target for AD. Human HEK293T cells were treated overnight following the same protocol as the APP-mediated wnt restoration assay described above. Each replicate was $n = 8$. The evaluated outcome was Dkk1 protein expression determined by an ELISA-

based measure of Dkk1 levels. Compounds conferring significant dkk blocking activity ($p < 0.05$ and Effect Size of 20%) were considered to be “hits” (Killick et al 2018).

Reduction of total and phosphorylated tau: Production and phosphorylation of tau is an established measurement of AD pathology and a key potential treatment target. CHO cells stably expressing Tau35 were treated for four hours with each compound in a 96-well format. Cells were fixed and stained for total tau, phosphorylated tau (Ser-396) and actin. Each replicate was $n = 8$. Total and phosphorylated tau as described previously (ref). Compounds achieving a significant reduction in tau phosphorylation ($p < 0.05$ and Effect Size of 20% reduction) were considered to be “hits” (Noble et al 2009).

Neurogenesis (cell proliferation and differentiation): This assay provides a model for studying molecular mechanisms regulating neurogenesis, a major question in potential AD treatment candidates. HPC03A/07 human hippocampal stem cell lines were treated for 48 hours (proliferation assay) and seven days (differentiation assay) respectively in a 96-well format. Cells were stained for stem-cell markers (Sox2, Nestin), proliferation (Ki67), apoptotic cell death (CC3), immature (Dcx) and mature (Map2) neurons and astrocytes (S100beta). The assay was analysed based on the percentage change from baseline with treatment on six readouts: (1) cell number, (2) cell proliferation, (3) Neurogenesis [(3a) neuroblasts, (3b) neurons, (3b) neurite outgrowth and (4) cell death. Compounds which increased proliferation of hippocampal progenitor cells or cells expressing stemness markers (Sox2 / Nestin), increased differentiation of hippocampal progenitor cells and decreased apoptotic cell death $p < 0.05$ on each of these measurements) were considered to be “hits” (Thuret et al 2013).

For all assays, the internal controls were amyloid alone, vehicle alone and compound plus vehicle.

Results

CMAP, LINCS and NMAP

Seventy-eight compounds from the 2 databases achieved an inverse correlation with a p value < 0.05 with at least one of the 3 AD signatures.

Figure 1
Compounds with $P < 0.05$ inverse correlation between AD Signatures and Drug Signatures
from CMAP and/or LINCS

CMAP LINCS											CMAP LINCS														
compound	ADsets	BRAAKsets	BRAAKmildsets	COGsets	5xFADsets	3xTGsets	ADsets	BRAAKsets	BRAAKmildsets	COGsets	5xFADsets	3xTGsets	compound	ADsets	BRAAKsets	BRAAKmildsets	COGsets	5xFADsets	3xTGsets	ADsets	BRAAKsets	BRAAKmildsets	COGsets	5xFADsets	3xTGsets
acetylsalicylic acid	20	48	81		64	44							ibuprofen							96					
alsterpaullone	7	3	3		30								imatinib	84	97	89				3			3	90	
altizide	32			39									indometacin											53	
ampicillin	6	1				92							isoniazid	6	42	91	2								
amrinone	47	60	95	31	91	70							kawain	39	66	37									
apigenin	1	1	34	5	4		67				3		kenpaullone							66					
astemizole	42		8			15							levonorgestrel	7	6	23	58	51	1	55					
atractyliside	2	6	5	11	13								L-methionine	41					29						
aztreonam	16	48											luteolin	5	2	52	18	15	47						26
bephenium	61	99	30										metformin	85	73				34					82	
bromopride	36		77										mitoxantrone	3	50	17	11		1	26				46	49
camptothecin	4	7	8	8	2						85		morantel	16	35	54	14								
capsaicin	23					9		65					nabumetone											90	
cefalotin				5					94				naftidrofuryl	62	67				12	43			62		
chenodeoxycholic acid	5	21	19	15	37								nalbuphine	77	75				58						
chlortetracycline						54							neostigmine	31	28	75			94						
clenbuterol		62				85			73	36			nifedipine			35		94	1						73
cotinine	37				41	97			78				norketamine						27	59					
cyanocobalamin	8	30					45						ofloxacin	16					9	6	2			30	
diclofenac						29	34						orphenadrine	59				67							
dilazep	94					21	45						oxaprozin	19		60	98	54	7	21					
diphenamil	35	98	97	31	91								oxolinic acid	8				6							
doxazosin	7	59	57	6	50								ozagrel											63	
ebselen		20		22									paracetamol	60	54										
escitalopram							34						paroxetine	2											
etomidate	19	17	50	36									phenazone	24			49								
famotidine	46	51	2		39								pheniramine	14				95	62						
fasudil					9	43							pyrantel	27	78										
fenbufen	15	16	55										ramipril			56									73
fenofibrate			25	66	57								risperidone	70											53
galantamine	26					90				13			rolipram	37	56										
gemfibrozil	41				88	97							ronidazole	22			89	45	74						39
genistein	7	8	18	56	9	61		76					tacrine	98	98	64	66	16				75			
ginkgolide A	1	33	5	89	30	32							thiostrepton	93	6			11			66			77	
GW8510	1	3	1	3									trioxysalen	4				94	42						
H-7	2	5	1	6					39				troleandomycin					72							
hesperidin	92						69						tropine		93										
hexestrol	45	40	9		76								yohimbine	17					20		69			54	
homatropine	12	22		61		35		46					zalcitabine	51	94			8	70			81		61	

p < 0.0001

p < 0.001

p < 0.01

p < 0.05

■ $p < 0.0001$
■ $p < 0.001$
■ $p < 0.01$
■ $p < 0.05$

Figure 1 footnote. The anti-correlating ranks of AD associated profiles against the CMAP and LINCS databases. AD profiles were based on: post mortem overt diseases versus control samples (ADsets); expression changes correlating with full BRAAK progression; expression changes correlating with mild BRAAK (≤ 3) progression; cognitive decline, based on Mini-Mental State Examination (MMSE) and *Clinical Dementia Rating* (CDR); sets of 5xFAD mouse brain samples; sets of 3xTG mouse model brain samples. The numbers given are the ranks of the given compound for the given query, where the rank is for the anti-correlation score. The drugs highlighted in yellow are those showing positive activity in at least two in vitro neuroprotection assays.

In Vitro Hits

Of the 78 compounds examined in the in vitro assays, 19 compounds fulfilled the criteria of reaching significance on two independent assays. The data for these compounds and a summary of their results in the in vitro studies are presented in table 1.

Table 1: Compounds which were “Hits” on at Least 2 *in vitro* Assays

Compound	Source	CMAP Correlation	A-beta cell death	Dendritic spine loss	P-tau reduction (CHO model)	APP-mediated Wnt restoration	DKK Blocking	Neurogenesis
Imatinib	LINCS	-0.18 (Early AD)	p=0.22 ES 27.5%	p=0.0267		p=0.02		
Mitoxantrone	CMAP	-0.27 (Mouse sig)		p=0.012	p<0.05, d>1	p=0.008		
Kenpaullone	LINCS	-0.2 (Mod AD)			p<0.05, d>1	p=0.00	p=0.00	
Tacrine	CMAP	-0.21 (Early AD)	p=0.007 ES 35.9%		p<0.05, d>1			
Apigenin	CMAP	-0.09 (Mouse sig)	p=0.0001 ES 72.5%		p<0.05, d>1			
Chlortetracyclin	CMAP	-0.11 (Mouse sig)	p=0.003 ES 32.1%			p=0.00		
Thiostrepton	CMAP	-0.16 (Early AD)	p<0.000 ES 46.8%			p=0.05		Flagged as possible negative impact

Compound	Source	CMAP Correlation	A-beta cell death	Dendritic spine loss	P-tau reduction (CHO model)	APP-mediated Wnt restoration	DKK Blocking	Neurogenesis
Trioxsalen	NMAP	-0.12 (Early AD)	p=0.0003 ES 41%			p=0.00		
Famotidine	NMAP	-0.17 (Early AD)	p=0.14 ES 23.8%				p=0.001	
Cyanocobalamin	CMAP	-0.29 (Early AD)	p=0.003 ES 39.1%					
Doxazosin Mesylate	NMAP	-0.16 (Early AD)	p=0.12 ES 51%					
Luteolin	CMAP	-0.09 (Mouse sig)		p=0.0079	p<0.05, d>1			
Hexestrol	NMAP	-0.12 (Mouse sig)		p=0.0086	p<0.05, d>1			
Metformin	CMAP	-0.8 (PD)			p<0.05, d>1			
Yohimbine	CMAP	-0.11 (PD)			p<0.05, d>1			

Compound	Source	CMAP Correlation	A-beta cell death	Dendritic spine loss	P-tau reduction (CHO model)	APP-mediated Wnt restoration	DKK Blocking	Neurogenesis
Pyrantel	CMAP	-0.17 (Early AD)			p<0.05, d>1			
Cotinine	CMAP	-0.58 (PD)				p=0.00	p=0.034	
Genistin	LINCS	-0.17 (Mod AD)				p=0.00	p=0.00	
Nabumetone	CMAP	-0.30 (PD)				P=0.03	P=0.000	

Of these 19 compounds six do not have published safety data, one is a veterinary drug that is not licensed for human use (thiostrepton) and one compound (tacrine) was developed as an Alzheimer's treatment but was withdrawn due to hepatotoxicity. Eleven of the compounds have good safety data and promising blood-brain barrier penetrance, all of which are licensed for use in humans. The characteristics of the identified compounds are shown in Table 3

Table 2 Characteristics of compounds which were hit an at least 2 in vitro assays

Compound	MeSH	BBB penetration	Novel	Safety	Drug-drug interactions	Proposed drug target
Imatinib	Tyrosine-kinase inhibitor	Yes	Yes	Side effects at dose used for chemotherapy	Risk with CYP3A4 inhibitors / inducers e.g. rifampicin	Kinase-independent inhibitor of the interaction between γ -secretase and the γ -secretase activating protein
Mitoxantrone	Anthracenedione antineoplastic agent	Unknown	Yes	Side effects at dose used for chemotherapy	Risk with antineoplastic agents, cardiotoxic drugs and immunosuppressants	Reduces /blocks A β (1-42) oligomerization, antifibrillogenic
Kenpaullone	GSK-3 β inhibitor	Unknown	No	Unknown	Unknown	Inhibition of cdk5/p25 formation and GSK-3
Tacrine	Anticholinesterase	Yes	No	Hepatotoxicity	Risk with anaesthesia	Inhibitor of acetylcholinesterase and butyrylcholinesterase
Apigenin	Flavone	Yes	No	Good	None established	NF- κ B transduction suppression, ERK/CREB/BDNF pathway reduction
Chlortetracyclin	Tetracycline antibiotic	Unknown	No	General (nausea, dysphagia), rare cases of hepatotoxicity	Avoid retinoids	Inhibitor of A β (1-42) aggregation, putative similar actions as other antibiotics
Thiostrepton	Oligopeptide antibiotic	Unknown	Yes	Unknown	Unknown	Proteasome inhibition
Trioxsalen	Furanocoumarin	Unknown	Yes	Possible photosensitivity	Possible ix with antibiotics	Unknown, interaction with DNA

Compound	MeSH	BBB penetration	Novel	Safety	Drug-drug interactions	Proposed drug target
		wn				
Famotidine	Histamine H2-receptor antagonist	Unknown	Yes	Good	None established	Histamine H2 receptor inhibitor
Cyanocobalamin	Synthetic vitamin B12	Unknown	No	Good	None clinically relevant	B12 replenishment, reversing hyperhomocysteinaemia
Doxazosin Mesylate	Adrenergic alpha-1 receptor antagonist	Yes	Yes	Possible hypotension	Avoid PDE-5 inhibitors	Antihypertensive effect in AD?
Luteolin	Flavonoid	Unknown	No	Good	Unknown	Free radical stabilisation, metal ion chelator, COX, LOX, iNOS, IL-6 inhibition
Hexestrol	Non-steroidal estrogen	Unknown	No	Unknown	Unknown	Unknown, DNA/RNA interaction
Metformin	Anti-diabetic	Yes	No	Hepatotoxicity	Relevant in diabetes	Possible BACE1 reduction
Yohimbine	Indole alkaloid	Yes	Yes	Avoid in psychosis, high CV risk, extrapyramidal	Avoid MAOIs and liver enzyme inducers	Modulation of the noradrenergic neurotransmitter system
Pyrantel	Antinematodal thiophene	Unknown	Yes	Unknown	Avoid AChE, levamisole, piperazine	Nicotinic receptor agonist
Cotinine	Alkaloid	Unknown	No	Unknown	Unknown	Akt agonist, signalling, GSK3 β inhibitor

Compound	MeSH	BBB penetration	Novel	Safety	Drug-drug interactions	Proposed drug target
Genistin	Isoflavone	Unknown	No	Unknown	Unknown	Unknown
Nabumetone	NSAID	Yes	No	Some GI / CV effects with long-term use	Possible interactions with anticoagulants etc as for all NSAIDs	COX2 inhibitor

Compared to the 19/78 compounds that were “Hits” based on transcriptional signatures, 0/15 compounds that were “non-hits” based on transcriptional signatures were *in vitro* hits (Fischer’s exact test $P=0.035$).

Discussion

The 3 stage approach in the current study developed a series of mRNA expression signatures for AD, used these signatures to identify compounds which were inversely associated with this signature in the CMAP and LINCS databases and then evaluated these compounds in a series of well validated in vitro assays focussing on target treatment mechanisms relevant to AD. Seventy eight compounds were identified which produced signatures which were significantly inversely correlated with the signatures for mild AD, moderate-severe AD or the signature of the 5Xfad AD mouse. Of these compounds, 19 (24%) were “hits” in at least two of the in vitro assays.

Firstly, the proportion of “CMAP hits” that were “hits” in the in vitro studies was significantly higher than the proportion of hits from CMAP non-hits (24% vs 0%, FET $P=0.035$). In addition, the 24% proportion of compounds identified as hits in in vitro studies compares favourably to reported drug discovery work using high throughput screening (refs). This suggests that mRNA expression signatures are potentially a very useful tool to triage compound libraries and increase the likelihood of identifying compounds with positive disease related effects in in vitro studies.

Eight of the “hits” are novel candidates, 3 which are known to be brain penetrant and 5 with properties indicating a likelihood of brain penetrance. Other hits confirm candidate drugs already highlighted as potential candidates for re-purposing, such as metformin and others highlight novel drugs within classes already highlighted as potential candidate treatments, such as the cox-2 inhibitor nabumetone and several flavonoids. Overall a number of potentially promising candidates for re-purposing have been identified and merit further evaluation.

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