Article

Anti-Cholinesterase Combination Drug Therapy as a Potential Treatment for Alzheimer's Disease

Hafsa Amat-ur-Rasool [1,2] , Mehboob Ahmed [2] , Shahida Hasnain [2] and Wayne G. Carter [1,*]

1 Royal Derby Hospital Centre, School of Medicine, University of Nottingham, Derby DE22 3DT, UK; hafsa.phd.mmg@pu.edu.pk
2 Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore 54590, Pakistan; mehboob.mmg@pu.edu.pk (M.A.); Shahida.mmg@pu.edu.pk (S.H.)
* Correspondence: wayne.carter@nottingham.ac.uk; Tel.: +44-(0)1332-724738

Abstract: Alzheimer's disease (AD) is a burgeoning social and healthcare problem. Cholinesterase inhibitors (ChEIs) are employed for symptomatic treatment of AD, but often elicit adverse drug reactions (ADRs). Herein, the potency of the ChEIs, donepezil, tacrine, berberine, and galantamine to inhibit human or *Torpedo californica* acetylcholinesterase (*tc*AChE) proteins were evaluated. The efficacy of dual-drug combinations to inhibit human AChE directly and within differentiated neurons was also quantified. ChEI potency was in the order: donepezil > tacrine > berberine > galantamine for both AChEs. Dual-drug combinations of berberine and tacrine (BerTac), berberine and galantamine (BerGal), and tacrine and donepezil (TacDon) all produced synergistic outcomes for AChE inhibition. Donepezil and berberine (DonBer) and tacrine and galantamine (TacGal) elicited antagonistic responses. Donepezil and galantamine (DonGal) was synergistic for human AChE but antagonistic for *tc*AChE. After application of dual-drug combinations to neuronal cells, BerTac, BerGal, DonGal, and DonBer all showed synergistic inhibition of AChE, TacDon additive, and TacGal antagonistic effects. BerGal produced the most potent synergism and reduced total drug dose by 72%. Individual ChEIs or dual-drug combinations were relatively non-toxic to neuronal cells, and only reduced cell viability at concentrations two–three orders of magnitude greater than that required to inhibit AChE. In summary, dual-drug combinations of ChEIs potentially represent a novel means of AD patient treatment, with reduced and more cost-effective drug dosing, and lowered likelihood of ADRs.

Keywords: Alzheimer's disease; cholinesterase inhibitors; drug synergism

1. Introduction

Approximately 50 million people globally suffer from dementia and the number is expected to triple by 2050 [1]. Alzheimer's disease (AD) represents the majority of dementia cases [2,3]. AD patients suffer from broad symptomology that includes confusion, periods of memory loss and cognitive decline, and with disease progression, AD patients lose the ability to perform daily activities and tasks with an associated loss of independence [4]. The reduced cognition aligns with a loss of neuronal transmission and functionality, as well as a loss of neuronal mass [5,6]. AD patients experience brain cortical atrophy primarily within the frontotemporal lobes, notably within the hippocampal and entorhinal cortex [5,6], consistent with the loss of cognitive function and memory attributed to these brain regions [7].

A reduction of signaling from the neurotransmitter acetylcholine (ACh) led to the concept of a "cholinergic hypothesis" to explain some of the cognitive deficits experienced by AD patients [8,9]. This cholinergic signaling decline provides the basis for the current first-line drug treatment for mild-to-moderate AD: employment of cholinesterase inhibitors (ChEIs) such as donepezil, rivastigmine, and galantamine [10–12]. ChEIs transiently inhibit the activity of acetylcholinesterase (AChE), which is predominantly localized to the pre-synaptic membrane in the CNS and the synaptic basal lamina of the neuromuscular

junction [13,14]. This (reversible) inhibition of AChE within the central nervous system (CNS) is therefore an attempt to prolong ACh signaling.

ChEIs are either of synthetic (e.g., donepezil and tacrine) or of plant origin (e.g., galantamine, huperzine A, and berberine). The current US Food and Drug Administration (FDA) approved ChEIs (donepezil, galantamine, and rivastigmine) have proved beneficial for patients with mild-to-moderate AD [15,16]; however, they are limited by induction of undesired side effects, and ultimately, only provide symptomatic rather than curative benefit. Nevertheless, the search continues for useful next-generation ChEIs with similar efficacy to the current drugs but with fewer adverse drug reactions (ADRs). Furthermore, additional beneficial properties such as provision of antioxidants, has garnered the use of certain plant extracts and phytochemicals as potential next-generation ChEIs [17–22].

To achieve efficacy, treatment with ChEIs often requires titration, typically with an increased drug dose after four weeks [15]. At the starting or increased drug dose there is still a risk of idiosyncratic ADRs. Furthermore, for chronically dosed patients, therapeutic effects may be limited (at a fixed dosing regimen) due to neurobiological adaptation and drug tolerance, promoting the need for further increased dosing. Therefore, the lowest drug dose possible that still achieves therapeutic efficacy will reduce the likelihood of induction of toxicity.

One approach to provide suitable drug efficacy to treat AD, but at a lower dosing regimen, is via combination drug therapy [23]. Combination drug therapy can provide a fixed-dose of two or more active pharmaceutical agents within a single dosing entity. In this study, the efficacy of individual ChEIs as well as two-drug combinations were assessed via their ability to directly inhibit AChE. The FDA-approved synthetic ChEIs, donepezil and tacrine, were investigated, as well as two plant alkaloids (phytochemicals), galantamine (also FDA approved) and berberine. Berberine was included as it is a phytochemical with documented AChE affinity and binding, antioxidant properties, and proposed as a potential anti-AD drug [24–26].

2. Materials and Methods

2.1. Chemicals and Reagents

Recombinant human acetylcholinesterase (hAChE), *Torpedo californica* acetylcholinesterase (*tc*AChE), acetylthiocholine iodide (ATCI), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), all-trans-retinoic acid, thiazolyl blue tetrazolium bromide (MTT), isopropanol, dimethyl sulfoxide (DMSO), and the drugs donepezil hydrochloride, MW = 415.95 g/mol; tacrine hydrochloride, MW = 234.72; berberine hydrochloride, MW = 371.82 g/mol; and galantamine hydrobromide, MW = 368.27 g/mol, were all purchased from Sigma-Aldrich (Poole, UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), albumin, and poly-D-lysine (PDL) were purchased from Thermofisher Scientific (Rochester, UK). Unless specified differently, all other chemicals were from Sigma-Aldrich (Poole, UK).

2.2. Enzymatic Activity Assays

A microtiter plate version of the Ellman's assay was used to determine the activity of human and *Torpedo californica* acetylcholinesterase enzymes [27]. Assays were undertaken in the presence of either individual drugs or two-drug combinations. Each assay data point within a 96-well plate contained 40 μL of 0.01 M DTNB, 46 μL of 0.1 M (pH 8.0) phosphate buffer, 2 μL of 0.075 M ATCI substrate, and 10 μL of drug or dual-drug combination. Drug concentrations were prepared according to Supplementary Table S1 [28]. Enzymatic cleavage of the ATCI substrate was initiated by addition of 2 μL of 0.5 U/mL AChE enzyme. The reaction was undertaken for 30 min at room temperature, protected from the light, before the color change was read at 412 nm using a Multiskan Spectrum plate reader (Thermo Electron Corporation, Vantaa, Finland). Under these assay conditions, a final color change was reached (results not included) such that an end-point assay measurement was taken [29].

Initially, assays were undertaken with a broad drug concentration range (0.1, 1, 10, 100 and 1000 μM) to determine an approximate concentration producing 50% inhibition of AChE (IC_{50}) (results not included). These scoping experiments enabled us to quantify an approximate IC_{50} for subsequent dose-response experiments. Two-fold serial dilutions at the approximate drug IC_{50} concentrations were prepared i.e., 0.25×, 0.5×, 1×, 2×, and 4× the IC_{50} concentrations. These concentrations were also used for dual-drug combination experiments. Experiments undertaken in the absence of inhibitor were designated as 100% enzymatic activity. Assays were performed in duplicates from which a mean was calculated.

AChE Assay Using Neuroblastoma Cells

Neuroblastoma SH-SY5Y cells (*passage* #15) were grown in DMEM containing 10% FBS in 6-well plates that had been pre-coated with 50 μg/mL PDL. Cells were grown to ≈50% confluency at 37 °C within an atmosphere of 5% CO_2 and 95% humidity. Growth media was replaced and cells differentiated in DMEM/F12 media (without choline or phenol red) containing 1% FBS and 10 μM all-trans-retinoic acid. Growth media was changed every other day and differentiation of neurons was monitored by light microscopy. After 6 days, neurons were fully differentiated, and then treated with drugs across the broad concentration range of 0.1, 1, 10, 100, and 1000 μM; either as individual drugs or in two-drug combinations. The cell-based Ellman's assay described by Li et al. (2017) [29] was modified for 6-well plates. A final reaction volume of 400 μL was used, comprised of 190 μL of 0.01 M DTNB, 200 μL of 0.1 M (pH 8.0) phosphate buffer and 10 μL of 0.075 M ATCI substrate. AChE present on the cell membranes of the differentiated neuronal cells provided the enzymatic activity to drive the hydrolysis of ATCI. Plates were placed in the 37 °C cell culture incubator for 30 min. The reaction mixture was then removed from the plate wells and transferred into 1.5 mL Eppendorf tubes. These were centrifuged at 12,000× *g* for 5 min to settle cellular debris. The supernatant was removed and optical density measured at a wavelength of 412 nm using a Multiskan Spectrum plate reader (Thermo Electron Corporation, Vantaa, Finland).

2.3. Computational Analysis

Percentage inhibition of AChE from in vitro assays in response to application of individual or dual-drug ChEIs were calculated using CompuSyn software (http://www.combosyn.com/index.html). The CompuSyn report provided IC_{50} and combination index (CI) values for individual drug or two-drug combinations and was used to plot dose-response effect curves and CI plots, respectively [30].

2.4. Cytotoxicity Assays

A MTT assay was used to assess the cytotoxicity of either the individual drugs or dual-drug combinations using the SH-SY5Y neuroblastoma cell line. SH-SY5Y cells were seeded in 96-well plates (passage #15) at a cell density of 3×10^4 cells/well in DMEM containing 10% FBS. Cells were grown until 80–85% confluent and then treated with drugs over a concentration range of 0.01, 1, 10, 100, and 1000 μg/mL in cell culture media. After an incubation of 48 h within the 37 °C incubator, cell culture media was removed and replaced with fresh growth media containing 0.5% (*w/v*) MTT. Plates were incubated for 4 h at 37 °C, before removal of the media and replacement with 1:1 (*v/v*) isopropanol:DMSO. Formation of formazan was quantified by color generation at a wavelength of 570 nm using a Multiskan Spectrum plate reader (Thermo Electron Corporation, Vantaa, Finland). Cell viability of cells treated with drugs was quantified by comparison to untreated control cells, set at 100% viability.

2.5. Statistical Analysis

Values generated for IC_{50} and for the dual-drug CI were determined using CompuSyn software. Cell viability data was calculated by non-linear regression (inhibitor vs. normal-

ized response—variable slope) using a model of best-fit values of four data points using GraphPad Prism V.7 (https://www.graphpad.com/scientific-software/prism/). For cell viability assays, a one-way ANOVA was performed to determine significant changes after treatments compared to control cell viability, set at 100%.

3. Results

3.1. Drug Inhibition of AChE Protein In Vitro

The drugs donepezil, tacrine, berberine, and galantamine (Figure 1) were incubated with recombinant human acetylcholinesterase (hAChE) and *Torpedo californica* acetylcholinesterase (*tc*AChE), and the level of enzymatic inhibition quantified. Dose-response effect curves were generated and a plot of fractional affect (Fa) 0.5 (representing the concentration producing 50% enzyme inhibition (IC_{50}) against dose plotted (Figure 2A,B). The inhibition curves showed that the potency of the drugs to inhibit AChE was in the order: donepezil > tacrine > berberine > galantamine for both the human (h) and *Torpedo californica* (*tc*) enzymes. Berberine and galantamine inhibited both species of AChE with similar potency. Donepezil was more potent an inhibitor of human AChE, and tacrine was ≈2.7-fold more potent for the *T. californica* enzyme, as detailed in Table 1.

The ability of two-drug combinations to inhibit human and *tc*AChE were then considered. Mixtures of berberine and tacrine (BerTac), berberine and galantamine (BerGal), tacrine and donepezil (TacDon), donepezil and galantamine (DonGal), donepezil and berberine (DonBer), and tacrine and galantamine (TacGal) were assayed at combinations of 0.25×, 0.5×, 1×, 2×, and 4×, as detailed in Supplementary Table S1. A combination index (CI) plot (from CompuSyn) of the different drug combinations was generated for the fractional affect (Fa) 0.5 (IC_{50} values) (Figure 2C,D). The central line with a CI of 1 reflects the point at which drug combinations had an additive effect. CI values below that line are indicative of synergism (CI < 1), and CI values above the line represent antagonism (CI > 1) [31].

The drug combinations, BerTac, BerGal, and TacDon produced synergistic outcomes, whereas DonBer and TacGal were antagonistic. DonGal was synergistic for human AChE but antagonistic for *tc*AChE (Table 1). The most potent synergistic combination to inhibit human or *tc*AChE was from a berberine and galantamine mixture (BerGal). This was in stark contrast to their individual drug potencies that displayed higher IC_{50} values than either of the synthetic ChEIs, donepezil or tacrine. If BerGal was used at a 1:1 combination, IC_{50} values were lowered to 1.101 μM for hAChE inhibition and 1.355 μM for *tc*AChE (Table 2).
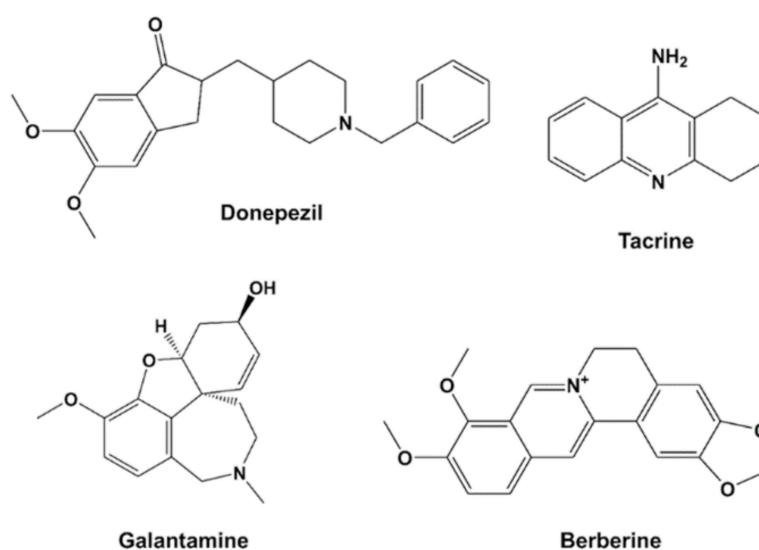

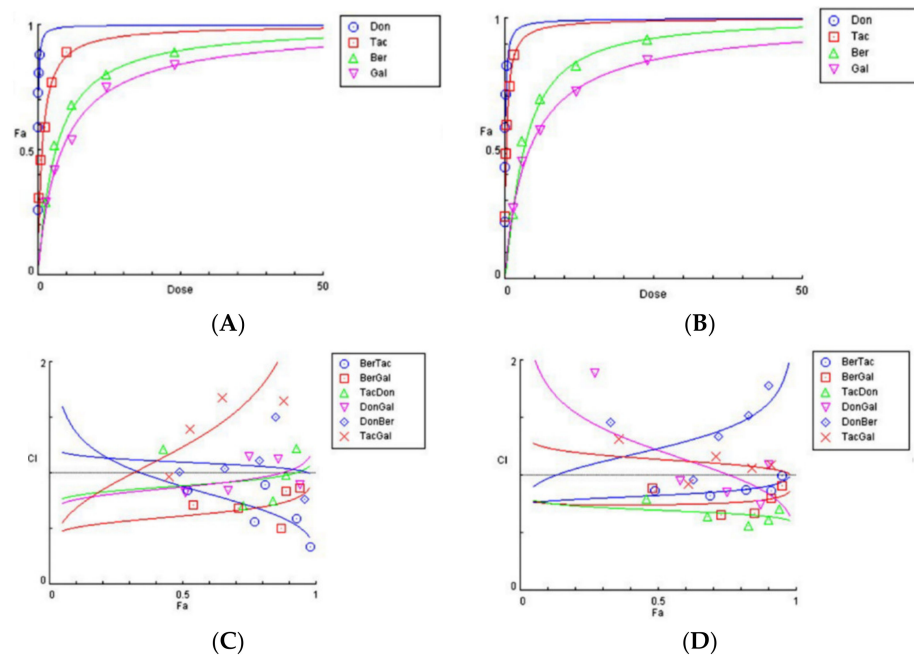**Figure 1.** Structures of the cholinesterase inhibitor (ChEI) drugs.

Figure 2. Drug inhibition of acetylcholinesterase (AChE) protein in vitro: Human AChE (**A**) or *Torpedo californica* AChE (**B**) were incubated with donepezil (Don), tacrine (Tac), berberine (Ber), and galantamine (Gal) and the level of cholinesterase inhibition quantified. Dose-response effect curves were generated as fractional affect (Fa) 0.5 (representing the concentration producing 50% enzyme inhibition (IC$_{50}$) against dose. Combination index (CI) plots for dual-drug inhibition of human AChE (**C**) and *Torpedo californica* AChE (**D**). BerTac = berberine and tacrine; BerGal = berberine and galantamine; TacDon = tacrine and donepezil; DonGal = donepezil and galantamine; DonBer = donepezil and berberine; TacGal = tacrine and galantamine.

Table 1. Quantitation of interactions between ChEI drugs, dual-drug combinations, and human and *Torpedo californica* AChE.

Drug or Drug Combination	CI Value	Acetycholinesterase Inhibition (IC$_{50}$) (nM)			
		Donepezil	**Tacrine**	**Berberine**	**Galantamine**
Don		57 (h) 91 (tc)			
Tac			726 (h) 271 (tc)		
Ber				3131 (h) 3234 (tc)	
Gal					4183 (h) 4173 (tc)
BerTac	0.880 (h) 0.826 (tc)		302 (h) 99 (tc)	1451 (h) 1486 (tc)	
BerGal	0.615 (h) 0.744 (tc)			1101 (h) 1355 (tc)	1101 (h) 1355 (tc)
TacDon	0.887 (h) 0.697 (tc)	27 (h) 29 (tc)	299 (h) 103 (tc)		
DonGal	0.876 (h) 1.216 (tc)	29 (h) 51 (tc)			1538 (h) 2729 (tc)
DonBer	1.099 (h) 1.233 (tc)	32 (h) 45 (tc)		1690 (h) 2393 (tc)	
TacGal	1.235 (h) 1.139 (tc)		489 (h) 156 (tc)		2349 (h) 2344 (tc)

Don = Donepezil; Tac = tacrine; Ber = berberine; Gal = galantamine. BerTac = berberine and tacrine; BerGal = berberine and galantamine; TacDon = tacrine and donepezil; DonGal = donepezil and galantamine; DonBer = donepezil and berberine; TacGal = tacrine and galantamine. (h) = human; tc = *Torpedo californica*. Combination Index values (CI): Synergism: CI < 1; Additive: CL = 1; Antagonism: CI > 1.

Table 2. Quantitation of interactions between ChEI drugs, dual-drug combinations, and neuronal AChE.

Drug or Drug Combination	CI Value	Acetylcholinesterase Inhibition (IC$_{50}$) (nM)			
		Donepezil	Tacrine	Berberine	Galantamine
Don		81			
Tac			1019		
Ber				4844	
Gal					7009
BerTac	0.686		348	1670	
BerGal	0.575			1648	1648
TacDon	0.963	41	461		
DonGal	0.804	40			2152
DonBer	0.834	36		1907	
TacGal	1.100		660		3169

Don = donepezil; Tac = tacrine; Ber = berberine; Gal = galantamine. BerTac = berberine and tacrine; BerGal = berberine and galantamine; TacDon = tacrine and donepezil; DonGal = donepezil and galantamine; DonBer = donepezil and berberine; TacGal = tacrine and galantamine. (h) = human; tc = *Torpedo californica*. Combination index values (CI); Synergism: CI < 1; Additive: CI = 1; Antagonism: CI > 1.

3.2. Drug Inhibition of AChE within Differentiated Neurons

Human derived neuroblastoma SH-SY5Y cells were differentiated with retinoic acid to produce neurons of a cholinergic phenotype [32–35], and then challenged with individual ChEIs and two-drug combinations, prepared according to Supplementary Table S1. Dose effect curves for the tested drugs were generated (Figure 3A), and showed that the relative drug potency observed for protein assays (Figure 2A,B) was mirrored with human neuronal cells grown in culture, with potency in the order donepezil > tacrine > berberine > galantamine. However, the cell-based assay curves were flatter and generated IC$_{50}$ values higher than those obtained with purified hAChE (Table 2).
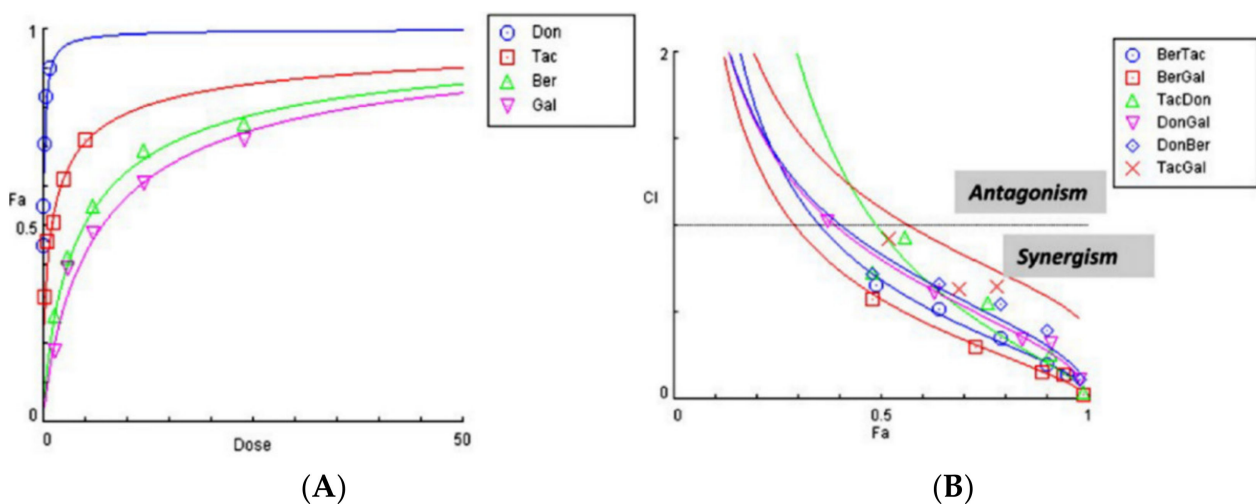

(A) **(B)**

Figure 3. Drug inhibition of AChE within SH-SY5Y cells: Human neuroblastoma cells were incubated with donepezil (Don), tacrine (Tac), berberine (Ber), and galantamine (Gal) and the level of cholinesterase inhibition quantified (**A**). Combination index (CI) plot of dual-drug inhibition of AChE (**B**). BerTac = berberine and tacrine; BerGal = berberine and galantamine; TacDon = tacrine and donepezil; DonGal = donepezil and galantamine; DonBer = donepezil and berberine; TacGal = tacrine and galantamine.

A CI plot of the different drug combinations against the fractional affect (Fa) 0.5 (IC$_{50}$ values) revealed that for the first assayed point (0.25×), only TacDon, DonGal, and TacGal generated values close to the central line, in keeping with additive effects, whereas BerTac, BerGal, and DonBer effects were all synergistic. However, at higher drug concentrations,

all of the tested combinations (BerTac, BerGal, TacDon, DonGal, DonBer, and TacGal) displayed synergistic effects (Figure 3B).

The CI values generated for the drug combinations are listed in Table 2, and show that the drug combinations BerTac, BerGal, DonGal, and DonBer all show synergistic inhibition of AChE at their IC$_{50}$ concentrations. The TacDon drug combination approached additive effects, while TacGal was antagonistic at IC$_{50}$ concentrations.

The BerGal drug combination had the most potent inhibitory effect (CI = 0.575). The individual IC$_{50}$ values for berberine and galantamine were 4.844 μM and 7.009 μM, respectively. Hence, a drug combination of 1.648 μM of each drug (IC$_{50}$ values for a 1:1 drug ratio) would in theory have similar efficacy as either drug, but with a substantial reduction of dose: 34% of the original berberine and 24% of galantamine. Figure 4A is a pictorial representation of this virtual BerGal combination pill and has a net reduction of 72% of total drug dose.
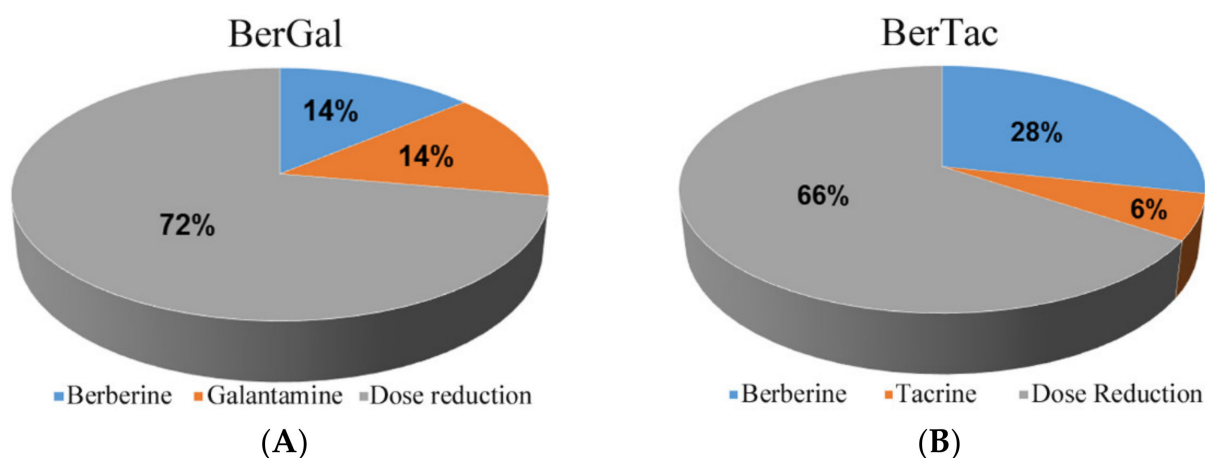

Figure 4. Virtual ChEI dual-drug combination pills: A virtual berberine and galantamine (BerGal) pill would have a 72% reduction of total drug dose (**A**). A virtual berberine and tacrine (BerTac) pill would have a 66% reduction of total drug dose (**B**).

Similarly, for the second most favorable drug combination of BerTac (CI = 0.686), there would be a beneficial reduction from their individual drug concentrations: IC$_{50}$ values of 4.844 μM and 1.019 μM reduced to 1.670 μM and 0.348 μM, respectively. A theoretical BerTac pill would only require 28% and 6% of the individual doses of berberine and tacrine, respectively, with a net 66% reduction of total drug dose (Figure 4B).

3.3. Cytotoxicity of Individual and Dual-Drug ChEI Combinations to Neuronal Cells

SH-SY5Y cells were treated with the ChEIs and two-drug combinations, and cell viability assessed using a MTT assay. Drugs were applied over a concentration range of 0.1, 1, 10, 100, and 1000 μg/mL for 48 h and cell viability compared with vehicle-treated control cells (set at 100% viability). The toxicity of the individual ChEIs to neuronal cells is shown in Figure 5. At the lowest concentration employed, 0.1 μM, none of the drugs were toxic to neurons (marked as non-significant changes from controls). A concentration of 1 μM (and above) of donepezil or berberine was able to induce toxicity, whereas at least 10 μM of tacrine or 100 μM of galantamine was needed to reduce cell viability. The drug (inhibitor) concentration that reduced cell viability by 50% (IC$_{50}$) was determined by non-linear regression, with values listed in Table 3. The order of toxicity of the ChEIs was donepezil > tacrine > berberine > galantamine. Dual-drug combinations were generally more toxic to neuronal cells with lower IC$_{50}$ values, apart from a BerGal combination that was relatively non-toxic (Figure 5 and Table 3).
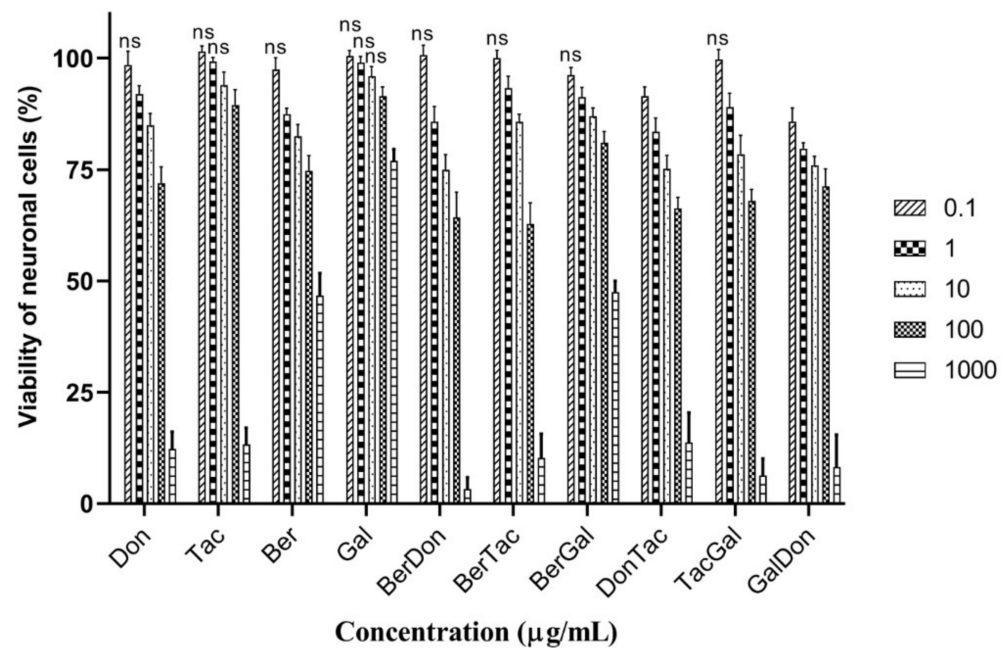
Figure 5. Cytotoxicity of individual and dual-drug ChEI combinations to SH-SY5Y cells: Human neuroblastoma cells were incubated with donepezil (Don), tacrine (Tac), berberine (Ber), and galantamine (Gal) or dual-drug combinations: BerTac (berberine and tacrine), BerGal (berberine and galantamine), TacDon (tacrine and donepezil), DonGal (donepezil and galantamine), DonBer (donepezil and berberine), and TacGal (tacrine and galantamine), and cell viability quantified using a MTT assay. Significant changes were quantified using a one-way ANOVA. ns = non-significant effect on cell viability. All other histograms not marked with ns, displayed significant changes from controls.

Table 3. Neurotoxicity of ChEI drugs and dual-drug combinations against SH-SY5Y neuroblastoma cells.

Drug or Drug Combination	Cell Cytotoxicity Log IC_{50} (μg/mL)
Don	2.31 ± 0.057
Tac	2.53 ± 0.091
Ber	2.98 ± 0.010
Gal	4.10 ± 0.041
DonBer	2.01 ± 0.043
BerTac	2.16 ± 0.031
BerGal	3.01 ± 0.046
TacDon	2.08 ± 0.038
TacGal	2.16 ± 0.052
DonGal	2.08 ± 0.059

4. Discussion

The drug treatment regimen for AD has not changed dramatically in over 20 years, with the current second-generation ChEIs, donepezil hydrochloride (FDA approved in 1997), rivastigmine tartrate (FDA approved in 2000), and galantamine hydrobromide (FDA approved in 2001), providing the mainstay treatment for mild-to-moderate AD. In addition, memantine (FDA approved in 2003), an antagonist of the N-methyl-D-aspartate (NMDA) receptor involved in excitatory glutamatergic neurotransmission, can also be used as a monotherapy, combination therapy, or adjunct to ChEI treatment. A drawback to the use of ChEIs is potential for ADRs as well as concerns regarding their cost-effectiveness [36,37]. Notably, tacrine, the first FDA approved ChEI (1993), was eventually removed as a prescription medication due to concerns with off-target toxicity effects, particularly hepatotoxicity [38]. To address this unmet need to improve dose efficiency, and thereby reduce

the likelihood of induction of undesired side-effects, and also to provide a more cost-effective medicine, we examined the potential benefits associated with novel dual-drug ChEI combinations.

When assessed as single drugs, the ChEIs, donepezil, tacrine, berberine, and galantamine, all proved useful and potent inhibitors of human or *Torpedo californica* AChE. Donepezil was the most effective ChEI and then tacrine, with these two inhibitors producing IC_{50} values in the nanomolar range, similar to those published for rat brain homogenates [39]. Substrate (AChE) binding by a ChEI may be to a similar site [39], but AChE can potentially accommodate two ChEI molecules simultaneously. The catalytic region of AChE (the esteratic site) is located at the bottom of a narrow gorge and contains three essential amino acids that provide the catalytic triad [24,40]. Anti-cholinergic drugs can inhibit AChE via direct binding to the catalytic site or via binding to a peripheral anionic site (PAS) primarily composed of aromatic amino acids. A number of ChEIs, including donepezil, tacrine, berberine, and galantamine, can bind at the PAS, and binding at this site can contribute to reduced AChE activity [24,41–44].

Screening of human or *Torpedo californica* AChE inhibition with dual-drug combinations revealed that BerTac, BerGal, and TacDon produced synergistic outcomes. For human AChE present within differentiated neurons, dual-drug synergism was also evidenced for these combinations as well as from DonGal, DonBer, and TacGal. The most potent synergism was attained via the BerGal combination and then the BerTac pairing. The potential benefit of synergistic inhibition by BerGal was a 72% reduction in total drug dose and a 66% reduction with BerTac. Reduced drug load would be expected to have a concomitant reduction of potential ADRs as well as improved cost-effectiveness. This is particularly pertinent for tacrine, given that excessive ADRs were responsible for its removal from the drug market [36,38].

To assess potential neurotoxicity, cell viability of differentiated neurons was quantified as single drug and dual-drug combinations. For the single drug exposures, the order of potency as a ChEI, donepezil > tacrine > berberine > galantamine, was mirrored with corresponding drug toxicity to cells (Tables 1 and 3). However, even for the most toxic drug, donepezil, (IC_{50} of 5.55 μM), the corresponding IC_{50} for AChE inhibition was only 0.081 μM (Table 2). Hence, therapeutic ChEI activity would still be efficacious without induction of significant neuronal toxicity.

Apart from BerGal, which was particularly well tolerated, each of the dual-drug combinations were of similar toxicity, and collectively, more toxic than donepezil (Table 3). Nevertheless, similar to application of individual drugs, the potency of dual-drug combinations towards AChE (IC_{50}s) were approximately two to three orders of magnitude lower than that for cell viability IC_{50}s; indicative of useful drug application within a therapeutic index.

The use of ChEIs for the treatment and management of AD may be improved through the use of dual-drug combinations or multi-drug targeting approaches [45–48]. The FDA approved concomitant use of memantine and ChEIs has primarily proved clinical efficacious and comparable to monotherapies, and for some studies provided additional benefit of reduced neurobehavioral symptoms [48].

In summary, there is an ongoing requirement for utilization of ChEIs with similar or improved clinical efficacy but with reduced ADRs. In the absence of newly available prescription drugs, deployment of current ChEIs (donepezil or galantamine), redeployment of a past ChEI (tacrine), or employment of a new ChEI (berberine) as dual-drug combinations, represent a potential means to achieve drug efficacy at lower drug doses able to diminish toxicity and increase drug cost-effectiveness. However, although dual-drug combinations are able to induce synergistic inhibition of AChE, individual drug pharmacokinetic profiles may yet hamper therapeutic utilization. For example, only donepezil and galantamine have relatively high oral bioavailability (compared to tacrine or berberine), and only the former drug has a half-life of over 24 h, facilitating a single daily dosing regimen [11,49–51].

Hence, in vivo studies are required to validate these in vitro results and the potential for implementation of coordinated dual-drug ChEI treatments for AD.

References

1. WHO. 10 Facts on Dementia, WHO Media Centre. 2009. Available online: https://www.who.int/features/factfiles/dementia/en/ (accessed on 26 December 2020).
2. GBD 2016 Dementia Collaborators. Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* **2019**, *18*, 88–106. [CrossRef]
3. Alzheimer's Association. Alzheimer's disease facts and figures. *Alzheimers Dement.* **2016**, *12*, 459–509.
4. Burns, A.; Iliffe, S. Alzheimer's disease. *BMJ* **2009**, *338*, b158. [CrossRef] [PubMed]
5. Frisoni, G.B.; Laakso, M.P.; Beltramello, A.; Geroldi, C.; Bianchetti, A.; Soininen, H.; Trabucchi, M. Hippocampal and entorhinal cortex atrophy in frontotemporal dementia and Alzheimer's disease. *Neurology* **1999**, *52*, 91–100. [CrossRef] [PubMed]
6. Dugger, B.N.; Dickson, D.W. Pathology of neurodegenerative diseases. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a028035. [CrossRef] [PubMed]
7. Bird, C.M.; Burgess, N. The hippocampus and memory: Insights from spatial processing. *Nat. Rev. Neurosci.* **2008**, *9*, 182–194. [CrossRef] [PubMed]
8. Terry, A.V.; Buccafusco, J.J. The cholinergic hypothesis of age and Alzheimer's Disease-related cognitive deficits: Recent challenges and their implications for novel drug development. *J. Pharm. Exp. Ther.* **2003**, *306*, 821–827. [CrossRef]
9. Schleibs, R.; Arendt, T. The cholinergic system in aging and neuronal degeneration. *Behav. Brain Res.* **2011**, *221*, 555–563. [CrossRef]
10. Grossberg, G.T. Cholinesterase inhibitors for the treatment of Alzheimer's disease: Getting on and staying on. *Curr. Ther. Res. Clin. Exp.* **2003**, *64*, 216–235. [CrossRef]
11. Colovic, M.B.; Krstic, D.Z.; Lazarevic-Pasti, T.D.; Bondzic, A.M.; Vasic, V.M. Acetylcholinesterase inhibitors: Pharmacology and toxicology. *Curr. Neuropharmacol.* **2013**, *11*, 315–335. [CrossRef]
12. National Institute for Health and Care Excellence (NICE). Available online: https://www.nice.org.uk/guidance/ta217/chapter/1-guidance (accessed on 26 December 2020).
13. Perrier, A.L.; Massoulie, J.; Krejci, E. PRiMA: The membrane anchor of acetylcholinesterase in the brain. *Neuron* **2002**, *33*, 275–285. [CrossRef]
14. Blotnick-Rubin, E.; Anglister, L. Fine Localization of Acetylcholinesterase in the Synaptic Cleft of the Vertebrate Neuromuscular Junction. *Front. Mol. Neurosci.* **2018**, *11*, 123. [CrossRef] [PubMed]

15. Ellis, J.M. Cholinesterase inhibitors in the treatment of dementia. *JAOA* **2005**, *105*, 145–158. [PubMed]
16. Birks, J.S. Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst. Rev.* **2006**, CD005593. [CrossRef]
17. Ahmed, F.; Ghalib, R.M.; Sasikala, P.; Ahmed, K.K. Cholinesterase inhibitors from botanicals. *Pharm. Rev.* **2013**, *7*, 121–130. [CrossRef]
18. Nwidu, L.L.; Elmorsy, E.; Thornton, J.; Wijamunige, B.; Wijesekara, A.; Tarbox, R.; Carter, W.G. Anti-acetylcholinesterase activity and antioxidant properties of extracts and fractions of Carpolobia lutea. *Pharm. Biol.* **2017**, *55*, 1875–1883. [CrossRef]
19. Nwidu, L.L.; Elmorsy, E.; Aprioku, J.S.; Siminialayi, I.; Carter, W.G. In vitro anti-cholinesterase and antioxidant activity of extracts of Moringa oleifera plants from Rivers State, Niger Delta, Nigeria. *Medicines* **2018**, *5*, 71. [CrossRef]
20. Jabir, N.R.; Khan, F.R.; Tabrez, S. Cholinesterase targeting by polyphenols: A therapeutic approach for the treatment of Alzheimer's disease. *CNS Neurosci. Ther.* **2018**, *24*, 753–762. [CrossRef]
21. Nwidu, L.L.; Alikwe, P.C.N.; Elmorsy, E.; Carter, W.G. An Investigation of potential sources of nutraceuticals from the Niger Delta Areas, Nigeria for attenuating oxidative stress. *Medicines* **2019**, *6*, 15. [CrossRef]
22. Amat-ur-Rasool, H.; Symes, F.; Tooth, D.; Schaffert, L.-N.; Elmorsy, E.; Ahmed, M.; Hasnain, S.; Carter, W.G. Potential nutraceutical properties of leaves from several commonly cultivated plants. *Biomolecules* **2020**, *10*, 1556. [CrossRef]
23. Cummings, J.L.; Tong, G.; Ballard, C. Treatment combinations for Alzheimer's disease: Current and future pharmacotherapy options. *J. Alz. Dis.* **2019**, *67*, 779–794. [CrossRef] [PubMed]
24. Amat-Ur-Rasool, H.; Ahmed, M. Designing second generation anti-Alzheimer compounds as inhibitors of human acetylcholinesterase: Computational screening of synthetic molecules and dietary phytochemicals. *PLoS ONE* **2015**, *10*, e0136509. [CrossRef] [PubMed]
25. Ji, H.F.; Shen, L. Berberine: A potential multipotent natural product to combat Alzheimer's disease. *Molecules* **2011**, *16*, 6732–6740. [CrossRef] [PubMed]
26. Cai, Z.; Wang, C.; Yang, W. Role of berberine in Alzheimer's disease. *Neuropsy. Dis. Treat.* **2016**, *12*, 2509–2520. [CrossRef]
27. Ellman, G.L.; Courtney, K.D.; Andres, V., Jr.; Feather-Stone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharm.* **1961**, *7*, 88–95. [CrossRef]
28. Chou, T.-C. The mass-action law based algorithms for quantitative econo-green bio-research. *Integr. Biol.* **2011**, *3*, 548–559. [CrossRef] [PubMed]
29. Li, S.; Huang, R.; Solomon, S.; Liu, Y.; Zhao, B.; Santillo, M.F.; Xia, M. Identification of acetylcholinesterase inhibitors using homogenous cell-based assays in quantitative high-throughput screening platforms. *Biotechnol. J.* **2017**, *12*, 1600715. [CrossRef]
30. Chou, T.; Martin, N. *CompuSyn for Drug Combinations: PC Software and User's Guide: A Computer Program for Quantitation of Synergism and Antagonism in Drug Combinations, and the Determination of IC50 and ED50 and LD50 Values*; ComboSyn: Paramus, NJ, USA, 2005.
31. Chou, T.-C. The combination index (CI < 1) as the definition of synergism and of synergy claims. *Synergy* **2018**, *7*, 49–50.
32. Encinas, M.; Iglesias, M.; Liu, Y.; Wang, H.; Muhaisen, A.; Cena, V.; Gallego, C.; Comella, J.X. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *J. Neurochem.* **2000**, *75*, 991–1003. [CrossRef]
33. Cheung, Y.T.; Lau, W.K.; Yu, M.S.; Lai, C.S.; Yeung, S.C.; So, K.F.; Chang, R.C. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* **2009**, *30*, 127–135. [CrossRef]
34. Agholme, L.; Lindstrom, T.; Kagedal, K.; Marcusson, J.; Hallbeck, M. An in vitro model for neuroscience: Differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. *J. Alzheimers Dis.* **2010**, *20*, 1069–1082. [CrossRef]
35. De Medeiros, L.M.; De Bastiani, M.A.; Rico, E.P.; Schonhofen, P.; Pfaffenseller, B.; Wollenhaupt-Aguiar, B.; Grun, L.; Barbé-Tuana, F.; Zimmer, E.R.; Castro, M.A.A.; et al. Cholinergic Differentiation of Human Neuroblastoma SH-SY5Y Cell Line and Its Potential Use as an In vitro Model for Alzheimer's Disease Studies. *Mol. Neurobiol.* **2019**, *56*, 7355–7367. [CrossRef] [PubMed]
36. Kröger, E.; Mouls, M.; Wilchesky, M.; Berkers, M.; Carmichael, P.H.; van Marum, R.; Souverein, P.; Egberts, T.; Laroche, M.L. Adverse drug reactions reported with cholinesterase inhibitors: An analysis of 16 Years of individual case safety reports from VigiBase. *Ann. Pharmacother.* **2015**, *49*, 1197–1206. [CrossRef] [PubMed]
37. Green, C.; Picot, J.; Loveman, E.; Takeda, A.; Kirby, J.; Clegg, A. Modelling the cost effectiveness of cholinesterase inhibitors in the management of mild to moderately severe Alzheimer's disease. *Pharmacoeconomics* **2005**, *23*, 1271–1282. [CrossRef] [PubMed]
38. Watkins, P.B.; Zimmerman, H.J.; Knapp, M.J.; Gracon, S.I.; Lewis, K.W. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *JAMA* **1994**, *271*, 992–998. [CrossRef] [PubMed]
39. Snape, M.F.; Misra, A.; Murray, T.K.; De Souza, R.J.; Williams, J.L.; Cross, A.J.; Green, A.R. A comparative study in rats of the in vitro and in vivo pharmacology of the acetylcholinesterase inhibitors tacrine, donepezil and NXX-066. *Neuropharmacology* **1999**, *38*, 181–193. [CrossRef]
40. Bajda, M.; Więckowska, A.; Hebda, M.; Guzior, N.; Sotriffer, C.A.; Malawska, B. Structure-based search for new inhibitors of cholinesterases. *Int. J. Mol. Sci.* **2013**, *14*, 5608–5632. [CrossRef]
41. Rydberg, E.H.; Brumshtein, B.; Greenblatt, H.M.; Wong, D.M.; Shaya, D.; Williams, L.D.; Carlier, P.R.; Pang, Y.-P.; Silman, I.; Sussman, J.L. Complexes of Alkylene-linked Tacrine dimers with Torpedo californica acetylcholinesterase: Binding of Bis (5)-tacrine produces a dramatic rearrangement in the active-site gorge. *J. Med. Chem.* **2006**, *49*, 5491–5500. [CrossRef]

42. Shan, W.-J.; Huang, L.; Zhou, Q.; Meng, F.-C.; Li, X.-S. Synthesis, biological evaluation of 9-N-substituted berberine derivatives as multi-functional agents of antioxidant, inhibitors of acetylcholinesterase, butyrylcholinesterase and amyloid-b aggregation. *Eur. J. Med. Chem.* **2011**, *46*, 5885–5893. [CrossRef]

43. Stavrakov, G.; Philipova, I.; Zheleva, D.; Atanasova, M.; Konstantinov, S.; Doytchinova, I. Docking-based design of galantamine derivatives with dual-site binding to acetylcholinesterase. *Mol. Inf.* **2016**, *35*, 278–285. [CrossRef]

44. Silva, M.A.; Kiametis, A.S.; Treptow, W. Donepezil inhibits acetylcholinesterase via multiple binding modes at room temperature. *J. Chem. Inf. Modeling* **2020**, *60*, 3463–3471. [CrossRef] [PubMed]

45. Mak, S.; Luk, W.W.; Cui, W.; Hu, S.; Tsim, K.W.; Han, Y. Synergistic inhibition on acetylcholinesterase by the combination of berberine and palmatine originally isolated from Chinese medicinal herbs. *J. Mol. Neurosci.* **2014**, *53*, 511–516. [CrossRef] [PubMed]

46. Kong, X.P.; Liu, E.Y.L.; Chen, Z.C.; Xu, M.L.; Yu, A.X.D.; Wu, Q.Y.; Xia, Y.-J.; Duan, R.; Dong, T.T.X.; Tsim, K.W.K. Synergistic Inhibition of Acetylcholinesterase by Alkaloids Derived from Stephaniae Tetrandrae Radix, Coptidis Rhizoma and Phellodendri Chinensis Cortex. *Molecules* **2019**, *24*, 4567. [CrossRef] [PubMed]

47. Benek, O.; Korabecny, J.; Soukup, O. A Perspective on Multi-target Drugs for Alzheimer's Disease. *Trends Pharmacol. Sci.* **2020**, *41*, 434–445. [CrossRef] [PubMed]

48. Kabir, M.T.; Uddin, M.S.; Mamun, A.A.; Jeandet, P.; Aleya, L.; Mansouri, R.A.; Ashraf, G.M.; Mathew, B.; Bin-Jumah, M.N.; Abdel-Daim, M.M. Combination Drug Therapy for the Management of Alzheimer's Disease. *Int. J. Mol. Sci.* **2020**, *21*. [CrossRef] [PubMed]

49. Forsyth, D.R.; Wilcock, G.K.; Morgan, R.A.; Truman, C.A.; Ford, J.M.; Roberts, C.J. Pharmacokinetics of tacrine hydrochloride in Alzheimer's disease. *Clin. Pharmacol. Ther.* **1989**, *46*, 634–641. [CrossRef] [PubMed]

50. Lou, G.; Montgomery, P.R.; Sitar, D.S. Bioavailability and pharmacokinetic disposition of tacrine in elderly patients with Alzheimer's disease. *J. Psychiatry Neurosci.* **1996**, *21*, 334–339.

51. Ye, M.; Fu, S.; Pi, R.; He, F. Neuropharmacological and pharmacokinetic properties of berberine: A review of recent research. *J. Pharm. Pharmacol.* **2009**, *61*, 831–837. [CrossRef]