Pharmacokinetic and pharmacodynamic properties of cholinesterase inhibitors, donepezil, tacrine and galantamine in aged and young Lister-hooded rats

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PK/PD of ChEIs in aged and young LH rats

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Abstract

Ageing accompanies physiological alterations that may change pharmacological response. Pharmacokinetic/pharmacodynamic (PK/PD) properties of cholinesterase inhibitors (ChEIs) used in the treatment of Alzheimer's disease, donepezil, tacrine and galantamine, were investigated in an aged Lister-hooded (LH) rat model. Intravenous and oral 6 h blood-sampling profiles in old (30-month old) and young (7-month old) rats revealed similar pharmacokinetic changes to humans with an approximately 40% increase in C$_{\text{max}}$ of galantamine and prolonged t$_{1/2}$ (1.4-fold) and MRT (1.5-fold) of donepezil. Tacrine disposition was maintained with age and AUC and clearance in old rats were similar to young rats for all drugs tested, as was bioavailability. Old rats showed a trend of increased pharmacodynamic sensitivity (< 20%) to ChEIs in cholinesterase activity assays which was attributed to pharmacokinetic effects since a trend of higher blood and brain concentrations was seen in the old rats though brain:blood ratios remained unaffected. Enhanced cholinergic-mediated behaviors such as tremor, hypothermia, salivation and lacrimation were also observed in the old rats which could not be accounted for by a similar magnitude of change in pharmacokinetics. A decrease in expression of muscarinic acetylcholine receptor subtype 2 detected in old rat brains was postulated to play a role. Greater age effects in both pharmacokinetics and pharmacodynamics of donepezil and tacrine were seen in previous studies with Fischer 344 rats, indicating a potential risk in over-reliance of this rat strain for ageing studies.
Introduction

The world population is ageing at an alarming rate with people aged 60 and above projected to triple, reaching nearly 2 billion from 2000-2050 (United Nations, 2009). The elderly, having an increased vulnerability to disease, consume a disproportionately larger share of medicines compared to any other age group. Changes in pharmacological response are known to occur on ageing (McLean and Le Couteur, 2004, Hilmer, 2008) and it is vital to understand and preferably predict and manage them to reduce incidents of adverse drug events or states of poor efficacious response.

The effect of age on pharmacokinetics has been extensively covered by various reviews (Klotz 2009, Cusack 2004, McLean and Le Couteur, 2004, Turnheim, 2003). The decline in glomerular filtration rate (GFR) on ageing is likely the most influential change, affecting excretion of most drugs. Ageing accompanies a decrease in liver size and blood flow which may reduce hepatic clearance of drugs but factors such as binding affinity with liver enzymes and plasma/tissue proteins also define drug uptake into liver, resulting in drug-specific alterations in metabolism and bioavailability. Cytochrome p450 (CYP450) enzymes involved in phase I metabolism have shown some dysregulation with age with varying trends in activity reported. Phase II enzymes involved in conjugation reactions in contrast are reportedly maintained on ageing. Drug distribution is known to change in the elderly due to body fat/water composition alterations and some reports on decreased transporter activity (e.g. P-glycoprotein) in the blood-brain-barrier on ageing could alter the brain permeability of drugs (Bartels et al., 2009, Tornliviet et al., 2006).

Pharmacodynamic changes with age are less clearly elucidated compared to pharmacokinetics. Investigations on pharmacodynamic changes should measure drug concentrations in blood/plasma to ensure that sensitivity alterations are not due to pharmacokinetics. The elderly are generally more sensitive to pharmacotherapy although bidirectional pharmacodynamic alterations have been observed (Mangoni and Jackson,
The central nervous system is especially vulnerable on ageing and centrally-acting drugs consistently shown to produce sensitized reactions in the elderly independent of pharmacokinetic changes include benzodiazepines (midazolam), opioids (morphine) and anticholinergic tricyclic antidepressants (imipramine, amitriptyline) (Villesen et al., 2007, Lotrich and Pollock, 2005, Albrecht et al., 1999). Mechanisms by which these pharmacodynamic alterations occur are still relatively unclear but are usually attributed to changes in drug-receptor interactions, altered post-receptor signalling and impaired homeostatic mechanisms (Shi et al., 2008).

Studying and establishing trends in the aged pharmacological response is fraught with various roadblocks. A key challenge lies with the heterogeneity in health status of the elderly population and confounding factors such as gender (as a higher proportion of women reach old age than men), pharmacogenetics, and environmental influences (e.g. smoking, diet, and co-administered drugs). The use of aged animal models provides an attractive alternative to studying the aged pharmacological response for their short life-span and close physiological resemblance to humans. Their functional decline on ageing mimics that of humans with some exceptions. Rats for example, experience a decline in CYP450 metabolism with age which does not occur with the same consistency in humans (Schwartz, 2007). In contrast, decreased GFR seen in the elderly is not apparent in rat, with the exclusion of some albino strains that undergo chronic progressive nephropathy (CPN), the manifestation of which is more dependent on strain and sex than age (Baylis and Corman, 1998, Goldstein et al., 1988). Nevertheless, prior pharmacokinetic/pharmacodynamic (PK/PD) studies carried out on anaesthetics and analgesics in aged animal models have shown good correlation to human responses (Jourdan et al., 2002, Hovinga et al., 1992).

An aged Lister-hooded (LH) rat model was employed to evaluate the PK/PD properties of cholinesterase inhibitors (ChEIs), donepezil (Aricept®), tacrine (Cognex®) and galantamine (Razadyne®) used in the treatment of Alzheimer’s disease (AD). AD is a
debilitating neurodegenerative disease characterised by cholinergic deficiency and ChEIs function to alleviate AD symptoms by preventing the breakdown of neurotransmitter, acetylcholine (ACh). Due to their prevalent use among the elderly, pharmacokinetic information is readily available comparing young and aged AD patients (Table 5, Jann et al., 2002, Farlow 2003, Pitrovsky et al., 2003). Similar information from rats has not been obtained. Oral and intravenous PK profiles and PD measurements in terms of ChE inhibition and induced cholinergic behaviours were therefore carried out in LH aged and young rats to evaluate the potential of this model in prediction of PK/PD changes with age of ChEIs in humans. Results were compared with similar studies done with aged Fischer 344 (F344) rats (Kosasa et al., 1999) to identify any issues with regard to strain selection in ageing studies.
Methods

Drugs and chemicals

Donepezil hydrochloride and galantamine hydrobromide salts were obtained from Manus Aktteva (Gujarat, India). Tacrine hydrochloride was purchased from Sigma Aldrich (St Louis, MO, USA). A proprietary GSK compound of similar characteristics to the compounds analysed was used as an internal standard (I.S.) in LC-MS/MS analysis (GlaxoSmithKline, Harlow, UK). Solvents acetonitrile, methanol, ethanol and ammonium acetate were obtained from Merck (Darmstadt, Germany).

Animal maintenance

Young (7 months old, 350-450g) and old (30 months old, 350-500g) male LH rats obtained from Harlan Laboratories (Loughborough, England) were approximately 3 months old on arrival. They were housed in groups of three to five in individual-ventilated cages at a controlled temperature (20 ± 1°C) and humidity (40 ± 2%) in a specified pathogen-free (SPF) facility till time of use. Rooms were on a twelve hour light/dark cycle, and animals were allowed ad libitum access to water and food (Teklad Irradiated Global 14% Protein Maintenance Diet, 2914, Harlan Laboratories). Old animals which developed tumours or signs of disease were not utilized in the study.

Animals that underwent cannulation surgery for pharmacokinetic studies were housed individually in clear cages and fitted with elastic harnesses attached to a counter-weighted swivel system allowing free movement (Instech Solomon, PA, USA). They were monitored daily for weight changes and their cannulas were flushed with 100 U/L heparinised saline (made with heparin sodium salt from Sigma Aldrich, St Louis, MO, USA) to prevent clotting. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee (protocol no.: 080342) and Singapore National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines for the use and care of animals for scientific purposes and GlaxoSmithKline animal research ethical standards.
Pharmacokinetic studies of donepezil, tacrine and galantamine in old and young rats

Animals underwent surgery where they were dosed subcutaneously with analgesic (Torbugesic®, 10 mg/kg, 0.05 ml/100g) and antibiotic (Baytril®, 1 mg/kg, 0.1 ml/100g) and hand-made cannulas (PE50 and PE10 polyethylene tubing, Beckton Dickinson, NJ, USA) were implanted into the right jugular and left femoral vein respectively under inhaled isofluorane anaesthesia. Following a day of recovery, rats were given a 1 h intravenous (i.v.) infusion of post-filtered drug solution dissolved in 2% DMSO (Sigma Aldrich, St Louis, MO, USA) and hydroxybetacyclodextrin (Acros Organics, Geel, Belgium) (10% w/v in 0.9% saline) at 1 mg/kg and 10 ml/kg/h via the femoral cannula with the aid of a syringe pump. A predose blood sample was taken from the jugular cannula followed by sampling at 15, 30, 45, 60, 65, 75, 90, 120, 150, 180, 240, 300 and 360 min time-points. To assess bioavailability, rats went through a one day wash-out period before being orally gavaged (3 mg/kg, 5 ml/kg, 1% methyl cellulose from Sigma Aldrich as vehicle) with the same drug they were dosed i.v. with before. Blood samples were collected at predose and 30, 60, 90, 120, 180, 240, 300 and 360 min following dosing. A blood volume of 120 µl was collected per time-point and diluted with an equal volume of water before being kept at -80°C till LC-MS/MS analysis.

Pharmacokinetic analysis

Pharmacokinetic analysis of the i.v. profiles were performed with WinNonlin® software (Pharsight, Lexington, KY, USA) using a non-compartmental model. Pharmacokinetic parameters obtained included peak concentration in blood (C_{max}), blood clearance (CL_{b}), volume of distribution (V_d), terminal half-life (t_{1/2}), mean residence time (MRT), area under the blood concentration-time curve per unit dose (AUC_{(0-t)}/Dose) with t being 6 h or time of last quantifiable concentration and AUC_{0-∞}/Dose representing AUC per unit dose extrapolated to infinity. AUCs were calculated using linear/log trapezoidal interpolation. Pharmacokinetic analysis for the oral profiles was performed in Microsoft Excel with AUC values calculated by
the linear trapezoidal rule. Additional pharmacokinetic parameters include time to reach \( C_{\text{max}} \) (\( T_{\text{max}} \)) and bioavailability (\( F_{\text{po}} \)) which was calculated by the equation:

\[
F_{\text{po}} \, (\%) = \frac{\text{AUC}_{\text{oral}} \times \text{dose}_{\text{iv}}}{\text{AUC}_{\text{iv}} \times \text{dose}_{\text{oral}}} \times 100
\]

where \( \text{AUC}_{\text{iv}} \) was obtained from the prior i.v. profile in the same rat unless stated otherwise.

**Terminal sampling of brain, blood and plasma for measurement of cholinesterase activity and drug concentration**

Young and old rats were dosed orally with the donepezil, tacrine, galantamine or vehicle alone (1% methylcellulose) at 3 mg/kg, 5 ml/kg (n=3). The rats were decapitated after 1 h and trunk blood collected into EDTA-coated tubes (BD Diagnostics, NJ, USA). A portion of blood was aliquoted into Eppendorf tubes and centrifuged to isolate plasma. Whole brains were extracted and briefly rinsed with water before being dissected in halves and stored in tubes. All samples were kept at -80°C till LC-MS/MS analysis.

**Sample preparation and LC-MS/MS analysis**

All brain tissues were weighed and homogenized in 1 vol of water with an ultrasonicating homogenizer (TOMTEC Autogiser, Receptor Technologies, Adderbury, Oxon, UK). Calibration standards of each compound were prepared from a 100 µg/ml stock in ethanol at concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml to a final volume of 50 µl. These were spiked with 100 µl of blood (diluted 1:1 with water) or 50 µl of brain homogenate from an undosed rat to account for matrix effects. Similarly, sample volumes of 100 and 50 µl of diluted blood and brain homogenate respectively were combined with 50 µl ethanol in 1.4 ml micronic tubes (Micronic, Lelystad, The Netherlands). Protein precipitation was carried out where 350 µl I.S. solution (100 ng/ml I.S. compound in 80% ACN with 20% 10 mM ammonium acetate) was added to samples and standards. The tubes underwent vigorous shaking for 20 min and centrifugation for 15 min at 3220 g at room
temperature before LC-MS/MS analysis was performed directly from the supernatant solution. A total blank (without analyte and I.S.) and blank (without analyte) were injected at the start of the standard line and again at the end, to ensure there was no carry-over of analytes. Washes were included between standards and samples and between different sample groups.

For LC-MS/MS analysis, samples were introduced with a CTC Analytics HTS Pal autosampler (Presearch, UK) to an Agilent 1100 series binary pump HPLC system (Waldbronn, Germany) interfaced with an API4000 triple quadrupole mass spectrometer equipped with a Turbo Ionspray (TIS) interface (Applied Biosytems, Ontario, Canada). All runs were carried out with the column thermostat set to 40°C, an eluent flow rate of 1 ml/min and 2 min run time, using mobile phases 10 mM ammonium acetate with 0.1% formic acid (Solvent A) and ACN (Solvent B). Positive ionization mode was used with TIS source temperature, 690°C; TIS voltage, 5500V; curtain gas, 20 psi; nebulising gas (GS1), 50 psi; TIS (GS2) gas, 70 psi; collision activated dissociation (CAD) gas, 4 psi. LC-MS/MS conditions and cassette groupings for the compounds analysed are listed in Table 1.

Peak quantification was carried out in Analyst® software (Applied Biosystems, CA, USA) and all standards had to pass ±20% acceptance criteria for runs to be accepted. Peak area ratios (analyte:IS) were quantified against the calibrated standard concentration line obtained using 1/x² weighted linear regression enabling calculations of the compound levels present in the samples.

**Cholinesterase (ChE) enzyme activity assay**

ChE activity was measured using Ellman’s colorimetric assay (1961) modified into a high-throughput 96-well plate format by Padilla et al. (1999). Half-brains collected previously from animals dosed with donepezil, tacrine, galantamine and vehicle were homogenized in 10 vol of 0.1 M sodium phosphate buffer (pH 8.0) and 1% Triton X-100 (Biorad) (homogenizing buffer). The homogenate was centrifuged for 10 min at 1000 g at 4°C and
protein from supernatant was quantified with a BCA (bicinchoninic) protein assay kit (Pierce, Rockford, IL, USA) to ensure all samples contain approximately similar protein levels. Supernatant was kept at -80°C in aliquots.

On the day of assay, neat plasma (5 µl) or brain preparations diluted 5X with homogenizing buffer (10 µl) were pre-incubated at 37°C for 10 min with 0.33 M DTNB (Sigma Aldrich, St Louis, MO, USA) in 0.1 M sodium phosphate buffer (pH 8.0) (working buffer). In wells where only AChE activity was to be measured, 10 µl of iso-OMPA (selective BChE inhibitor, Sigma Aldrich) was included (final concentration in well: 0.1 mM). After pre-incubation, 10 µl of substrate, acetylthiocholine iodide (ACthI, Sigma Aldrich), was added to the wells (final concentration in well: 1 mM). The plate was immediately put in a microplate reader (Infinite®F500, Tecan, Switzerland) heated to 37°C and read in a kinetic format with the plate set to shake for 5 s followed by a read at 412 nm, this process repeating every 2 min up to 12 min (6 reads). Tissue and substrate blanks were included for every plate which excluded brain homogenate/plasma and ACthI respectively. All samples were done in triplicate wells. Final volume in each well: 200 µl.

For calculation of activity, the mean of the slopes (change in absorbance per min, \( \Delta A/\text{min} \)) for each triplicate set of samples was taken and converted to µmol/min/ml (plasma) or µmol/min/g (brain) with the following equation:

\[
\text{Activity} = \frac{[(\text{Average } \Delta A - \Delta A_{\text{blank}})/\text{min}]}{\text{factor(}\Delta A/^/-\text{SH nmol}) / \text{sample vol (ml)} / \text{sample dilution (1 for plasma, 55 for brain)} / 1000 (\text{nmol to µmol})}
\]

\textit{where factor is an instrument-specific calculation factor determined by establishing a free sulphydryl standard curve that measures } \Delta A \text{ per unit of } -\text{SH.}

BChE activity was calculated by taking total ChE activity minus residual activity in the presence of the specific BChE inhibitor (i.e. AChE activity).

\textit{Observation of cholinergic-mediated behaviour in tacrine-dosed animals}
Young and old animals (n=6) were dosed orally with tacrine at 10 mg/kg and 30 mg/kg at 5 ml/kg dose volume with 1% methyl cellulose vehicle that was given alone to a control group of animals. Doses were given in a random fashion and grading of behaviour was made by a person unaware of the treatment administered. Behavioural grading of tremor and lacrimation was carried out on a 0-3 scale as shown in Table 2 which was devised previously by Dronfield et al. (2000). Temperature was taken by means of a rectal probe (Bioseb, Chaville, France) inserted by 5 cm into the rectum which gave a steady reading within 10 s that was promptly recorded. Salivation was measured by collecting saliva on a pre-weighed cotton bud by wiping the bud in and around the mouth for 6 s and reweighing it. Responses were recorded prior to dosing and 0.5, 1, 2, 4 and 6 h after dosing.

**Immunoblotting experiments to quantify expression of muscarinic acetylcholine receptor (mACHR) subtypes in rat brains**

Six young and six old rat half-brains were dissected free of meninges and white matter before gentle homogenization with a glass and mortar (ten plunges) in 1x CST lysis buffer (Cell Signalling Tech Inc., Beverly, MA, USA) with protease inhibitors, AEBSF (Sigma Aldrich) and one complete mini Roche tablet (Roche Applied Science, IN, USA). Sample buffer (Laemmli Sample buffer, Bio-rad, CA, USA) with 2-mercaptoethanol was then added to the samples, followed by boiling at 100°C for 3-5 min to denature proteins. The samples and a precision plus dual colour protein standard (#161-0374, Bio-rad, 0-250 kDa range) were loaded in 10% polyacrylamide gels and electrophoresis was carried out at 120 V for 1 h. Transfer took place onto nitrocellulose membranes and these were blocked in 10 mM phosphate buffered saline (pH 7.4), 0.1% Tween 20 and 5% skim milk (PBSTM) before immunoblotting with primary antibody in PBSTM with 5% bovine serum albumin at 1:1000 overnight at 4°C. Primary antibodies used for M1 mACHRs (rabbit polyclonals) were from Alomone labs (Jerusalem) while M2-M4 mACHR antibodies (rabbit polyclonals) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Following washings in
PBSTM and incubation with horseradish peroxidase conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch Inc., West Grove, PA), immunoreactive bands on the membranes were detected by enhanced chemiluminescence and quantified by an image analyzer (UVItc, Cambridge, UK). Membranes were then stripped and re-blotted with anti-β-actin (1:5,000, mouse monoclonal, Sigma-Aldrich) to control for sample loading across lanes. Normalized immunoblot optical densities are expressed in arbitrary units.

**Statistical analysis**

Data are represented as mean ± S.D. All statistical analysis was performed using Statistica software (StatSoft Ltd, Bedford, UK). Comparisons between young and aged groups were made using unpaired/independent t-tests (two-tailed). A one-way ANOVA followed by a Dunnet post-hoc test was used to compare variance of treatment groups from control group (cholinesterase activity). For repeated measurements (e.g. cholinergic grading or i.v./p.o. pharmacokinetic profiles), a repeated measure ANOVA was employed with planned comparisons made between groups to demonstrate treatment or age effects. p values < 0.05 were considered significant.
Results

Pharmacokinetic profiles of ChEIs dosed i.v.

Out of the three drugs tested, only galantamine showed a notably different concentration-time profile on 1 h i.v. infusion at 1 mg/kg and 10 ml/kg (fig. 1C). Significantly higher blood concentrations of drug were reached in the old rats from 0.25 to 2 h with a Cmax 1.4-fold greater than that reached in the young group. Following which, drug concentrations declined to similar levels as seen in the young rats. For the other drugs, concentration-time profiles were similar between young and old rats, with slight but significant differences in concentrations seen at certain time-points (fig. 1A and B).

Pharmacokinetic parameters obtained from non-compartmental analysis of the i.v. concentration-time profiles are represented in table 3. Despite the higher Cmax observed in galantamine-dosed old rats, changes in AUC, t1/2 and Clb were not significant due to the greater variability among the old rats. A decrease in Vd in old rats however, reached significance (p<0.01). In contrast with galantamine, a significantly higher Vd of donepezil was found in the old rats (p<0.01). This was accompanied by a significant prolongation of t1/2 and MRT by 1.4 and 1.5-fold respectively (p<0.01). No significant change in AUC or Clb was observed. Pharmacokinetic parameters of tacrine showed no significant differences between young and old rats.

It should be noted that larger extrapolations were made for the concentration-time profile of donepezil as it did not reach baseline levels at 6 h. Pharmacokinetic parameters derived should therefore be subject to caution on interpretation.

Pharmacokinetic profiles of ChEIs dosed p.o.

Oral dosing commonly results in a more variable concentration-time profile due to differing extents of first-pass effect between animals. This was observed in the p.o. pharmacokinetic profiles where higher standard deviations were obtained compared to i.v. pharmacokinetic profiles. Old rats dosed with donepezil and tacrine showed significant but
slight increase of blood drug concentrations at certain time-points with a delayed return back to basal levels more so for donepezil than tacrine (fig, 1D and E). A shift in $T_{\text{max}}$ can be seen in the average blood concentration-time curves of donepezil but as a prolonged $T_{\text{max}}$ (up to 4-fold of the median) was only seen in 1 old rat out of 5, the median $T_{\text{max}}$ in both age groups were the same (table 3). There was a trend of slightly elevated blood concentrations of galantamine in the old rats during the first 2 h following dosing with similar levels attained after 3 h (fig. 1F). No significant changes were detected with age for all pharmacokinetic parameters obtained, including bioavailability ($F_{\text{po}}$) (table 3).

**Cholinesterase activity in plasma and brain of young and old rats**

ChE activity levels were 20-fold higher in the brain (12-15 µmol/min/g) compared to plasma (0.4-0.8 µmol/min/ml) (fig. 2). A major proportion of total ChE activity in brain was derived from AChE (AChE:BChE ratio ~12.2) whereas equivalent levels of AChE and BChE were found in plasma (AChE:BChE ratio ~1.2). In the aged animals, total plasma ChE activity levels appeared significantly enhanced (by ~60%) with BChE activity showing a greater elevation (2.1-fold) than AChE (1.2-fold) when compared to the respective ChE activity in young rat plasma. Brain ChE activity levels in contrast were not significantly different between young and aged animals.

**Effects of donepezil, tacrine and galantamine on cholinesterase activity in plasma and brain of young and old rats**

Plasma and brain isolated from young rats 1 h following treatment with the various ChE inhibitors (3 mg/kg) showed significant inhibition of total ChE activity only after donepezil treatment (fig. 3). This inhibition was greater in the plasma than in the brain (81.6% vs. 92.6%). Most part of donepezil’s inhibition was derived from AChE inhibition rather than BChE inhibition. Tacrine was observed to have the greatest BChE-inhibiting effect among the drugs which is significantly discernable in the plasma rather than the brain as BChE is found at higher levels there.
ChE inhibition showed a trend of enhancement in the old rats for both plasma and brain tissues (fig. 3). Absolute ChE activity levels in the plasma however, remained higher in the old rats compared to the young (not shown). The percentage enhancement ranged from 3.2% increase in tacrine-induced brain AChE inhibition to 10.5% increase in galantamine-induced brain AChE and total ChE inhibition to 18.0% increase in donepezil-induced plasma total ChE inhibition (table 5). Although these enhancements were slight, they led to significant inhibition of total ChE in the old rats which were previously undetected.

**Brain and blood concentrations of donepezil, tacrine and galantamine**

Brain and blood concentrations together with brain:blood ratios of donepezil, tacrine and galantamine at 1 h post-dose can be seen in table 4. Corrected brain concentration refers to measured brain concentration minus residual blood volume present in brain which is 15 µl/g as derived from literature (Brown et al., 1986). Apart from a significantly higher brain concentration of donepezil detected in the old rats (p<0.05) at a magnitude of 1.8-fold greater than the young rats, other changes in concentrations were non-significant. However, there was a trend observed of higher blood and brain drug concentrations in the old rats compared to the young rats across treatments. Brain:blood ratios were found to be similar in young and old rats for all drugs.

**Cholinergic-mediated behaviour in tacrine-dosed young and old rats**

To further investigate ageing changes in pharmacodynamic response to ChEIs, a simple assessment of cholinergic-mediated behaviour (tremor, hypothermia, lacrimation and salivation) in response to tacrine administration (p.o., 10 mg/kg and 30 mg/kg) was carried out in young and old rats. The results are shown in figure 4..

Higher baseline cholinergic activities were observed in the old rats, indicated by the presence of subtle tremor in limbs and consistent hypothermia in vehicle-dosed old rats. For all effects, response to tacrine was significantly prolonged in the old rats compared to the young rats. This was more striking at the 10 mg/kg dose level where responses in the young
rats tended to recover to baseline levels by 6 h whereas old rats continued exhibiting cholinergic-mediated behaviour. With regard to magnitude of response, salivation underwent the most observable enhancement at both dose levels with old rats producing almost three times the amount of saliva compared to tacrine-dosed young rats. The latter of which reported no significant differences in salivation from the vehicle-dosed group (fig. 4D).

At the 30 mg/kg dose level, lacrimation was not seen in the young rats till 2 h post-dose while old rats exhibited signs of lacrimation at 0.5 h (fig. 4C). In contrast, tremor and hypothermia manifested more slowly in the old rats compared to the young. This was only discernable at the 10 mg/kg dose level where significant treatment effect in terms of tremorigenic response was seen at 2 h in the old rats ($p < 0.017$) compared to 1 h in the young rats ($p < 0.004$). Similarly, a significant decrease in temperature in response to 10 mg/kg tacrine was detected at 1 h in the old rats and 0.5 h in the young rats.

**Expression of mAChR subtypes in old and young LH rat brain**

Enhanced cholinergic responses may arise from an increase in mAChR-mediated response or decline in mAChR autoregulation of ACh release. Expression of mAChR subtypes in brain was determined for young and old rats by immunoblotting and results can be found in figure 5. It was found that only M2 mAChR expression decreased significantly while M1, M3 and M4 receptor subtypes showed no significant change in expression with age. The expression of muscarinic M2 receptors in the old rats was decreased to a level approximately two thirds of that in the young.

**Discussion**

Human studies investigating the effect of age on the disposition of the ChEIs used in this paper found that only donepezil and galantamine underwent significantly different pharmacokinetics in the elderly. A prolonged $t_\text{1/2}$, $T_{\text{max}}$ and MRT of donepezil attributed to a significantly larger $V_d$ as oral clearance was maintained, was observed in 6 elderly patients.
(68-82 years) versus 12 young healthy adults (20-27 years) following a single 2 mg oral dose (Ohnishi et al., 1993). For galantamine, $C_{\text{max}}$ was 30-40% higher in older AD patients than healthy younger adults although AUC and $t_{1/2}$ remained unchanged (Jaan et al., 2002). A population pharmacokinetic modelling study utilizing data from 15 clinical trials revealed a 30% reduction in galantamine clearance with age when comparing healthy subjects (median age: 25) with AD patients (median age: 75), with hepatic impairment having a greater impact on clearance over renal insufficiency (Pitrovsky et al., 2003).

Pharmacokinetic studies of ChEIs carried out in old and young LH rats saw no change in tacrine disposition, a significant prolonging of $t_{1/2}$ and MRT of donepezil and a significant 1.4-fold (~40%) increase in $C_{\text{max}}$ of galantamine in the old rats following i.v. infusion of the respective drugs at 1 mg/kg. No differences in clearance or AUC were observed for all drugs whereas alterations in $V_d$ of galantamine and donepezil were detected in contrasting directions. The smaller $V_d$ of galantamine observed in old rats likely contributed to the greater $C_{\text{max}}$ achieved during the initial phase of drug absorption (0.25-2 h) as the decreased distribution into tissue compartments would result in higher blood concentrations. Donepezil on the other hand, having a larger $V_d$ in the old rats, takes a longer time to penetrate and dissociate from tissue compartments to be subsequently cleared, resulting in a longer $t_{1/2}$ as well as a prolonged $T_{\text{max}}$ seen in one old rat after oral administration. The different directional changes in $V_d$ may be attributed to galantamine having a lower lipophilicity (clogP: galantamine 1.02 vs. donepezil 4.60) and slightly higher polar surface area than donepezil (PSA: galantamine 41.9 vs. donepezil 38.8). Galantamine would therefore associate less extensively to fatty tissue compartments compared with donepezil and bind to polar regions with greater affinity. As body fat content is known to increase with age while body water declines (Beaufrere and Morio, 2000), contrasting directional movements of these drug levels in the blood and tissues may result. Pharmacokinetic parameters obtained from oral profiles of the ChEIs were similar between age groups although old rats exhibited slightly elevated
blood concentrations of donepezil and tacrine at certain time-points. Calculated bioavailability was maintained with age for all drugs, indicating a sustained metabolic clearance with age of the ChEIs in the LH rat.

The lower clearance of galantamine reported in the elderly AD patient group from Pitrovsky’s study which was not observed in the aged LH rat may have stemmed from the greater proportion of females used in this group (57.5% as opposed to 25.3% in the young healthy subject group). Females experience a reduced rate of renal and metabolic clearance as these functions are components of body weight (Pitrovsky et al., 2003). The LH aged rat therefore provides a fairly robust model for estimating alterations in pharmacokinetics of donepezil, tacrine and galantamine in aged humans.

To measure pharmacodynamic responses to the ChEIs, ChE activity assays were carried out in plasma and brain tissue of LH rats. Without drug interference, ChE activity in plasma was enhanced in old rats by nearly 60% whereas brain ChE activity remained unchanged. This was in line with previous findings in F344 rats (Kosasa et al., 1999). In elderly subjects, plasma BChE activity was enhanced though AChE activity was maintained (Hubbard et al., 2008). Upon dosing with the ChEIs, donepezil exerted the greatest inhibitory effect on total ChE derived mostly from AChE inhibition. This correlates with its higher potency and greater selectivity for AChE over BChE in contrast with tacrine, which has similar affinities for both ChEs resulting in the greater incidence of peripheral side effects associated with its use (Ogura et al., 2000). Galantamine had the least effect on ChE inhibition due to its lower potency (Geerts et al., 2005). In the old rats, ChE inhibition showed a trend towards enhancement in plasma and brain tissues with some significant differences reported in magnitudes of < 20%. Concentrations of the drugs in blood and brain from the same rats were mostly maintained with age aside from donepezil, which was present at 1.8-fold higher concentrations in brain of the old rats. In addition, blood:brain ratios remained unchanged between age groups indicating a maintenance of blood-brain-
barrier integrity with age. A trend of increased blood and brain concentrations of the ChEIs observed in the old rats however, makes it difficult to rule out pharmacokinetic changes as a factor for the slightly enhanced pharmacodynamic response seen.

Interestingly, prior investigations in F344 rats revealed equivalent plasma but lower brain ChE activity levels compared to LH rats (F344: 5-8 vs. LH: 12-15 µmol/min/g). Furthermore, donepezil (2.5 mg/kg) inhibited total ChE activity by 40% 1h-postdose in F344 rats and enhancement of ChE inhibition by ~30% was seen in old vs. young F344 rats (Kosasa et al. 1999). Donepezil concentrations in brain and plasma were 3 to 4-fold higher in the old F344 rats accompanied by increased brain:plasma ratios. These findings demonstrate that differences in ageing physiology can occur between strains; F344 rats for example undergo CPN which could lead to lower levels of drug clearance. This exacerbates concerns regarding the overuse of the F344 strain for ageing studies (Weindruch and Masoro, 1990).

Behaviours such as tremor, hypothermia, lacrimation and salivation induced by tacrine administration result from an over-stimulation of mAChRs by ACh accumulation. Prior pharmacological studies using centrally and peripherally-acting mAChR agonists and antagonists have established that tremor and hypothermia are centrally-mediated effects while salivation and lacrimation are mediated in the peripheral nervous system (Dronfield et al., 2000). The old LH rats exhibited 1) a trend of increased baseline cholinergic responses with consistent hypothermia and presence of subtle limb tremor, 2) enhanced peripherally-mediated cholinergic behaviour (salivation, lacrimation) and less sensitive centrally-mediated cholinergic behaviour (tremor, hypothermia) in terms of response time and magnitude of effect during the initial 2 h post-dose with tacrine and 3) prolonged cholinergic response with protracted return back to baseline in the older animals following dosing.

Observations 2 and 3 may be ascribed to pharmacokinetics but prior pharmacokinetic p.o. profiles of tacrine revealed similar T_{max} (0.5-1 h) in both age groups with blood
concentrations in old rats returning to similar levels as the young rats after 6 h. A disparity in
dose levels in cholinergic behaviour studies compared with p.o. profiles (10 and 30 mg/kg vs.
3 mg/kg) could be a possible source of contention as a higher dose can overwhelm
clearance mechanisms, leading to greater tacrine accumulation in the bloodstream.
Nevertheless, Pedigo et al. (1984) observed that intracerebroventricular injection of
oxotremorine (muscarinic agonist) also produced an age-related hypothermic sensitivity,
suggesting other mechanisms may be involved.

The decrease in M2 mAChR expression detected in aged LH rat brain may play a role
in the altered pharmacodynamics of tacrine observed in the old rats. From previous knock-
out studies in mice, the M2 mAChR subtype was identified to be critical for mediating fine
whole body tremors and hypothermia (Bymaster et al., 2003). Its age-related decline in
expression may therefore account for the slower manifestation and slightly smaller
magnitude of central responses in old rats compared to young rats (observation 2).
Conversely, M2 mAChRs also act as autoreceptors inhibiting ACh release in the
hippocampus and cortex, its down-regulation could therefore contribute to observation 1 and
3 which manifest at high ACh levels. Interestingly, lower baseline body temperatures have
similarly been reported in the elderly, attributed mostly to impaired homeostatic mechanisms
(Sund-Levander, 2009).

Currently, determining the aged pharmacological response of a drug is achieved
through controlled clinical trials prior to it entering the market. The underlying problem with
this is that a general under-representation of the elderly often occurs. For cancer drugs in
particular, it was reported that only 22-36% of patients 65 years and older took part in clinical
trials although they represent nearly 60% of the population with cancer (Lewis et al., 2003).
Aside from exclusion criteria based on the presence of comorbidities and concurrent drug
intake, other factors involving logistic and financial impositions, poor compliance, lack of
social support and wide-spread ageist behaviours of physicians who associate older age with
poorer outcomes, act as deterrents for the recruitment of elderly into clinical trials (Siu, 2007). The lack of appropriate evidence-based profiling of drug responses in the elderly consequently contributes to an increased incidence of adverse drug events or a dampened efficacious response among the aged once the drug enters the market, especially so for drugs with a narrow therapeutic range.

The aged LH rat model therefore provides a useful alternative for studying age-related changes in pharmacokinetics and pharmacodynamics. Further testing with drugs of varying pharmacological properties however would be required to ascertain its translational potential.
Authorship Contribution

Participated in research design: Goh, Browne and Chen.

Conducted experiments: Goh, Chiu and Lee.

Performed data analysis: Goh.

Wrote or contributed to the writing of the manuscript: Goh, Browne and Chen.
References


Footnotes

a) This work was supported by GlaxoSmithKline, R&D China.

b) This work was part of an M.Sc (Pharmacology) thesis submitted to the National University of Singapore in 2009 titled “Investigating the influence of age on pharmacokinetic and pharmacodynamic characteristics of gold standard Alzheimer’s disease drugs in the Lister-hooded rat.” Author: Catherine. W. Goh

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Email: catgoh@live.com
Legends for figures

FIG. 1. Blood concentration-time profiles of donepezil, tacrine and galantamine dosed i.v. in a 1 h infusion (A, B and C) and p.o. (D, E and F) in young (●) and old (△) LH rats. Points represent mean ± S.D., n=3 except donepezil old group, n=5 and galantamine i.v.-dosed young group, n=2. Repeated measure ANOVA was performed and planned comparisons between young and old groups reported significance (p < 0.05) at certain time-points (*).

FIG. 2. Total cholinesterase (ChE), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in plasma (A) and brain (B) of young and old LH rats. Data expressed as mean ± S.D., n=3. ** p < 0.01, *** p < 0.001.

FIG. 3. Total cholinesterase (ChE), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition in plasma (A) and brain (B) 1 h after oral dosing with donepezil, tacrine and galantamine represented as % activity of vehicle-dosed rats. Values are mean ± S.D., n=3. † p < 0.05 vs. respective control (Dunnet post-hoc test) and * p < 0.05 vs. the corresponding young group (independent t-test).

FIG. 4. Cholinergic-mediated behaviours induced by tacrine 6 h following oral dosing at 10 mg/kg and 30 mg/kg in young (●) and old (△) LH rats. Dashed lines show response of vehicle-dosed rats. A, tremor. B, rectal temperature. C, lacrimation. D, salivation. Lacrimation scores of vehicle-dosed rats were zero and therefore not shown. Data represented as mean and above S.D. bars (n=6, n=12 for vehicle groups).

FIG. 5. Muscarinic acetylcholine receptor subtype expression in young and old LH rat brain. A, representative western blots of M1-M4 receptor subtypes from four old and four young
rats. B, graphical representation of normalized expression. Data represented as mean ± S.D., n=6. *p < 0.05 vs. young group (independent t-test).
<table>
<thead>
<tr>
<th></th>
<th>Donepezil</th>
<th>Tacrine</th>
<th>Galantamine</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRM transition</td>
<td>380.10 → 91.00</td>
<td>198.92 → 170.31</td>
<td>288.04 → 213.30</td>
<td>339.18 → 257.20</td>
</tr>
<tr>
<td>Declustering potential (V)</td>
<td>86</td>
<td>81</td>
<td>56</td>
<td>86</td>
</tr>
<tr>
<td>Collision energy (V)</td>
<td>63</td>
<td>143</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>Collision exit potential (V)</td>
<td>8</td>
<td>16</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>HPLC conditions</td>
<td>Casette analysis</td>
<td>Casette analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(donepezil+tacrine+I.S.)</td>
<td>Discovery® Cyano HPLC column</td>
<td>Hypurity C18 column (5cm x 4.6mm, particle size 5μm, Thermo Scientific, MA, USA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5cm x 4.6mm, particle size 5μm, Supelco, Sigma Aldrich, MO, USA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient elution</td>
<td>1 → 90% B (0-1.2min),</td>
<td>1 → 90% B (0-1.2min),</td>
<td>90% B (1.2-1.6min),</td>
<td>90% B (1.2-1.6min),</td>
</tr>
<tr>
<td>conditions</td>
<td>90% B (1.2-1.6min),</td>
<td></td>
<td>1% B (1.61-2min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1% B (1.61-2min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection volume (ul)</td>
<td>3</td>
<td></td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

MRM, multi-reaction monitoring.

B, organic mobile phase, ACN.
**TABLE 2**

*Grading scale for cholinergic-mediated behaviours of tremor and lacrimation*

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tremor</strong></td>
<td>no tremor</td>
<td>obvious tremor in two limbs or slight tremor in four limbs</td>
<td>obvious tremor in four limbs</td>
<td>severe tremor in four limbs (shaking movements)</td>
</tr>
<tr>
<td><strong>Lacrimation</strong></td>
<td>no lacrimation</td>
<td>lacrimation in one eye</td>
<td>lacrimation in both eyes (watery but not flowing)</td>
<td>severe lacrimation in both eyes</td>
</tr>
</tbody>
</table>
**TABLE 3**

*Pharmacokinetic parameters in blood after intravenous and oral administration of donepezil, tacrine and galantamine*

Values represent mean ± S.D., n=3 except for donepezil old group (n=5). For galantamine i.v.-dosed young group (n=2), both values were shown. $T_{\text{max}}$ expressed as median and range. $F_{\text{po}}$ calculated for galantamine-dosed young rat 3 using average AUC$_{iv}$ from young rat 1 and 2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Donepezil</th>
<th>Tacrine</th>
<th>Galantamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Young</td>
</tr>
<tr>
<td>Intravenous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µM)</td>
<td>$0.32 \pm 0.04$</td>
<td>$0.30 \pm 0.02$</td>
<td>$0.70 \pm 0.06$</td>
</tr>
<tr>
<td>$C_{l}$ (ml/min/kg)</td>
<td>52 ± 7</td>
<td>43 ± 5</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>$V_{d}$ (L/kg)</td>
<td><strong>9.2 ± 0.6</strong></td>
<td><strong>11.8 ± 0.8</strong></td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td><strong>2.5 ± 0.3</strong></td>
<td><strong>3.6 ± 0.3</strong></td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>MRT (h)</td>
<td><strong>3.0 ± 0.4</strong></td>
<td><strong>4.6 ± 0.5</strong></td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>AUC$_{0-t}$/Dose (min.kg/L)</td>
<td>15.9 ± 1.7</td>
<td>16.4 ± 1.6</td>
<td>10.2 ± 1.0</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$/Dose (min.kg/L)</td>
<td>19.5 ± 2.9</td>
<td>23.7 ± 3.0</td>
<td>10.8 ± 1.0</td>
</tr>
<tr>
<td>% extrapolation</td>
<td>18 ± 3</td>
<td>30 ± 3</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Oral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µM)</td>
<td><strong>0.18 ± 0.06</strong></td>
<td><strong>0.20 ± 0.02</strong></td>
<td><strong>0.36 ± 0.13</strong></td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0 (0.5-1.5)</td>
<td>1.0 (0.5-4.1)</td>
<td>0.5 (0.5-1.0)</td>
</tr>
<tr>
<td>AUC$_{0-t}$ (µM·h)</td>
<td>0.76 ± 0.27</td>
<td>0.91 ± 0.09</td>
<td>0.70 ± 0.15</td>
</tr>
<tr>
<td>AUC/Dose (min.kg/L)</td>
<td>5.8 ± 2.1</td>
<td>6.9 ± 0.8</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>$F_{\text{po}}$ (%)</td>
<td>36 ± 10</td>
<td>43 ± 8</td>
<td>27 ± 7</td>
</tr>
</tbody>
</table>

* $p < 0.05$; ** $p < 0.01$
TABLE 4

* Blood, brain concentrations and brain:blood ratios of donepezil, tacrine and galantamine at 1 h following oral administration *

Values represent mean ± S.D.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Blood concentration (μM)</th>
<th>Corrected brain concentration (μM)</th>
<th>Brain:Blood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Young</td>
</tr>
<tr>
<td>Donepezil</td>
<td>0.13 ± 0.01</td>
<td>0.21 ± 0.11</td>
<td><strong>0.57 ± 0.09</strong></td>
</tr>
<tr>
<td>Tacrine</td>
<td>0.11 ± 0.03</td>
<td>0.19 ± 0.12</td>
<td>1.2 ± 0.30</td>
</tr>
<tr>
<td>Galantamine</td>
<td>0.42 ± 0.02</td>
<td>0.58 ± 0.21</td>
<td>0.73 ± 0.04</td>
</tr>
</tbody>
</table>

* p < 0.05
### TABLE 5

**Pharmacokinetic properties of donepezil, tacrine and galantamine in human and changes in PK parameters of the ChEIs with age in human, F344 rats and LH rats.**

<table>
<thead>
<tr>
<th></th>
<th>Pharmacokinetic parameters (human)</th>
<th>Mode of Metabolism / Excretion</th>
<th>Change in PK parameters with age?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donepezil</strong></td>
<td>F: 100%</td>
<td>Hepatic (CYP2D6/3A4):</td>
<td>Humans: AUC ↔ Brain/plasma: AUC ↔</td>
</tr>
<tr>
<td>pMW: 379.504</td>
<td></td>
<td>57% excreted in urine, 15% in faeces</td>
<td>F344 Rats: t1/2 ↑, MRT ↑</td>
</tr>
<tr>
<td>clogP: 4.60</td>
<td></td>
<td></td>
<td>LH Rats: t1/2 ↑, MRT ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tacrine</strong></td>
<td>F: 17-37%</td>
<td>Hepatic (CYP1A2):</td>
<td>Humans: ↔ Brain/plasma: ↔</td>
</tr>
<tr>
<td>pMW: 198.270</td>
<td></td>
<td></td>
<td>F344 Rats: concentration ↑</td>
</tr>
<tr>
<td>clogP: 3.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Galantamine</strong></td>
<td>F: 85-100%</td>
<td>Hepatic (CYP2D6/3A4):</td>
<td>Humans: AUC ↔ No data: AUC ↔</td>
</tr>
<tr>
<td>pMW: 287.362</td>
<td></td>
<td></td>
<td>F344 Rats: Cmax ↑ available</td>
</tr>
<tr>
<td>clogP: 1.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Figure 1

(A) Donepezil (i.v.)

(B) Tacrine (i.v.)

(C) Galantamine (i.v.)

(D) Donepezil (p.o.)

(E) Tacrine (p.o.)

(F) Galantamine (p.o.)

Blood concentration vs. Time (h) for different drugs and administrations.
Figure 3

A

ChE Inhibition (Plasma)

Total ChE

AChE

BChE

% Vehicle

Young

Old

Vehicle

Donepezil

Tacrine

Galantamine

Vehicle

Donepezil

Tacrine

Galantamine

Vehicle

Donepezil

Tacrine

Galantamine

B

ChE Inhibition (Brain)

Total ChE

AChE

BChE

% Vehicle

Young

Old

Vehicle

Donepezil

Tacrine

Galantamine

Vehicle

Donepezil

Tacrine

Galantamine

Vehicle

Donepezil

Tacrine

Galantamine
Figure 5

A

B

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