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FK962 and donepezil act synergistically to improve cognition in rats: Potential as an add-on therapy for Alzheimer's disease☆,☆☆

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ABSTRACT

FK962 is a member of a novel class of compounds that promote somatostatin production in the brain, and is being developed as a treatment for patients with Alzheimer's disease. As acetylcholinesterase inhibitors such as Aricept© (donepezil) are widely used to treat these patients, it is important to confirm that potential new medicines in this disease area can be co-administered with drugs such as Aricept. To study the effect of FK962 in combination with donepezil, touchscreen methodology was used to measure the effect on cognition in rats. Doses of FK962 and donepezil were identified that resulted in minimal cognition enhancement when given separately. There was strong evidence ($p = 0.002$) of a treatment difference between the combination of FK962/donepezil and FK962 alone: the estimated treatment difference is 5.47 (95% CI: 2.19–8.75). There was also evidence ($p = 0.017$) of a treatment difference between the combination of FK962/donepezil and donepezil alone: the estimated treatment difference is 4.01 (95% CI: 0.77–7.26). Therefore, a combination of low doses of FK962 and donepezil showed a significantly greater effect on cognition than low doses of either compound alone. This is the first time that FK962 has shown activity in a reward-based model of cognition. In addition, these data suggest that this compound could beneficially be given in addition to Aricept to treat Alzheimer's disease patients.

1. Introduction

Somatostatin (SST) has been implicated in Alzheimer's disease (AD) pathology and aetiology for a number of years. Levels of SST are depressed in the frontal cortex region of the brain of AD patients, and the degree of the deficit correlates with the cognitive decline (Viollet et al., 2008). In addition, polymorphisms in the SST gene are associated with the risk of developing AD, a finding that has been observed in two different populations (Vepsäläinen et al., 2007; Xue et al., 2009). It has also been shown that infusion of SST can improve cognitive performance in animal models (Ohno et al., 1994) and in humans (Craft et al., 1999), although this latter finding is somewhat controversial. Finally, recent research has shown that SST promotes the release of neprilysin, a protease that degrades β-amyloid peptides in the brain: age-related decreases in neprilysin activity have been proposed as one of the events that could trigger development of AD in older subjects (Saito et al., 2005).

This body of evidence supports a role for SST in AD aetiology; therefore, SST biology provides opportunities for novel treatments of AD. FK962 is a compound with a novel mechanism of action that promotes the release of SST in the brain, and improves cognitive performance in a number of animal models (Tokita et al., 2005). The compound is undergoing clinical development for treating the cognitive deficits in AD patients, although an initial small clinical study in AD patients did not establish efficacy. Mild to moderate AD patients are usually treated with Aricept© (donepezil), an acetylcholinesterase inhibitor which gives symptomatic improvement, although the underlying disease progression is unaffected (Ibach and Haen, 2004). Therefore, it is important to examine the effects of combining FK962 with donepezil. Another member of the same class of compounds as FK962 (called FK960) had previously shown good evidence for additivity with cholinesterase inhibitors in pre-clinical testing (Tokita et al., 2002); however, these findings need to be replicated with FK962 to support its use in any clinical studies in combination with Aricept.

Previous pre-clinical testing of FK962 has used conventional cognition models based on avoidance of aversive stimuli, such as

Abbreviations: SST, Somatostatin; AD, Alzheimer's disease; ITI, Inter-trial interval.

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passive avoidance tests or the Morris swim task. Understanding of cognition and the deficits in Alzheimer's has progressed significantly in recent years, leading to the development of improved methods of assessing cognitive behaviour in pre-clinical models, with greater relevance to human disease. These new assessments are more representative of cognitive deficits seen in patients; in many cases, virtually identical paradigms and methodologies in the animal model and the human participant can be used. One of these new approaches was used for this current study, to expand the pre-clinical models in which FK962 has been tested to encompass these reward-based systems. The cognitive testing in the present study utilised touchscreen methodology (Bussey et al., 1994). This method has a number of advantages compared with conventional methods: animals can be tested on tasks very similar to tasks used to test human populations (see for example Robbins et al., 1994); the tasks are appetitive (food reward) rather than aversive, thus minimising confounding effects due to pain and stress (although other confounders may affect food response); and the automated nature of the method minimises experimenter contact with the animals during testing. Although some concerns have been raised about this paradigm, for example the possibility of suboptimal learning rates (Minini and Jeffery, 2006), such concerns are not relevant to the optimized version of the method used here.

In this study, the effect of FK962 – with or without donepezil – on the cognitive behaviour of rats was tested using a touchscreen-based visual discrimination method. Touchscreen-based visual discriminations have recently been shown to be impaired in animals with blockade of either NMDA or muscarinic receptors in perirhinal cortex (Winters et al., 2010), one of the first regions to deteriorate in AD, and therefore is highly appropriate for testing of potential treatments for AD.

2. Methods

2.1. Subjects

The subjects were male Lister Hooded rats (Harlan Olac, Bicester, UK), weighing approximately 270–300 g at the start of testing: group sizes of 16 were used. The rats were housed on a reversed 12 h light/12 h dark cycle (lights on 19:00), in groups of four. All behavioural testing was conducted during the dark phase of the cycle. Rats were food deprived to 85–90% of their free feeding weight throughout, while water remained available ad libitum. All experimentation was conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.2. Apparatus

Animals were trained using an automated touchscreen apparatus as previously described (Talpos et al., 2009; Winters et al., 2010). Briefly, the apparatus consisted of an operant chamber (Med Associates, Vermont, USA; height = 23 cm, width = 30 cm, depth = 25 cm) with clear Perspex walls and a metal frame, and a floor consisting of metal bars spaced 1 cm apart. The floor of the operant chamber was mounted around 3 cm above a waste receptacle lined with paper inserts. A touch-sensitive liquid crystal display monitor was mounted at one end of the chamber. The monitor was covered by a black Perspex 'mask' (38 × 30 cm) which had 2 open response windows (height = 15.2 cm, width = 9.3 cm, spaced 2.5 cm apart) in which the stimuli were displayed. The mask was attached to the screen leaving around a 0.5 cm gap so that it would not trigger the touchscreen. A spring-hinged 'shelf' (6 × 20.5 cm) was attached 15 cm above the grid floor. This shelf was at a 90° angle to the mask and was designed to encourage the rats to pause, rear up and look at the stimuli before responding.

A food magazine, served by a pellet dispenser and equipped with a light and infrared beam to detect rats' head entries into the magazine, was located on the wall opposite to the touchscreen. A house light (3 W) and a tone generator were situated above the food magazine. Each setup was placed inside a sound-attenuating chamber and ventilated by a small fan. Each box and touchscreen computer screen was controlled by an IBM computer running custom software written in Visual Basic 6.0 (Microsoft Corp., Redmond, Washington, USA).

2.3. Behavioural pre-training

At the start of training, animals were given one session of combined habituation and Pavlovian training. In this session, training stimuli (40 stimuli varying in brightness, shape, and pattern) were displayed on the screen in any position, one at a time, for a period of 30 s. Food pellets (0.045 g Purified Rodent Tablet, Testdiet, Richmond, Indiana, USA) were dispensed automatically following stimulus offset, accompanied by a tone and illumination of the magazine light. However, if during stimulus display the animal made a nose-poke in the response window in which the stimulus was displayed then three pellets were dispensed. This session ended when the rat had received 100 pellets or when 60 min had elapsed, whichever came first.

After this session, there were three stages of training. In the first stage, on a given trial, one training stimulus was displayed on the screen in any position. If the rat responded to the stimulus by nose-poking it directly on the touchscreen, it received a food pellet accompanied by the light and tone; there were no consequences for responding to any other location. The inter-trial interval (ITI) was 20 s. Animals were required to complete 100 trials in 60 min to move to the next stage.

The second stage was the same as the first stage except that animals were required to initiate trials by nose-poking the illuminated magazine. Initiation became available after an ITI of 20 s. Again the criterion was completion of 100 trials in 60 min.

The third stage was the same as the second stage, with the exception that touching anywhere on the screen other than the stimulus triggered a time-out period in which the house light was extinguished for 5 s. Touching the stimulus was scored as correct; responding elsewhere was scored as incorrect. In this stage, the animals were required to get 95 correct out of 100 trials within 45 min. As soon as animals reached criterion on this stage of pre-training, they moved onto two-choice visual discrimination training.

2.4. Two-choice visual discrimination training

Once the rats had completed pre-training, they were then trained on a two-choice visual discrimination (Bussey et al., 1997; Winters et al., 2010). Each session consisted of a maximum of 100 trials, or as many trials as were completed in 60 min. The ITI was 20 s, and rats were required to initiate each trial by responding at the magazine, as in the pre-training. Following trial initiation, a pair of stimuli would appear on the screen, one in each of the two response areas defined by the mask; one stimulus was the correct S+ and the other the incorrect S−. A nose-poke to the S+ resulted in a tone, magazine light, and a reward pellet. Incorrect responses resulted in a 5 s time-out period followed by the start of the correction procedure, intended to prevent the development of side biases (see Bussey et al., 1997). The correction procedure consisted of the same stimulus configuration being presented continuously on successive trials until the animal made a correct response. Responses on correction trials were not counted in the overall percent correct score.

The S+ and S− were simple white shapes on a black background. Rats had previously been shown to have no inherent bias for this particular stimulus set, identified as "spider" and "plane" (stimulus set 2 from Bussey et al., 2008; see Fig. 1, panels a and b). Both discriminative stimuli were presented simultaneously on each trial. The left–right arrangement of the stimuli was determined pseudo-randomly across trials, with a constraint that a given stimulus could

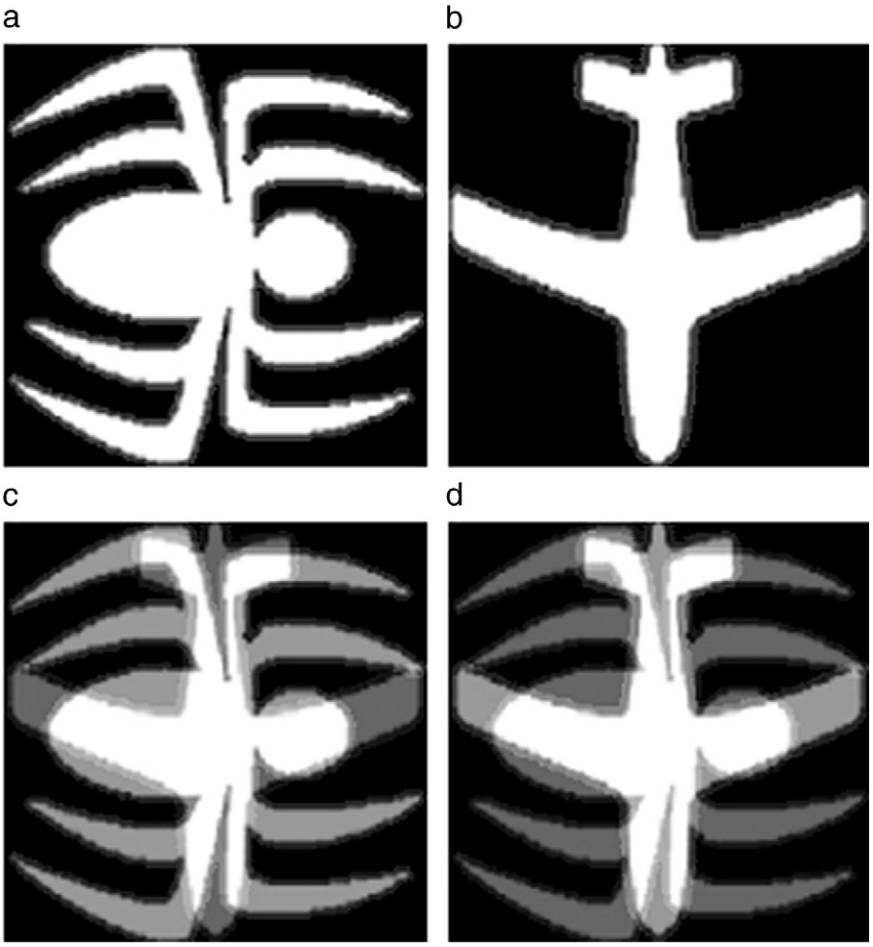

Fig. 1. Stimuli used in the visual discrimination. a) "spider" and b) "plane," training stimuli; c)"spider morph" and d) "plane morph," testing stimuli.

not appear on the same side of the screen on more than three consecutive trials. These stimuli were novel to the animals at the start of two-choice visual discrimination training and did not resemble any of the stimuli used in the pre-training stages. The experiment was counterbalanced such that half of the rats in each group received one stimulus in the pair as the S+, and the other half received the other stimulus as the S+. Animals were trained until they were stably proficient in this task: at this time, the animals were achieving approximately 90–95% correct responses.

2.5. Two-choice "morph" visual discrimination testing

Once stable performance had been achieved on the two-choice visual discrimination training, animals were moved onto the testing stimuli. The test stimuli were "morphed" (blended) versions of the training stimuli (see Fig. 1 panels c and d), "plane morph" containing 60% plane and 40% spider, and "spider morph" containing 60% spider and 40% plane. The rats were tested for at least 5 trials to ensure stable performance. Under these conditions, the animals achieved a lower correct response rate of approximately 65–75% correct responses, which reduced the effect of potential ceiling effects on performance enhancement. During drug dosing, animals performed 100 trials, or as many as they could within a 60 min time limit.

2.6. Compound and dosing

After training, each animal was tested for each compound and dose (including vehicle) using a Latin square design. Animals were dosed between 30 and 60 min before testing, and there was at least 48 h (>6 half-lives: Matsui et al., 1999; Astellas Pharma Inc., unpublished information) between successive compound testing days. All compound dosing was intraperitoneal, and the compounds were dissolved in saline solution.

To study the effect of a combination of drugs on cognition enhancement, it is important to use low doses of the two compounds to avoid problems due to ceiling effects in the experimental models if the compounds are showing strong efficacy when dosed separately. Therefore, before testing the effect of a combination of FK962 and donepezil, different doses of each compound were examined to determine doses showing barely detectable activity for each compound: these doses were then taken forward to a combination experiment. Combined dosing of the two compounds was undertaken to look for additivity of effect.

2.7. Data analysis

For each compound, three doses, namely 0.1, 0.3 and 1 mg/kg, were compared to vehicle and suitable doses were chosen by visual inspection of dot plots. In the combination testing, treatment differences were estimated using the basic estimators approach (Senn and Hildebrand, 1991). For each contrast of interest, crossover differences (basic estimators) were calculated and means determined for each treatment sequence. It was assumed for each rat, that the crossover difference reflects three elements: 1) the treatment contrast being estimated, 2) a difference between periods, which depends on sequence, and 3) random variation. By averaging estimators within a sequence group, the relative importance of the random element was reduced while preserving the treatment and period effects. Under the

assumptions of additive period effects, no treatment by period interaction and balanced sequences, taking the mean over the four sequences eliminates the effect of period differences. It is noted that in the current study, the wash-out period was greater than six half-lives, minimising any effect of carry-over, and balanced sequences were provided by the Latin Square design.

The estimation of treatment differences by the basic estimators approach was achieved through the application of ordinary least squares, in which the basic estimator is regressed on sequence. The variance of the estimated treatment effect was obtained by adjusting for the counts of rats in each sequence and the number of sequence groups. To correct for multiple testing, the Sidak step-down method of adjustment was used. The descriptive plots, tabulation and the estimation analysis were all conducted using R v2.7.2 (R Development Core Team, 2008).

3. Results

To achieve accuracy levels appropriate for studying enhancement of cognition, the standard test symbols were "morphed" (blended) to make the stimuli harder to differentiate. Under these conditions, the animals could achieve accuracy levels of 65–75%. Group sizes of 16 rats were used for all experiments: for each study, results were obtained for at least 15 animals. There was some weak evidence for a ceiling effect, as defined by a negative correlation between baseline performance and treatment difference. Upon closer inspection, however, the effect was driven by a single rat with low baseline performance and large improvement upon taking combination (data not shown).

Compound testing took place in two stages. The first stage tested the effect of FK962 (at different dose levels) and a single concentration of donepezil as a control. Based on results in other cognition models, FK962 was given at doses of 0.1, 0.3 or 1.0 mg/kg (Tokita et al., 2005), and donepezil was given as a single dose of 1 mg/kg (Ogura et al., 2000). On the basis of the results (Fig. 2), a dose level of 1.0 mg was selected to yield efficacy for the experimental compound albeit at a level lower than that of donepezil (1.0 mg/kg). In the second stage, to select an appropriate dose of donepezil for the combination study, lower doses were studied in a separate experiment (Fig. 3). Based on these data, a dose of 0.3 mg/kg donepezil was selected as having minimal detectable effect and thus offering protection against ceiling effects.

The selected doses of FK962 and donepezil (1 and 0.3 mg/kg, respectively) were further tested singly and in combination. This study was carried out in two separate groups of rats ($n=16$ per group) trained in the visual discrimination task. The results of the two separate studies were essentially the same: the pooled data from the two studies are shown in Fig. 4.

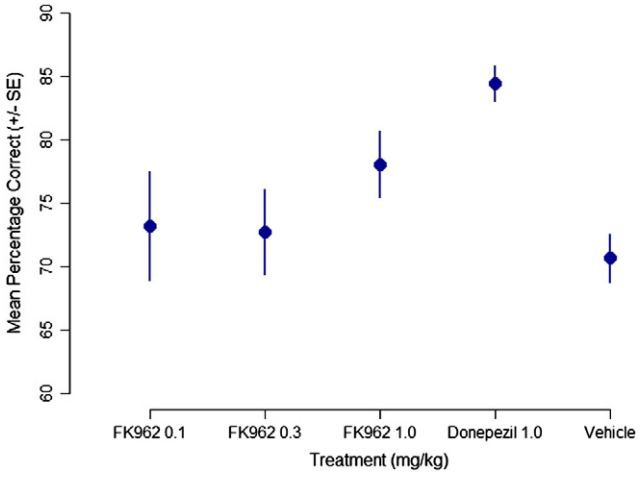

Fig. 2. Effect of FK962 or donepezil on visual discrimination accuracy. Dot plot showing the effect of different doses (in mg/kg) of FK962 and of donepezil on visual discrimination.

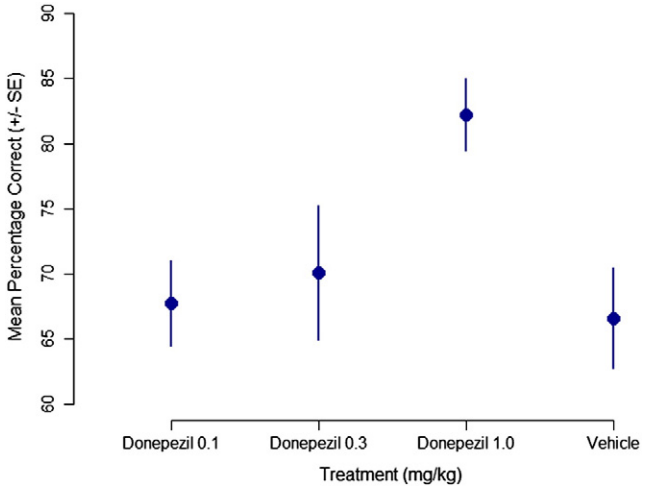

Fig. 3. Effect of different doses of donepezil on visual discrimination accuracy. Dot plot showing the effect of different doses (in mg/kg) of donepezil on visual discrimination.

Using the basic estimators approach, the combination showed significantly greater efficacy than either FK962 (raw $p=0.002$: estimated treatment difference is 5.47; 95% CI: 2.19–8.75) or donepezil alone (raw $p=0.017$: estimated treatment difference is 4.01; 95% CI: 0.77–7.26). As three comparisons were made during the statistical analysis, correction for multiple testing using the Sidak step-down method gave adjusted $p=0.006$ for the combination vs. FK962, and adjusted $p=0.034$ for the combination vs. donepezil alone.

4. Discussion

This study tested the ability of low doses of FK962 to improve cognition in young male rats in the presence of a low dose of donepezil. Acquisition of the touchscreen visual discrimination used in the present study has been shown to be dependent on cholinergic and glutamatergic function within the perirhinal cortex (Winters et al., 2010). This part of the brain, which is known to be involved in visual memory in rats (Prusky et al., 2004; for review see Winters et al., 2008) and primates (Meunier et al., 1993; Davachi and Goldman-Rakic,

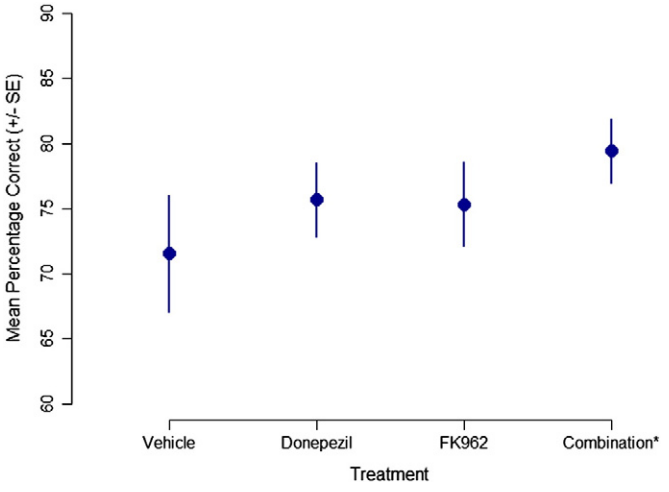

Fig. 4. Effect of FK962, donepezil (singly or combined) on visual discrimination in rats. Dot plot showing the effect of a combination of FK962 and donepezil on visual discrimination. The doses used were as follows: FK962, 1 mg/kg; donepezil, 0.3 mg/kg. $^{*}p<0.05$ for the combination compared to either drug on its own.

2001), is also affected in very early stages of AD (Braak and Braak, 1991). Indeed, defects in visual memory can be detected in subjects with mild cognitive impairment many years before overt cognitive symptoms are apparent (Lu et al., 2005).

Studies such as this one which look for enhancement of cognitive performance require the animals to be achieving a defined range of scores, particularly in two-choice tasks such as that used here, where chance will produce a 50% correct score. If the scores are too low (i.e. near 50%), they are not different from chance, with the risk of poor sensitivity. However, if the scores are too high (e.g. >75% correct), there is little opportunity for enhancement to be observed due to ceiling effects. In this study, animals were trained, and task parameters varied (by "morphing" the stimuli) until their baseline performance was in the range 65–75% correct choices. Similarly, if the compounds are dosed at too high a dose exhibiting significant activity in their own right, then there is little opportunity for a combination of the two drugs to show enhanced activity.

A combination of the two compounds was significantly more effective than the individual compounds dosed at the selected concentrations when dosed alone. While there are no published reports to indicate why a SST modulator and an acetylcholinesterase inhibitor should complement each other, there are a number of suggestions in the literature that SST and acetylcholine pathways interact. For example, Rakovska et al. (2002, 2003) showed that SST promotes release of acetylcholine from striatal cells. Based on these observations, it is possible to speculate that the SST release induced by FK962 enhances acetylcholine release, an effect that would be potentiated in the presence of cholinesterase inhibitors.

It should be noted that the focus of this study was on establishing the efficacy of the two compounds in combination, requiring testing of the compounds at doses with low or minimal activity. Therefore, the top end of the dose response has not been explored for either compound, so it is not known if the two compounds have comparable maximal activity in this task, or if the combination would have a greater maximal activity than either compound alone. Also, while a clear positive interaction was seen between FK962 and donepezil in the visual discrimination task, further work would be needed to define the exact nature of the interaction between the two compounds.

These data show that FK962 is able to combine positively with donepezil to enhance cognitive performance in normal animals. This is the first time that FK962 has shown activity in this type of reward-based model of cognitive behaviour, and is an important addition to the existing data which show that the compound improves performance in aversive tests such as passive avoidance tasks and the Morris swim task (Tokita et al., 2005). Finally, these data provide compelling evidence that an adjunct clinical protocol, where FK962 is dosed with Aricept, is a promising study design to explore the activity of FK962 in Alzheimer's patients.

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