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LONG-TERM inhibition of dipeptidyl peptidase-4 in Alzheimer’s prone mice

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Running title: DDP-4 inhibition and Abeta pathology in mice

Key Words: Alzheimer’s, mice, dipeptidyl peptidase-4, sitagliptin, beta-amyloid, interleukin-1β
Abstract

We tested here the impact of a long-term inhibition of dipeptidyl peptidase-4 (DPP-4) with sitagliptin on the deposition of amyloid beta within the brain and deficits in memory-related behavioral paradigms in a model of Alzheimer’s disease (AD): double transgenic mice B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J. Mice began to receive sitagliptin at 7 months of age. Three different dose of sitagliptin (5, 10 20 mg/kg), were administered daily for 12 weeks by gastric gavage.

The treatments counteracted: i) the memory impairment in the contextual fear conditioning test; ii) increased the brain levels of GLP-1; iii) produced significant reductions of nitrosative stress and inflammation hallmarks within the brain, as well as iv) a significant diminution in the ultimate number and total area of βAPP and Aβ deposits. All these effects much more evident for the dose of 20 mg/kg sitagliptin. The long-term inhibition of the endogenous DPP-4 enzymes with sitagliptin can significantly delay some forms of AD pathology, including amyloid deposition, when administered early in the disease course of a transgenic mouse model of AD.
1. Introduction

AD is a complex neurodegenerative disorder, with aging, genetic and environmental factors contributing to its development and progression. The complexity of AD presents substantial challenges for the development of new therapeutic agents. AD is typified by pathological depositions of beta-amyloid peptides and neurofibrillary tangles within the diseased brain (Jiang et al., 2008). It has also been demonstrated to be associated with a significant microglia-mediated inflammatory component, dysregulated lipid homeostasis and regional deficits in glucose metabolism within the brain. AD also shares some common pathophysiological hallmarks, such as amyloid beta (Abeta), phosphorilation of tau protein, and glycogen synthase kinase-3 with type 2 diabetes mellitus (Li, 2007), and thus some drugs acting in diabetes to reduce the incidence of the adverse action of these mediators also promise to be useful in the prevention/reduction of Alzheimer’s. This is the case for example of the incretin Glucagon-like peptide-1 (GLP-1). In patients with type 2 diabetes GLP-1 increases pancreatic islet beta-cell proliferation and glucose-dependent insulin secretion; it lowers blood glucose and food intake. GLP-1 has cAMP coupled receptors within the brain of rodents and humans, thus, suggesting a central role for the endogenous peptide in the regulation of proper neuronal function as it is plasticity, cell survival, neurite outgrowth and protection against excitotoxic cell death and oxidative injury (Perry and Greig, 2002). Moreover, from experimental studies GLP-1 was shown to reduce endogenous levels of amyloid-beta peptide (Abeta) in mouse brain and to reduce levels of beta-amyloid precursor protein (betaAPP) in neurons (Perry and Greig, 2003) overall these data suggesting that GLP-1 peptide may beneficially affect a number of therapeutic targets associated with AD.

Unfortunately, GLP-1 peptide usually have short half-life in presence of functionally intact endogenous DPP-4 enzymes. These enzymes usually metabolize GLP-1 within minutes of release to inactive metabolites (Drucker, 2003; Hansen et al., 1999) resulting in very short half-life of intact hormone (about 2 minutes) (Drucker, 2003; Vilsboll et al., 2003) with the consequence that the
small amounts of active GLP-1 that survive this initial cleavage act on receptor sites producing the proper responses of the peptide (Drucker, 2006; Meier and Nauck, 2004). By blocking the activity of the endogenous DPP-4 enzymes one would stabilize the levels of the bioactive GLP-1 (Brown et al., 2004; Knecht et al., 2006) and in theory its effects. Based on this contention, and although much remains to be elucidated with regards to the downstream signaling pathways involved in the properties of GLP-1, we have investigated here an alternative strategy to prevent/treat AD. We aimed to increase the bioactivity of endogenous GLP-1 with a chronic orally administered specific DPP-4 inhibitor, and investigate the effects of this continuous activation of the endogenous incretin axis on the deposition of Aβ deposits within the brain and deficits in memory-related behavioural paradigms typical of AD.

Among the DPP-4 inhibitors we selected sitagliptin recently approved for the treatment of type 2 diabetes with a glucose-lowering effect best obtained after long-term treatment (Lamont and Drucker, 2008). Sitagliptin (Januvia®, Merck Pharmaceuticals) is effective in lowering HbA1c, and fasting as well as postprandial glucose in monotherapy and in combination with other oral antidiabetic agents. It stimulates insulin secretion when hyperglycemia is present and inhibits glucagon secretion (Gallwitz, 2007). Recent studies underline that the continuous administration of des-fluoro-sitagliptin in the food likely achieves optimal GLP-1 control by a potent and sustained 24-h inhibition of DPP-4 activity especially relative to the twice-daily administration of others DPP-4 inhibitors (Lamont and Drucker, 2008; Larsen et al., 2001, Raun et.al., 2007,) with an end-point being the optimal stimulation of incretin axis and incretin activities.

2. Methods

2.1 Animals

The animals used were the double transgenic mice B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J from the Jackson Laboratory (Bar Harbor, USA). These transgenic mice express a chimeric
mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) both directed to CNS neurons. Both mutations are associated with early-onset (usually 6-7 months) Alzheimer's disease. By nine months of age, histological examination of brain tissue reveals numerous amyloid deposits within the hippocampus and cortex (Jankowsky et al., 2004) resembling those observed in the brains of patients with AD. The number of amyloid deposits increases dramatically between the ages of 10 and 12 months, with an accompanying profound inflammatory component being apparent. Mice (B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J, and lean littermates; n = 6 per group) of 7 months of age were used for experiments. They were fed with Purina 5002 rodent chow and drinking water. Sitagliptin was dissolved in normal saline for p.o. administration (Beconi et al., 2007). Vehicle (saline) and sitagliptin, at three different doses chosen in the therapeutic range of 11 mg/kg likely being 5, 10, 20 mg/kg, were daily administered by gastric gavage in a 100 microliter volume/10 g body weight. On completion of the study (after 12 weeks of drug treatment) mice were killed by cervical dislocation 2 h after their final morning sitagliptin or vehicle administration and the glucose assay repeated.

In a separate set of experiments exendin (9-39) was also tested. It was continuously administered through Alzet micropumps (Alza, Palo Alto, CA) delivering a rate of 2 pmol/kg/min (Cani et al., 2006) or vehicle (0.9% NaCl) for 12 weeks starting at 7 months of age. Animal care and handling was performed according to the local ethics committees.

2.2 Tissue preparation

At the time of sacrifice, the mice were transcardially perfused with heparinized sodium chloride (0.9%). The brains were removed and brain regions were dissected from one hemisphere. One hemisphere was placed into an acryl brain block lined up with the interaural line at 0 mm. Subsequently, coronal slices were cut to obtain cortical, hippocampal, cerebellar and brainstem sections, which were dissected, snap-frozen in liquid nitrogen and stored at –80°C until further
analysis. The remaining brain hemisphere was either fixed in 4% paraformaldehyde followed by paraffin embedding according to standard protocols and sectioning for immunohistochemistry.

2.3 Immunohistochemistry

Serial sagittal sections of paraffin-embedded brain tissues were cut (7 µm thick, Leica microtome 2155 or Leica Cryostat CM3050S) and mounted (poly-L-lysine-coated slides, Histobond adhesion slides, Marienfeld, Germany). Retrieval of antigen sites, blocking of endogenous peroxidase activity and blocking of non-specific binding sites were performed according to standard protocols. After washing in phosphate-buffered saline (PBS), sections were incubated overnight at 4°C with primary antibodies. The antibodies used were: (i) rabbit pAb against Aβ1–42 (Biosource International, Inc., USA); and anti-β-APP (Santa Cruz); anti-IL-1β (Santa Cruz); anti-nitrotyrosine (Santa Cruz).

For image analysis of brain immunostaining, serial sagittal sections of one brain hemisphere were examined. β-APP, Aβ1-42, immunostaining was evaluated on sagittal brain sections of six animals from each group. For each animal, antigens were detected in 10 parallel sections having a defined distance of 70 µm and showing both the hippocampus and brain cortex. In each section, the hippocampus and the frontal cortex were evaluated. The total stained area (real area) and integral staining density (sum of all individual optical densities of each pixel in the area being measured) were determined and given as percentage of stained surface per region. All images were acquired using a standard light and immunofluorescence microscope (Nikon, Eclipse E-800) connected to a digital camera (Sony, model DXC-9100P, Sony, Köln, Germany) and to a PC system with LUCIA imaging software (LUCIA 32G, version 4.11; Laboratory Imaging, Düsseldorf, Germany). For each animal, average values from all sections were determined.
2.4 Brain amyloid assay

The individual hemisphere was homogenized in 6.5 volumes of ice-cold buffer containing 20 mM Tris–HCl (pH 8.5) and a cocktail of proteinase inhibitors (Roche Mannheim, Germany), using a small Potter-type mechanical homogenizer. After centrifugation (135 000 g for 1 h at 4°C), a portion of the supernatant was centrifuged again (200 000 g for 2 h at 4°C) and soluble amyloid peptides were quantified by enzyme-linked immunosorbent assay (ELISA; IBL international, Hamburg, Germany; assay range 1.56-100 pg/ml).

In another set of experiments brain GLP-1 of B6.Cg-Tg(APPswe.PSEN1dE9)85Dbo/J mice was assayed by using a commercially available ELISA kit (Linco Research, St. Charles, MO; 2pM sensitivity).

2.5 Statistical analysis

The data shown are the means ± S.D. Statistical comparisons for significance between mice with different treatments were performed using the Student’s t test and ANOVA followed by the Tukey post hoc test.

2.6 Behavioural testings

Open-field test

This test was applied as previously described (Onozuka et al., 2008). Briefly, mice were placed into the corner of a wooden box 50 X 50 X 40 cm and allowed to freely explore for 10 min. The floor of the field was divided into 25 identical squares so that the ambulation of animals could be measured. The ambulation of the mice was measured by counting the number of times that the animals crossed from one square to another. The scorer of the behavioral experiments was blind to treatment group.

Contextual fear conditioning
In the training session, mice were placed into a chamber with metal grids floor. Mice were allowed to freely explore for 2 min, and were given an electric shock (2 s, 0.7 mA) from a metal grid floor at the end of the 2 min. The 2 min/2 s shock paradigm was repeated for a total of two shocks. After the last shock, animals were allowed to explore the context for an additional 1 min prior to removal from the training chamber. The freezing time (seconds), defined as cessation of all but respiratory movement, was measured by observing the animals every 5 min. Baseline freezing was established for the first 2 min of the training session. Freezing time was also recorded during the last 3 min of the training session (training). In the test session performed 24 h after training, mice were placed into the conditioned chamber for 5 min and freezing time was measured manually (test).

3. Results

3.1 Inhibition of DPP-4 does not affect ambulation counts

Since B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice began to exhibit a number of amyloid deposits at 9 months of age (Jankowsky et al., 2004), 7 month-old transgenic mice were daily administered sitagliptin for 12 weeks. We carefully monitored the general health of the mice throughout the course of the treatment with sitagliptin and did not observe any adverse changes nor did we observe significant weight changes. After 12 weeks of the daily administration of sitagliptin we first examined the effects of sitagliptin on ambulation in the open field test. In this test, the number of crossed squares was similar between the vehicle-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J and sitagliptin-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J with no statistical significance between them (Figure 1), suggesting that treatment with sitagliptin did not affect general behaviour in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice.

3.2 Inhibition of DPP-4 with sitagliptin counteracts the memory impairment in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice
To examine the effects of sitagliptin on learning and memory, we next used the contextual fear conditioning task, in which animals learn to associate a normally innocuous context with an aversive stimulus (Kim et al., 1991, Atkins et al., 1998). In the training session, there were no significant differences in the freezing time between the group vehicle-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J and the sitagliptin-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J. In contrast, the test session performed 24 h after training showed that treatment with sitagliptin (20 mg/kg) significantly improved the memory impairments in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice (p<0.01) with the respect to vehicle-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice, by increasing the freezing behavior of the animals against the aversive stimulus (Figure 2). The freezing time was ~ 67% longer than the vehicle. Other lower doses of sitagliptin 5 and 10 mg/kg had no effect on this, with a calculated increase in percentage of freezing of ~ 15% for the dose of 10 mg/kg (Figure 2).

3.3 Long-term inhibition DPP-4 increases brain endogenous levels of GLP-1

Figure 3 shows that B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice had increased levels of the incretin GLP-1 within the brain, following treatment with sitagliptin. This increasing effect of sitagliptin was maximally present after the dose of 20 mg/kg.

3.4 DPP-4 inhibition improves βAPP and Abeta pathology in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice

All mice were sacrificed after completion of the behavioral tests, and their brains were isolated and processed for neuropathological or biochemical evaluations. To determine the effects of the long-term administration of sitagliptin on amyloid beta deposition, we immunostained the brain sections
from vehicle- and sitagliptin-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice with anti-amyloid beta antibody. At the age of 10 months, B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice expressed a considerable number of amyloid deposits throughout the cortex and hippocampus. Since the contextual fear conditioning paradigm has been previously shown to be hippocampal-dependent (Logue et al., 1997), we analyzed the βAPP and Abeta deposition in the hippocampus. Notably, daily treatment with sitagliptin (10 and 20 mg/kg) for 12 weeks reduced both βAPP and Abeta deposition in the hippocampus of B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J (Figure 4 A and B). Our analysis demonstrated, as for example, that the treatment with sitagliptin resulted in about 60% decrease in the number of neurons within the area involved in the Abeta deposition as well as the amount of immunoreactivity compared with vehicle-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J. In contrast exendin (9-39) treatment did not affect βAPP and βamyloid deposition in the brain (Figure 4A and B). It is noteworthy that exendin (9-39) is specific for the GLP-1 receptor and at the rate studied here, no nonspecific effect could be envisioned as previously described with higher rate (Knauf et al., 2005).

We next analyzed quantity of Abeta with ELISA to investigate the effects of the DPP-4 inhibitor sitagliptin on Abeta levels in the brain. Administration of sitagliptin for 12 weeks significantly decreased levels of Abeta in the brain. As for example the levels of Abeta within the hippocampus was 12±1.6 pg/mg protein in vehicle-treated mice while it was 6.2±1.1 in sitagliptin- (20 mg/kg, p<0.01) treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J. Taken together with the results of immunohistochemical analysis, long-term treatment with sitagliptin reduced the amount of Abeta and inhibited formation of amyloid plaques in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice.

3.5 Reduced expression of nitrosative stress and inflammation hallmarks within the brain of B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice following DPP-4 inhibition
As shown by the representative immunohistochemistry of the brain, treatment of B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice with sitagliptin caused a substantial reduction of the inflammation and nitrosative stress within the brain associated with the reduced amyloid deposition described earlier. This effect was much more evident for the dose of 20 mg/kg sitagliptin (shown here by Figure 5). 10 mg had slight reductive action (data not shown) while 5 mg/kg had no significant effect.

4. Discussion
Epidemiological study suggests that 7.1% of all deaths for year were attributable to Alzheimer, placing it on a par with cerebrovascular disease as the third leading cause of death (Singer, 2005). Recent study suggests that patients with AD had decreased survival compared with survival in the general U.S. population (Larson et al., 2004): men had a median survival of 4.2 years from their initial diagnosis, and women had a median survival of 5.7 years. Moreover, patients with AD were more likely to have cardiac left ventricular wall motion abnormalities, left ventricular hypertrophy, and reduced ejection fraction compared with patients affected by vascular dementia (Reitz et al., 2006).

Brain dysfunction in AD is characterized by loss of neocortical neurons (Blennow et al., 2006) and focal amyloid deposits, which consist of the locally expressed amyloid ß protein (AßP). Formation of AßP by neurons is generally thought to be a prime trigger of the pathogenesis of AD. The generation of AßP is initiated by a protease that cleaves a larger precursor protein, the ß-amyloid precursor protein (ß-APP), at the N terminus side of the A peptide. ß-secretase or ß-site APP-cleaving enzyme (BACE1) was cloned and identified as a transmembrane aspartyl protease (Blennow et al., 2006). BACE1 deficiency precludes AßP formation in transgenic mice (Mohajeri et al., 2004) and does not cause or promote any neurological or phenotypic abnormalities. Moreover, BACE1 inactivation rescues memory deficits in transgenic mice (Lesnè et al., 2006),
strongly supporting the importance of BACE1 as a therapeutic target in AD. However, recent objectives have been focused on a new strategy based on the reinforcement of the incretin axis by direct stimulation of it with administration of incretins or analogues or indirectly by blocking their degradation with dipeptidyl peptidase-4 inhibitors. The rationale of using these new classes of drugs in AD may resides in the fact that several studies nowadays have ascertained common pathophysiologic hallmarks, such as amyloid beta (Abeta) with type 2 diabetes mellitus (Li et al, 2007), and have ascertained some diabetic drugs also useful in the prevention/reduction of Alzheimer’s. This is the case for example of the incretin GLP-1 or DPP-4 inhibitors.

While many reports are present in literature on the use of the incretin GLP-1 or its analogues in AD, no study has investigated the second possibility that of use DPP-4 inhibitor in order to prevent amyloid deposits within the brain, thus preventing AD progression. DPP-4 is an enzyme that is localized vastly in the endothelium and can also be measured in the blood. DPP-4 cleaves peptides with an N-terminal alanine or proline amino acid residue (Mentlein et al., 1993). In case of the incretin GLP-1 the two GLP-1 fragments resulting from DPP-4 activity are both biologically inactive; the fragment GLP-1(9-36) amide has even been described as having GLP-1 antagonistic properties in some studies (Knudsen and Pridal, 1996). DPP-4 inhibition raises intact GLP-1 plasma concentrations to levels observed in the stimulated state after a meal. Further results in support come from mice deficient in DPP-4/CD26. These animals are resistant to STZ-induced diabetes and have elevated circulating plasma concentrations of intact GLP-1 and other incretin hormones (Marguet et al., 2000).

We report here that long-term treatment with the DPP-4 inhibitor sitagliptin increases the endogenous levels of GLP-1 and reduces the levels of amyloid-beta peptide (Abeta) and amyloid precursor protein (APP) in the brain in vivo. This result in agreement with the concept that GLP-1 reduces the levels of amyloid-beta peptide in the brain in vivo and reduces the levels of amyloid precursor protein (APP) in cultured neuronal cells, and with data that GLP-1 and exendin-4 (a GLP-
1 analogue) protect cultured hippocampal neurons against death induced by Abeta and iron, an
oxidative insult (Perry et al., 2003). Bearing in mind that endogenous GLP-1 possesses neurotropic
properties (Lamont and Drucker, 2008) there is, therefore, a possibility that with the introduction of
a DPP-4 inhibitor the successful management of GLP-1 release is maintained while still allowing
GLP-1 activity to go unaltered and potentially act to positively reduce Aβ concentrations. In other
word a DPP-4 inhibitor as it is sitagliptin would maintain the natural action of GLP-1 on the Aβ
degradation and may add positive effects in term of reducing the cognitive impairment caused by
AD.

On another note, we show here that DPP-4 inhibition is associated with a reduction of the
inflammation and nitrosative stress within the cerebral structures. This is in agreement with many
evidence that i) an inflammatory process in the central nervous system is believed to play an
important role in the pathway leading to neuronal cell death (Kubis and Janusz, 2008), and ii)
proinflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor are
increased in AD (Bryan et al., 2008).

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**Figure Legends**

**Figure 1.** Effects of sitagliptin (5, 10 and 20 mg/kg) on ambulation counts in the open-field test in B6.Cg-Tg(APPswe,PSEN1dE9)85DbO/J mice. The ambulation counts were evaluated by the number of squares crossed by the mice during a 3-min period of time. Each value represents the mean of 6 animals per group. V=vehicle; sita=sitagliptin.
Figure 2. Effects of sitagliptin (5, 10 and 20 mg/kg) on freezing time (seconds) in the contextual fear conditioning test in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice. Each value represents the mean±s.e. of 6 animals per group. P<0.01 was considered statistically significant. V=vehicle; sita=sitagliptin.

Figure 3. Effects of sitagliptin (5, 10 and 20 mg/kg) on brain GLP-1 levels in B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice. Each value represents the mean±s.e. of 4 animals per group. P<0.05 and P<0.01 were considered statistically significant. Sita=sitagliptin.

Figure 4. Representative hippocampal immunostaining (X200) for A) βAPP and for B) βamyloid within the brain of mice treated with vehicle, sitagliptin (20 mg/kg) or exendin (9-39; 2 pmol/kg/min). Evident is the major immunoreactivity at levels of hippocampal neurons of vehicle-treated and exenedin B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice compared with sitagliptin-treated B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice.

Figure 5. Representative hippocampal immunostaining (X200) for A) nitrotyrosine and B) IL1-β within the brain of B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J mice treated with vehicle or sitagliptin (20 mg/kg).
Figure 1
Figure 2
Figure 3

Bar graph showing GLP-1 levels (pmol/l) with different treatments:
- vehicle
- sita 5
- sita 10
- sita 20

Significance levels indicated by asterisks: * and **