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Blockade of enzyme activity inhibits tissue transglutaminase-mediated transamidation of α -synuclein in a cellular model of Parkinson's disease

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ABSTRACT

Transamidation of α -synuclein by the Ca^{2+} -dependent enzyme tissue transglutaminase (tTG, EC 2.3.2.13) is implicated in Parkinson's disease (PD). tTG may therefore offer a novel therapeutic target to intervene in PD. Here we first evaluated the potency and efficacy of three recently developed irreversible active-site inhibitors of tTG (B003, Z006 and KCC009) to inhibit tTG activity *in vitro* and in living cells. *In vitro*, all compounds were found to be full inhibitors of tTG activity showing a rank order of potency (defined by IC₅₀ values) of Z006 > B003 > KCC009. Upon Ca^{2+} ionophore (A23187) induced activation of cellular tTG (measured by incorporation of the tTG-specific amine substrate 5-(biotinamido)pentylamine (BAP) into cellular proteins) in neuroblastoma SH-SY5Y cells, only Z006 (0.3–30 μM) retained the capacity to completely inhibit tTG activity. Under these conditions B003 (3–300 μM) only partially blocked tTG activity whereas KCC009 (3–100 μM) failed to affect tTG activity at any of the concentrations used. Z006 (30 μM) also blocked the tTG mediated incorporation of BAP into α -synuclein monomers and SDS-resistant multimers *in vitro* and in α -synuclein overexpressing SH-SY5Y cells exposed to A23187 or the PD mimetic 1-methyl-4-phenylpyridine (MPP⁺). Moreover, Z006 (30 μM) substantially reduced formation of SDS-resistant α -synuclein multimers in SH-SY5Y cells exposed to A23187 or MPP⁺ in the absence of BAP. We conclude that α -synuclein is a cellular substrate for tTG under conditions mimicking PD and blockade of tTG activity counteracts α -synuclein transamidation and aggregation *in vitro* and in living cells. Moreover, our cell model appears an excellent readout to identify candidate inhibitors of intracellular tTG.

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1. Introduction

Parkinson's disease (PD) is a neurological movement disorder, characterized by progressive degeneration of catecholaminergic neurons in various brain areas, in particular the substantia nigra pars compacta (SNpc) and locus coeruleus (Braak et al., 2003). Another hallmark of PD is the presence of large intraneuronal proteinaceous inclusions called Lewy bodies (LBs) and Lewy neurites (LNs). These inclusions consist primarily of highly aggregated forms of the α -synuclein protein (Spillantini et al., 1997). Similar α -synuclein containing inclusions occur in a number of other neurodegenerative disorders, including dementia

with Lewy Bodies (DLB) and multiple system atrophy, which, together with PD, are commonly referred to as synucleinopathies (Galvin et al., 2001).

α -Synuclein is a relatively small acidic protein consisting of 140 amino-acids with an apparent size of about 14 kDa. While its physiological function remains largely elusive, α -synuclein is associated with the presynaptic nerve terminal, where it is involved in priming and recycling of synaptic vesicles (Chandra et al., 2005; Gitler et al., 2008; Larsen et al., 2006). α -Synuclein belongs to the family of so-called intrinsically disordered proteins and has been reported to exist in an unfolded conformation *in vitro*, which, upon negatively charged phospholipid binding, adopts an α -helical conformation (Weinreb et al., 1996; Eliezer et al., 2001; Ulmer et al., 2005). This unfolded conformation makes α -synuclein prone to misfolding, allowing it to readily form multimers and protofibrils that eventually fibrillize into insoluble aggregates which constitute the core of LBs and LNs (Conway et al., 2000).

The importance of α -synuclein for PD pathogenesis is emphasized by the fact that increased α -synuclein expression

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induced by multiplication of the α -synuclein gene can cause rare familial forms of PD, whereas missense point mutations that stimulate protein misfolding, such as A30P, A53T and E46K, give rise to autosomal dominant early-onset forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Chartier-Harlin et al., 2004; Ibanez et al., 2004). In addition, several other factors have been implicated in α -synuclein aggregation, such as the presence of polycations and divalent metal ions, and post-translational modifications, including oxidation, phosphorylation, nitrosylation and transamidation (reviewed in (Uversky, 2007)).

Tissue transglutaminase (tTG, EC 2.3.2.13), also known as TG2, is the best characterized member of the transglutaminase (TG) family of enzymes, which consists of eight catalytically active members and one inactive member (Griffin et al., 2002; Lorand and Graham, 2003). Like other TGs, tTG is a thiol-sensitive, calcium-dependent enzyme that catalyzes several reactions, in particular the acyl-transfer between the γ -carboxamide group of a polypeptide bound glutamine and the ϵ -amino group of a polypeptide bound lysine or low molecular weight (poly)amines such as putrescine, spermine and spermidine (Lorand and Graham, 2003; Griffin et al., 2002). This so-called transamidation reaction results in the formation of covalent, highly protease resistant ϵ -(γ -glutamyl)lysine isopeptide cross-links or (γ -glutamyl)polyamine bonds, respectively. Besides the increased expression of tTG protein in the SNpc and elevated levels of tTG protein in cerebrospinal fluid (CSF) of PD patients (Andringa et al., 2004; Vermes et al., 2004), tTG induced cross-links in soluble α -synuclein monomers and (small)multimers are also detected in the SNpc of PD patients and cross-links are found in LBs from patients suffering from PD and DLB (Andringa et al., 2004; Junn et al., 2003; Nemes et al., 2009). These findings strongly implicate tTG activity in α -synuclein aggregation and thus in the pathogenesis of synucleinopathies.

Alike the situation in the PD brain, tTG activity induces crosslinks in α -synuclein, *in vitro* (Konno et al., 2005; Schmid et al., 2009; Segers-Nolten et al., 2008). Interestingly, intra-molecularly cross-linked α -synuclein has recently been shown to function as a seed that initiates and promotes α -synuclein misfolding (Nemes et al., 2009). As a result of all these findings, tTG is increasingly recognized as a potential therapeutic target to counteract protein misfolding in PD (Wilhelmus et al., 2008). Surprisingly, however, although established in a test-tube setting, tTG-catalyzed transamidation of α -synuclein in more relevant, i.e. cellular model systems, is still subject of debate (Junn et al., 2003; Suh et al., 2004). Moreover, the lack of selective tTG inhibitors has seriously hampered evaluation of tTG as a potential therapeutic target. With the recent synthesis of a number of selective pepidergic irreversible active-site inhibitors of tTG, this problem has largely been overcome (Choi et al., 2005; Hausch et al., 2003; McCounoughy et al., 2010; Schaertl et al., 2010).

The SH-SY5Y neuroblastoma is a well-characterized catecholaminergic cell line often used as a neuronal model in PD research (Schule et al., 2009). Upon treatment with all-trans-retinoic acid (RA), SH-SY5Y cells stop dividing and show signs of neuronal differentiation and express a large amount of tTG protein (Pahlman et al., 1984; Zhang et al., 1998). These properties make the cell line ideally suited to identify targets of tTG activation in a live cell setup. Therefore, in the present study we used RA treated wild-type and α -synuclein overexpressing SH-SY5Y cells to study the effect of a number of recently developed tTG inhibitors on tTG activity and transamidation of α -synuclein. Our results demonstrate that α -synuclein is an intracellular substrate for tTG and that a relatively simple cellular model can be used to identify promising drugs to counteract this pathogenetically important interaction.

2. Materials and methods

2.1. Materials

Poly-Horseradish Peroxidase (HRP) was obtained from Sanquin (Amsterdam, The Netherlands). Dithiothreitol (DTT) was purchased from Promega (Leiden, The Netherlands). 5-(Biotinamido)pentylamine (BAP), streptavidin coupled to agarose and the Biotinoninonic acid (BCA) Protein Assay Kit were purchased from Pierce (Rockford, IL, USA). Dimethylsulfoxide (DMSO) was purchased from Riedel-de Haën (Seelze, Germany). Standard 96-well and Maxisorp immuno 96-well flat-bottom plates were obtained from Nunc (Roskilde, Denmark). 12-well culture plates were obtained from Corning (Corning, NY, USA) and black 96-well plates were purchased from Greiner (Alphen aan den Rijn, The Netherlands). SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Manassas, CA, USA). Fetal Bovine Serum (FBS) was obtained from Cambrex (Verviers, Belgium). All other cell culture media and supplements were obtained from Invitrogen/Gibco (Paisly, UK). Bovine serum albumin (BSA), o-phenylenediamine dihydrochloride (OPD), 1-methyl-4-phenyl-pyridinium (MPP⁺), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other reagents were obtained from Sigma (St. Louis, MO, USA). Mouse monoclonal antibody 211 directed against human α -synuclein was purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal antibodies Ab-2 (clone TG100) and Ab-3 (clones CUB 7402 + TG100) directed against guinea pig tTG, were obtained from Thermo Scientific (Fremont, CA, USA). Goat derived polyclonal antibody directed against guinea pig tTG was purchased from Millipore (Haarlerbergweg, Amsterdam, The Netherlands). Mouse monoclonal antibody AC-15 directed against amino acids 1–15 of *Xenopus laevis* β -actin was purchased from Abcam (Cambridge, MA, USA). B003 (Boc-DON-Gln-Ile-Val-OMe) and Z006 (Z-DON-Val-Pro-Leu-OMe) were purchased from Zedira GmbH (Darmstadt, Germany). Cbz-Gln-tyrosyl-halo-dihydrosoxazole KCC009 was a kind gift of Alvine Pharmaceuticals, Inc. (San Carlos, CA, USA).

2.2. Cell culture

SH-SY5Y cells were cultured at 37 °C, under 5% CO₂ in air in a 1:1 mixture of Eagle's minimum essential medium and Ham's F12 nutrient mixture, containing 10% FBS, 2.5 mM L-glutamine, 1/100 non-essential amino acids and 1 mM sodium pyruvate. Cells were plated in 12-well plates (25,000 cells/cm²). After 24 h, the medium was removed and replaced by cell culture medium containing 3% FBS and 20 μ M RA (Sigma). Six days after RA administration, cells were used for cellular tTG activity measurements.

2.3. *In vitro* tTG activity assay

In vitro tTG activity was measured essentially as described by Jeitner et al. (2001), with few modifications. In short, reaction buffer (100 mM HEPES-HCl, 20 mM DTT, 40 mM CaCl₂, pH 8.0) was incubated in a black 96-well plate at 37 °C for 30 min. Freshly prepared monodansylcadaverine (CAD-DNS, Sigma), in HEPES-HCl, pH 8.0 and 1-N-(carboxybenzoxy-L-glutamyl)glycyl-5-N-(5'-N'-dimethylaminonaphthalenesulfonyl) diamidopentane (CGG-DNS, Zedira) was added to each well to a final concentration of 10 μ M and 200 μ M, respectively. The mixture was incubated 15 min prior to the addition of inhibitors. Finally, 3 μ g/ml human recombinant tTG (Zedira) in a 100 mM HEPES buffer (containing 10 mM DTT, 0.5 mM EDTA and 10 μ g/ml BSA, pH 8.0) was added. The relative fluorescence enhancement was measured kinetically in a fluorimeter (BMG, Germany) for 4 h at 260 nm (excitation) and 530 nm (emission) at 37 °C. Results were corrected for background fluorescence, which was measured in samples where tTG was omitted from the reaction.

2.4. Cellular tTG activity assay

For cellular tTG activity measurements, incorporation of BAP into proteins was determined in SH-SY5Y cells, as described previously by Zhang et al. (1998) with slight modifications. All cell incubation steps were performed at 37 °C and under 5% CO₂ in air. SH-SY5Y cells were washed once with phosphate buffered saline (PBS) and incubated thereafter for 4 h with culture media containing 1 mM BAP. Subsequently, without changing medium, the cells were preincubated for 15 min with various tTG inhibitors (dissolved in dimethyl sulfoxide (DMSO) as 0.1 M stock solutions), or solvent at appropriate concentrations, prior to addition of 10 μ M of the Ca²⁺ ionophore A23187 (Sigma) for 40 min, or, depending on the experiment, 1–5 mM MPP⁺ for 24 h. The cells were then carefully washed twice with cold PBS and sonicated (Branson sonifier, Danbury, CT, USA) in 400 μ l ice-cold homogenizing buffer (50 mM Tris, 150 mM NaCl, pH 7.4, containing 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 μ g/ml of aprotinin/pepstatin). The protein concentrations of the lysates were determined with the BCA assay and either immediately used for detection of tTG activity, or stored at –20 °C. The use of frozen samples did not affect any of the results in the following detection method (data not shown).

For detection of tTG activity, 96-well Maxisorp plates were incubated with 50 μ l of coating buffer (50 mM Tris, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, pH 7.4), followed by 1 μ g of cell lysate and incubated overnight at 4 °C.

After this step, 200 μ l of incubation buffer (50 mM Tris–Cl, 80 mM NaCl, 2.5% BSA, 0.01% sodium dodecyl sulfate (SDS) and 0.01% Tween-20, pH 7.4) was added. Incubation was continued for 2 h at 37 °C. After this period, wells were washed three times with 200 μ l washing buffer (50 mM Tris–Cl, 80 mM NaCl, 0.5% BSA, 0.01% Tween-20, pH 7.4), followed by incubation with 100 μ l of 0.1 μ g/ml streptavidin conjugated Poly-HRP (Sanquin), diluted in wash buffer over 1 h at room temperature (RT). Subsequently, wells were washed three times with washing buffer and incubated in OPD-solution (0.6 mg/ml OPD, 35 mM citric acid, 50 mM Na₂HPO₄, pH 5.0) containing 0.01% hydrogen peroxide for 30 min at RT. The reaction was stopped by adding 1 M H₂SO₄. Absorbance was measured on a microplate reader (SPECTRAMax 250, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 490 nm and was corrected for background, which was defined as the absorbance in cellular extracts obtained from experiments where BAP had been omitted from the culture medium.

2.5. *In vitro* transamidation of α -synuclein by tTG

In vitro tTG catalyzed transamidation reactions were performed with 1 μ M recombinant human tTG (Zedira) and 50 μ M recombinant human α -synuclein (rPeptide, Bogart, GA, USA) in buffer containing 1 mM BAP, 10 mM Tris–Cl, 50 mM NaCl, 1 mM CaCl₂ and 10 mM DTT, pH 7.4 for 30 min at 37 °C. The transamidation reaction was started by the addition of tTG. If drugs were used (EDTA or Z006), they were added 5 min prior to addition of tTG. The reaction was stopped by the addition of TBST (10 mM Tris–Cl, 150 mM NaCl, 0.1% Tween-20, pH 7.4), containing 1 mM EDTA. BAP labelled protein species were precipitated with 25 μ l streptavidin coupled to agarose slurry (Pierce) for 1 h at RT. After 5 subsequent washing steps with TBST, the samples were heated for 10 min at 95 °C in Laemmli sample buffer (SB) (Laemmli, 1970) containing 10 mM DTT.

2.6. Cellular transamidation of α -synuclein by tTG

SH-SY5Y cells stably overexpressing α -synuclein (SH-SY5Y/Syn) were generated as described (Gerard et al., 2010) and cultured using the same procedure and cell density as used for wild type (WT) SH-SY5Y cells in 6-well plates for a total of 6 days. Thereafter, cells were, either incubated with solvent, or treated for 24 h with 250 nM A23187 or 5 mM MPP⁺, with or without the simultaneous addition of 30 μ M Z006. After two washing steps with PBS, cells were lysed in RIPA buffer (50 mM Tris–Cl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, pH 7.4) containing 0.1 mM PMSF and 1 μ g/ml of aprotinin/pepstatin and subjected to sonication to disrupt DNA strands. Protein concentrations were then determined with BCA and lysates were boiled in SB for subsequent immunoblot analysis.

For biotin labeling experiments, 1 mM BAP was added simultaneously with 250 nM A23187 or 5 mM MPP⁺ in the presence or absence of 30 μ M Z006 for 24 h. After lysis, 400 μ g protein per condition was used for subsequent streptavidin precipitation as described for the *in vitro* BAP labeling experiments.

2.7. SDS-PAGE and immunoblot analysis

Protein fractions obtained from cell lysates or streptavidin precipitations were subjected to 12% SDS-poly acrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes via immunoblotting. After blocking the membranes for 30 min in TBST containing 5% non-fat skimmed milk (ELK) (Campina, Woerden, Utrecht, The Netherlands), membranes were probed with primary antibodies 211, Ab-3 and AC-15, where appropriate, in TBST containing 2.5% non-fat skimmed milk. Secondary goat anti-mouse HRP-coupled antibody (DAKO, Heverlee, Belgium) was used as secondary antibody. After washing the membranes three times in TBS (10 mM Tris–Cl, 150 mM NaCl, pH 7.4), proteins were visualized with the Supersignal West Dura extended detection substrate (Pierce) and imaged using the Chemidoc XRS imaging system (Bio-Rad, Veenendaal, The Netherlands).

2.8. tTG sandwich enzyme-linked immunosorbent assay (ELISA)

In order to measure cellular tTG concentrations in SH-SY5Y/Syn cells that were, either incubated with solvent, or treated for 24 h with 250 nM A23187 or 5 mM MPP⁺, in the presence or absence of 30 μ M Z006, cells were lysed in RIPA and subjected to ELISA essentially as described by us (Breve et al., 2008). In short, 96-well maxisorp plates (NUNC) were incubated with goat derived polyclonal antibodies directed against guinea pig tTG (Millipore) at a dilution of 1/1000 for 16 h at 4 °C in coating buffer (50 mM Na₂CO₃, pH 9.2). Plates were rinsed once with wash buffer (0.5 M NaCl, 10 mM Na₂HPO₄, 0.1% (v/v) Tween-20, pH 7.2) and subsequently blocked for 1 h at RT with 200 μ l/well wash buffer containing 0.5% BSA. After rinsing the plates three times with wash buffer, 100 μ l/well of 10 μ g/ml cell lysate, diluted in wash buffer, was incubated overnight at 4 °C. After washing, 100 μ l/well wash buffer containing 1 μ g/ml monoclonal antibody Ab-2 (Thermo Scientific) was added for 1 h at RT. Subsequently, wells were incubated with wash buffer containing 100 μ l of 1 μ g/ml biotinylated goat anti-mouse IgG (H + L)

(Jackson, Newmarket, Suffolk, England), followed by incubation with 100 μ l wash buffer containing 0.1 μ g/ml streptavidin poly-HRP (Sanquin), each for 1 h at room temperature. After final washing steps, 100 μ l/well OPD-solution containing 0.01% hydrogen peroxide was added and incubated at RT for 15 min. The reaction was stopped by the addition of 100 μ l/well 1 M H₂SO₄ and absorbance values were determined at 490 nm using a SPECTRAMax 250 microplate reader.

2.9. Cell viability assay

Under normal growth conditions, SH-SY5Y cells were cultured in 96-well cell culture plates (Nunc). A final concentration of 0.5 mg/ml MTT was added to each well and was allowed to incubate for 45 min at 37 °C under 5% CO₂ in air. Thereafter, the medium was removed and replaced by 200 μ l of DMSO to which 0.5% (v/v) FBS had been added. After formazan solubilization by vibration on a plate shaker, the absorbance of each well was measured at 540 nm using the SPECTRAMax 250 microplate reader, as described previously (Drukarch et al., 1996). Absorbance data were calculated by subtracting the mean of background readings obtained from identical incubations in the absence of cells.

2.10. Statistics

To calculate IC-50 values, concentration response curves were fitted by nonlinear regression using a four-parameter hill slope equation with the software package Graphpad Prism 5 (La Jolla, CA, USA). Statistical analysis was performed with the software package Graphpad Prism 5, using one-way analysis of variance (ANOVA) with Bonferroni's correction of P values for multiple comparison. For MTT analysis, Student's t-test was used. A P-value less than 0.05 was considered statistically significant.

3. Results

3.1. *In vitro* and cellular inhibition of tTG activity

In the current study, we used recently developed peptide based irreversible active-site inhibitors of tTG i.e. B003, Z006 and KCC009 (Choi et al., 2005; Schaertl et al., 2010). Whereas both B003 and Z006 employ a 6-diazo-5-oxo-norleucine (DON) reactive group, KCC009 has a dihydro-isoxazole group for active-site inhibition of tTG (Fig. 1A). The results from our *in vitro* test, which is based on the tTG mediated formation of the highly fluorescent reaction product CGG-DNS–CAD-DNS from CGG-DNS and CAD-DNS, show that all three drugs inhibited tTG activity with equal efficacy, but with different potency. Thus, under the experimental conditions used, Z006 displayed an IC-50 of ± 0.04 μ M, reaching maximal inhibition at ± 0.3 μ M (Fig. 2A). Compared to Z006, B003 was found to be less potent (IC-50 ± 0.3 μ M), with a maximally effective concentration of ± 3 μ M (Fig. 2A). KCC009, however, was the least potent with an IC-50 of ± 1 μ M and a maximally effective concentration of ± 10 μ M (Fig. 2A).

In contrast to the *in vitro* data, the results from our cellular model clearly distinguished between the different drugs on both potency and efficacy of tTG inhibition. In fact, to our knowledge, our data are among the first to establish a clear concentration–effect relationship of pharmacologic tTG inhibition in cells, although using a different experimental setup, effects of inhibitors on tTG activity in SH-SY5Y cells have been reported previously (Beck et al., 2006; Lesort et al., 2003; Singh et al., 2003). This model, originally characterized by Zhang et al. (1998), is based on detection of tTG-mediated covalent incorporation of the amine donor BAP into cellular proteins following activation of transamidation activity by a rise in intracellular Ca²⁺ concentration as a result of exposure of the cells to Ca²⁺ ionophores like A23187.

Under these experimental conditions, Z006 induced a concentration-dependent inhibition of cellular tTG activity with an IC-50 of ± 0.7 μ M and near complete blockage of BAP incorporation at a concentration of 10 μ M (Fig. 2B). Although similar to the *in vitro* measurements, B003 was found to be approximately 10-fold less potent than Z006 in inhibiting tTG activity in SH-SY5Y cells (IC-50 ± 7 μ M), it demonstrated considerable less efficacy in the cell model, reaching 75% enzyme inhibition at a maximally effective

Fig. 1. Molecular structure of tTG inhibitors used in this study and setup of tTG activity measurements. (A) The reactive moieties that irreversibly bind to tTG are in gray. B003 and Z006 rely on a 6-diazo-5-oxonorleucine group, whereas KCC009 contains a 3-halo-4,5-dihydro-isoxazole reactive group. (B) Schematic overview of cellular determination of tTG activity, as described in Section 2. RA (20 μ M) differentiated SH-SY5Y cells, which express high cellular tTG levels (Zhang et al., 1998), are incubated with BAP. Upon cellular stimulation with calcium ionophore A23187, the cellular Ca^{2+} concentration is increased and tTG is activated resulting in the incorporation of BAP into proteins.

concentration of 300 μ M (Fig. 2B). In contrast to the DON based inhibitors Z006 and B003 and the *in vitro* measurements, the dihydroisoxazole based compound KCC009 failed to significantly affect cellular tTG activity in concentrations up to 100 μ M (Fig. 2B). At higher concentrations of KCC009, the amount of DMSO (>1%) required to completely dissolve the compound interfered with the cellular tTG assay.

In the absence of A23187, only small amounts of BAP bound to proteins. This non-specific (i.e. non tTG-mediated) BAP incorporation was not influenced by the tTG inhibitors, who also did not affect cellular viability at any of the concentrations tested (data not shown). Moreover, BAP incorporation due to exposure to A23187 was completely blocked by co-incubation with the Ca^{2+} chelating agent EDTA at a concentration of 1 mM (data not shown).

3.2. α -Synuclein is a substrate of tTG *in vitro*

The above mentioned data indicated that (1) cellular BAP incorporation properly reflected tTG activity in A23187-treated cells, (2) the assay method differentiates between alleged tTG inhibitors in their potency and efficacy to block intracellular tTG activity, and (3) protein-bound BAP is an indicator of newly formed cross-links. We therefore wondered whether we could use this polyamine to develop a cellular assay that measures the effect of

inhibition of tTG activity on α -synuclein transamidation. For this purpose, we first investigated BAP incorporation into human recombinant α -synuclein in the presence of Ca^{2+} activated recombinant human tTG *in vitro*. Immunoblot analysis, following streptavidin precipitation of BAP bound α -synuclein, revealed both α -synuclein monomers as well as SDS-stable multimers (Fig. 3), in line with previous *in vitro* studies that only used tTG and α -synuclein (Junn et al., 2003; Konno et al., 2005; Lashuel et al., 2002; Nemes et al., 2009; Segers-Nolten et al., 2008). Moreover, BAP incorporation into α -synuclein was completely blocked via co-incubation with the tTG inhibitor Z006 (30 μ M), or by Ca^{2+} chelation with EDTA (1 mM) (Fig. 3), whereas no BAP incorporation occurred in the absence of tTG (data not shown).

3.3. α -Synuclein is a cellular substrate of tTG

In an additional set of experiments, we used lentiviral transduced SH-SY5Y cells that stably express human WT α -synuclein (characterized previously by Gerard et al. (2010)) and treated these cells with A23187 in the presence of BAP, following differentiation with RA. Upon a short treatment (40 min) of these cells with A23187 (10 μ M), i.e. the paradigm that was used for determination of the cellular concentration–response curves of tTG inhibitors, no biotinylated α -synuclein could be detected.

Fig. 2. *In vitro* and cellular tTG activity measurements in the presence of irreversible active-site inhibitors. (A) *In vitro* concentration–response curves of tTG inhibitors B003 ($\diamond$), KCC009 ($\square$) and Z006 ($\circ$) were determined by measuring tTG activity in the presence of increasing concentrations of inhibitors. tTG activity in the presence of DTT (10 mM) and Ca^{2+} (10 mM) was defined as maximum activity and set to 100%. Data were then plotted on a log-scale with Graphpad Prism 5 statistical software package and expressed as percentage of maximum activity. (B) The cellular concentration–response curves of the compounds described above were determined by measuring tTG activity in RA differentiated SH-SY5Y cells in the presence of increasing inhibitor concentrations. Cellular tTG activity in cells treated with A23187 (10 μ M, 40 min) was defined as maximum activity and set to 100%. tTG activity in the presence of inhibitors was plotted on a log-scale with Graphpad Prism 5. Data is expressed as percentage of maximum tTG activity. Background correction was performed as described in Section 2. All experiments were performed three times in triplicate and data are depicted as mean + SEM.

Fig. 3. tTG transamidates α -synuclein monomers and multimers *in vitro*. Human recombinant tTG (1 μ M) was incubated in the presence of human recombinant α -synuclein (α S) (50 μ M) and BAP (1 mM). Biotinylated proteins were precipitated and analyzed on immunoblot (IB, monoclonal antibody 211). Enzymatic activity of tTG was blocked when Z006 (30 μ M) or EDTA (1 mM) was co-incubated during the transamidation reaction. As control for unspecific conjugate binding, empty streptavidin coupled to agarose (beads) was used. Experiment was repeated 3 times with similar results. Typical experiment is shown.

Therefore, a longer incubation time with BAP (24 h), in combination with reduced A23187 (250 nM) concentrations, was used. Under these circumstances, BAP incorporation could be readily detected in α -synuclein monomers and a variety of SDS-stable α -synuclein immunoreactive multimers (Fig. 4). Moreover, in the presence of Z006 (30 μ M), a large reduction in biotinylated α -synuclein was observed (Fig. 4).

3.4. tTG-dependent α -synuclein transamidation is induced by the PD mimetic MPP⁺

Given the fact that ionophore-induced calcium mobilization in differentiated SH-SY5Y/Syn cells readily resulted in tTG-dependent α -synuclein transamidation and multimerization, we questioned whether this phenomenon also occurs under conditions more relevant to PD. Therefore, we treated the cells with the mitochondrial complex I inhibitor MPP⁺, an active metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) that is commonly used as a toxin to induce PD-like phenomena in several well-established cellular and *in vivo* PD models (Burns et al., 1983; Heikkilä et al., 1984). In line with previous work by Beck et al. (2006), we found that incubation with MPP⁺ (1–5 mM) for 24 h induced a concentration-dependent activation of tTG activity in RA-differentiated WT SH-SY5Y cells (Fig. 5A). More importantly, following treatment of RA-differentiated SH-SY5Y/syn cells with MPP⁺ (5 mM) and BAP (1 mM) for 24 h and subsequent precipitation of biotinylated proteins, α -synuclein monomers and SDS-stable α -synuclein immunoreactive multimers could be discerned on immunoblot (Fig. 5B), in a pattern similar to that observed upon treatment with A23187 (Fig. 4). Moreover, in the presence of MPP⁺, co-incubation with 30 μ M Z006 reduced the levels of biotinylated α -synuclein below the detection limit of the assay (Fig. 5B).

Fig. 4. Cellular Ca²⁺ influx induces tTG mediated α -synuclein transamidation. RA differentiated SH-SY5Y/Syn cells were incubated with 250 nM A23187 for 24 h with (Z6) or without (–) the presence of Z006 (30 μ M) as detailed in Section 2. All incubations were performed in the presence of BAP (1 mM). Biotinylated proteins were precipitated and subjected to immunoblot (IB) analysis. α -Synuclein (α S) was visualized using monoclonal antibody 211. As control for unspecific conjugate binding, empty streptavidin coupled to agarose (beads) was used. For details see Section 2. Experiment was repeated 3 times with similar results. Typical experiment is shown. Although all samples originate from the same IB, a shorter illumination time was required for human recombinant α -synuclein.

3.5. α -Synuclein multimerisation is not dependent on BAP

In the above set of data, in order to determine whether activation of tTG by either A23187 or MPP⁺ resulted in newly formed transamidated α -synuclein species, we used BAP incorporation as a specific marker for tTG catalyzed crosslinks. A limitation of this approach may be that the presence of polyamines itself is reported to induce α -synuclein multimerisation (Antony et al., 2003). To rule out the possibility that α -synuclein multimerisation and the effect thereon of tTG inhibition is dependent on the presence of the polyamine BAP, we treated SH-SY5Y/Syn cells with A23187 (250 nM) or MPP⁺ (5 mM) for 24 h in the presence or absence of Z006 (30 μ M). Importantly, BAP was omitted from these experiments. As depicted in Fig. 6A (upper panel), although absent in untreated cells, treatment with A23187 or MPP⁺ resulted in the formation of α -synuclein positive, SDS-resistant multimers and larger aggregates that did not migrate further than the stack of the SDS-PAGE gel. Co-incubation with Z006 (30 μ M), however, strongly reduced α -synuclein multimer and aggregate formation. In fact, in cells treated with Ca²⁺ ionophore or MPP⁺, only mild α -synuclein multimerization and aggregate formation was noted in the presence of the tTG inhibitor (Fig. 6A, upper panel).

Finally, to rule out that the inhibitory effect of Z006 on α -synuclein transamidation induced by A23187 or MPP⁺ following prolonged incubation was due to an effect of Z006 on tTG protein expression or processing rather than activity, we quantified total tTG levels in the aforementioned SH-SY5Y/Syn cells with two

Fig. 5. Cellular tTG-mediated α -synuclein transamidation is induced by MPP⁺ treatment. (A) RA differentiated SH-SY5Y cells were treated with increasing concentrations of MPP⁺ (1–5 mM) for 24 h in the presence of BAP (1 mM). BAP incorporation into proteins was quantified as described in Section 2. tTG activity in untreated cells was defined as basal activity and set to 100%. Data were subjected to one-way ANOVA and Bonferroni's Multiple Comparison Test when means were significant. The experiment was performed three times in triplicates and data are depicted as mean $\pm$ SEM. (B) RA differentiated SH-SY5Y cells overexpressing α -synuclein (α S) were exposed to MPP⁺ (5 mM) for 24 h with (Z6) or without (–) Z006 (30 μ M) in the presence of BAP (1 mM). Biotinylated proteins were precipitated and analyzed on immunoblot (IB) with α -synuclein specific monoclonal antibody 211. As control for unspecific conjugate binding, empty streptavidin coupled to agarose (beads) was used. Experiment was repeated 3 times with similar results. Typical experiment is shown.

different approaches. First, tTG levels were estimated on immunoblot, using β -actin as a loading control (Fig. 6A, lower panel). Second, tTG concentrations were determined with a previously in-house established tTG specific sandwich-ELISA (Breve et al., 2008) (Fig. 6B). Even though a small decrease in tTG protein levels in A23187 and MPP⁺ treated cells was noted, co-incubation with Z006 (30 μ M) did not significantly alter tTG protein levels (Fig. 6A, lower panel and Fig. 6B). It is important to note that incubation with Z006 (30 μ M) did not affect cellular viability of SH-SY5Y/Syn cells, as measured with MTT (Fig. 6C).

4. Discussion

Post-translational modification by tTG is increasingly recognized as an important stimulatory factor in the aggregation of α -synuclein and formation of LBs, as observed in PD and related synucleinopathies (Nemes et al., 2009; Junn et al., 2003; Andringa et al., 2004). Therefore, tTG may represent an attractive target to counteract α -synuclein misfolding in neurodegenerative diseases. In the present study, we used a cell model to study the effect of pharmacological inhibition of enzyme activity on the interaction between tTG and α -synuclein. We show here for the first time that in neuronally differentiated, α -synuclein overexpressing cells, active tTG is able to transamidate cellular α -synuclein, resulting in formation of modified monomeric and multimeric α -synuclein species which appear similar to those detected by us and others *in vitro* (Junn et al., 2003; Konno et al., 2005; Schmid et al., 2009; Segers-Nolten et al., 2008) and in PD brain (Andringa et al., 2004). These results underscore the validity of our model. Moreover, our data demonstrate that this interaction between tTG and α -synuclein was abolished not only *in vitro* but also in our cell system in the presence of a recently developed cell-membrane permeable irreversible tTG active-site inhibitor. We also show that this effect

of tTG inhibition is not due to drug-induced reduction of tTG protein levels or altered processing of the enzyme. As such, they support and add to previous data reported by Junn et al. who showed blockade of α -synuclein aggregation in tTG and synuclein overexpressing cells exposed to high concentrations of the classic but non-specific, competitive tTG inhibitor cystamine (Junn et al., 2003). Therefore, although the methods used do not allow us to conclude that blockade of tTG activity will prevent all α -synuclein aggregation under the conditions prevailing in the diseased brain, our results do suggest that the contribution of tTG to this process is amenable to effective pharmacological intervention.

Under physiological circumstances, at least in cultured cells, cytoplasmic Ca^{2+} levels are too low to induce the transamidation activity of cellular tTG and the enzyme remains catalytically silent (Zhang et al., 1998). This is in line with our results demonstrating no detectable enzymatic interaction between tTG and α -synuclein under control, i.e. non-stimulated, condition. However, under pathological conditions such as prevail in PD, cytoplasmic Ca^{2+} concentration rises to levels which enable tTG to interact with its substrates (Zhang et al., 1998). To specifically identify tTG modified forms of α -synuclein under experimental conditions mimicking PD, we activated intracellular tTG either through stimulation of Ca^{2+} influx with the ionophore A23187 (Fesus et al., 1981) or inhibition of mitochondrial respiration with the PD mimetic MPP⁺ (Beck et al., 2006). Simultaneously, we incubated the cells with the well-characterized labelled synthetic tTG substrate polyamine BAP (Zhang et al., 1998), followed by enrichment of BAP-positive α -synuclein protein. Taking into consideration the highly stable (i.e. covalent), protease and detergent resistant nature of tTG-mediated transamidation products (Schmid et al., 2009; Segers-Nolten et al., 2008), this method in principle allows for sensitive determination of all tTG modified α -synuclein species into which BAP is incorporated within the time frame of the experiment. However,

Fig. 6. α -Synuclein multimerization is independent of BAP or changes in tTG concentration. (A) RA differentiated SH-SY5Y cells overexpressing α -synuclein (α S) were exposed to A23187 (250 nM) or MPP⁺ (5 mM) for 24 h with (Z6) or without (–) Z006 (30 μ M). Cell lysates were analyzed with immunoblotting (IB) and probed with antibodies 211, Ab-3 and AC-15. Experiment was repeated 3 times with similar results. Typical experiment is shown. (B) Determination of the tTG concentration by ELISA was performed as described in Section 2. The tTG concentration in untreated RA differentiated SH-SY5Y cells overexpressing α -synuclein was 497 ± 59 ng per mg total protein. Co-incubation of Z006 (30 μ M) with MPP⁺ (5 mM) or A23187 (250 nM) treated cells had no significant (N.S.) effect on cellular tTG protein levels (one-way ANOVA and Bonferroni's Multiple Comparison Test, $P > 0.05$). (C) Cellular viability of SH-SY5Y cells overexpressing α -synuclein treated with Z006 or vehicle-matched control (DMSO) as measured with MTT. Formazan absorbance of vehicle-matched cells was set to 100%. Z006 treatment had no significant effect on cellular viability (Student's t -test, $P > 0.05$). Experiments represented in panels B and C were performed four times in triplicates and data are depicted as mean + SEM.

also using BAP incorporation in α -synuclein overexpressing and RA-differentiated SH-SY5Y cells, Suh and coworkers failed to detect any interaction between tTG and α -synuclein following a 20 min treatment with the potent, but highly toxic Ca^{2+} ionophore maitotoxin (Suh et al., 2004). In our opinion, the most likely explanation for this apparent difference in results is the relatively short incubation time with Ca^{2+} ionophore used by Suh et al. Also in our hands, extended periods of exposure to A23187 or MPP⁺ were required in order to reach detectable amounts of biotinylated α -synuclein.

Naturally occurring polyamines, like spermine and spermidine, have been shown to stimulate α -synuclein aggregation, albeit thus far only *in vitro* (Antony et al., 2003). In our *in vitro* setting, in the absence of tTG, neither spontaneous incorporation of BAP into α -synuclein, nor α -synuclein multimerization was detected (data not shown). Moreover, in our cell model omission of BAP did not prevent α -synuclein aggregation induced by exposure to A23187 or MPP⁺, nor altered the ability of the tTG inhibitor Z006 to substantially inhibit this effect, thereby ruling out that effects observed in the presence of BAP were independent of tTG. Of course, in contrast to the experiments in which BAP was used, in this set of experiments where BAP was omitted, we cannot exclude the possibility that Z006 exerted its effect on α -synuclein aggregation indirectly, rather than directly, e.g. via blockade of the interaction between tTG and another protein which influences α -synuclein folding. Nevertheless, these data do support the notion that activation of tTG stimulates α -synuclein multimerization. On one hand, tTG can transamidate α -synuclein directly

resulting in large multimers, consisting only of α -synuclein, or α -synuclein complexed with other proteins. Another explanation is that transamidated α -synuclein monomers acts as seeds that recruit other α -synuclein molecules thereby inducing multimerisation of α -synuclein, as proposed by Nemes et al. (2009).

The irreversible tTG active-site inhibitors used in this study were found to have excellent *in vitro* enzyme activity blockade characteristics with apparent potencies in the rank order Z006 > B003 > KCC009, which compare well with data provided previously by others applying different assays (Madi et al., 2005; McConoughey et al., 2010; Schaertl et al., 2010). As expected, although maintaining similar rank order, quantitative measurement of tTG activity in living RA-differentiated wild-type SH-SY5Y cells showed that both Z006 and B003 had a reduced potency, i.e. approximately 20-fold lower IC₅₀ values compared to the *in vitro* measurements. In our view, this may be considered as a relatively small decline in light of the large differences in experimental setup. In contrast to B003, however, Z006 retained the ability to (almost) completely inhibit tTG activity inside cells in low micromolar concentrations. Data published recently by the groups of Schaertl and McConoughey confirm our observation, that in a cellular environment Z006 shows better pharmacological characteristics as a tTG inhibitor than B003 in terms of apparent potency (McConoughey et al., 2010; Schaertl et al., 2010). Interestingly, Schaertl et al. reported a much higher cellular IC₅₀ value for both Z006 and B003 as compared to our results. Although this may be attributed simply to the fact that the group of Schaertl used a somewhat different assay procedure, in which incorporation of

radiolabelled polyamine putrescine into the cellular protein fraction is measured in tTG overexpressing HEK cells, it could also point to cell-type dependent characteristics of tTG inhibition. To our knowledge, this issue has not been considered before but, in light of its potential impact on the ongoing development of tTG inhibitors for treatment of diseases affecting a large variety of cell types (Choi et al., 2005; Griffin et al., 2008; Hausch et al., 2003; McConoughey et al., 2010; Schaertl et al., 2010; Siegel and Khosla, 2007; Watts et al., 2006), deserves further consideration in future experiments.

In contrast to Z006 and B003, KCC009 failed to affect cellular tTG activity in concentrations up to 100 μ M. For reasons discussed in Section 3, we were unable to test higher concentrations of the compound. In other studies using cultured cells KCC009 has been claimed to block tTG activity in concentrations of 100 μ M and above, although actual measurements of effect on ongoing tTG activity in living cells were not made (Siegel et al., 2008; Yuan et al., 2005). If correct, these data suggest that the lack of effect of KCC009 on cellular tTG activity in the present study is explained by the concentration range in which the compound was tested. However, considering our data, this would also imply that, in comparison to Z006 and B003 who show an approximately 20-fold decline between cellular and *in vitro* measurements (see above), a far higher reduction in apparent potency would occur for KCC009 under the experimental conditions used. KCC009 is highly lipophilic and is expected to readily pass the cell membrane. Indeed, exposure of cultured cells to KCC009 followed by cell extraction and *in vitro* measurement of tTG activity showed inhibition of tTG activity by residual KCC009 retained in the cell extract (Siegel et al., 2008). Therefore, the apparent failure to inhibit cellular tTG activity in concentrations up to 100 μ M in our experimental setup is unlikely to be due (only) to insufficient uptake. More likely, it is caused by a combination of compound characteristics, perhaps the relative instability of the reactive dihydroisoxazole moiety in KCC009 as compared to the DON group in both Z006 and B003, and prevailing conditions in the cell interior, like high concentrations of tTG protein and/or enzyme substrates. This conclusion is in line with observations made in an *in vivo* mouse model for chronic liver disease in which KCC009 clearly reduced overall liver injury, an effect which may be caused by interaction of the compound with tTG present at the cell surface or in the extracellular matrix (Akimov et al., 2000; Akimov and Belkin, 2001), but had no effect on tTG regulated formation of Mallory Bodies inside hepatocytes (Strnad et al., 2006). Within this context, it is also curious to note that Schaertl and coworkers reported a virtual lack of effect on intracellular enzyme activity for the large majority of tTG inhibitors tested by them (Schaertl et al., 2010). Thus, further investigation of the factor(s) determining intracellular efficacy of tTG inhibitors is clearly warranted.

Finally, tTG is increasingly recognized as a promising target to counteract protein aggregation occurring inside cells in a number of degenerative diseases. Besides α -synuclein in PD and other synucleinopathies this includes huntingtin in Huntington's disease (Karpuj et al., 1999; Lesort et al., 1999), tau in Alzheimer's disease and related tauopathies (Johnson et al., 1997; Singer et al., 2002) and keratins in chronic liver disease (Zatloukal et al., 1992). In light of this rising interest, the selection of suitable, i.e. intracellularly active drug candidates by the use of relatively simple and reproducible cell models will become more important. In this way, large numbers of compounds may be screened before further testing in dedicated but more time-consuming cellular- and animal models. That the non-radioactive, quantitative cellular BAP protein incorporation assay as applied by us may be used for this purpose is underscored by the observation, that the maximally effective concentration of Z006 identified in this manner (i.e. 30 μ M) was shown to reduce BAP incorporation into α -synuclein

monomers and multimers and α -synuclein aggregation below the detection limits of the much more laborious immunoblotting assays. Therefore, taken together, our work adds to the growing body of literature on tTG inhibition as a promising target for disease modifying therapeutic strategies in PD and other protein misfolding diseases.

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