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# Development of fluorine-18 labeled peptidic PET tracers for imaging active tissue transglutaminase

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ABSTRACT

*Introduction:* The protein–protein crosslinking activity of the enzyme tissue transglutaminase (TG2; EC 2.3.2.13) is associated with the pathogenesis of various diseases, including celiac disease, lung-, liver- and kidney fibrosis, cancer and neurodegenerative diseases. This study aims at developing a TG2 PET tracer based on the peptidic irreversible TG2 inhibitor **Z006**.

*Methods:* Initially, the carbon-11 labeling of **Z006** at the diazoketone position was explored. Subsequently, a set of analogues that allow for fluorine-18 labeling was synthesized. Two potent analogues, **6f** and **6g**, were radiolabeled with fluorine-18 and biodistribution and metabolite analysis in Wistar rats was performed. The identity of the main metabolite of [$^{18}$F]**6g** was elucidated using LC–MS/MS. *In vitro* binding to isolated TG2 and *in vitro* autoradiography on MDA-MB-231 breast cancer tissue using [$^{18}$F]**6g** was performed.

*Results:* [$^{18}$F]**6f** and [$^{18}$F]**6g** were obtained in 20 and 9% yields, respectively. Following administration to healthy Wistar rats, rapid metabolism of both tracers was observed. Remarkably, full conversion to just one single metabolite was observed for one of the tracers, [$^{18}$F]**6g**. By LC–MS/MS analysis this metabolite was identified as C-terminally saponified [$^{18}$F]**6g**. This metabolite was also found to be a potent TG2 inhibitor *in vitro*. *In vitro* binding to isolated TG2 and *in vitro* autoradiography on MDA-MB-231 tumor sections using [$^{18}$F]**6g** demonstrated high specific and selective binding of [$^{18}$F]**6g** to active TG2.

*Conclusions:* Whereas based on the intensive metabolism [$^{18}$F]**6f** seems unsuitable as a TG2 PET tracer, the results warrant further evaluation of [$^{18}$F]**6g** *in vivo*.



## 1. Introduction

Tissue transglutaminase (TG2) is an enzyme known for the calcium dependent catalysis of the transamidation reaction, forming crosslinks between the γ-carboxamide of a glutamine acyl-acceptor substrate and the ε-amine of a lysine acyl-donor substrate [1–3]. The resulting γ-glutamyl-ε-lysine bonds, commonly referred to as isopeptide bonds, are highly stable to proteolysis as these particular sidechain bonds are not easily recognized by proteases. Protein crosslinking only occurs when TG2 adapts the open conformation, favored by a high calcium concentration, in which the catalytic site is exposed [4]. In the closed conformation, promoted by high guanosine diphosphate (GDP) concentrations, two consecutive β-barrels sterically prevent substrates from reaching the active site [5]. Furthermore, it has been shown that transglutaminase activity is only possible when the 230–231 cysteine-cysteine disulfide bond is reduced [6]. Increased TG2 cross-linking activity has been strongly associated with various disease processes, such as cancer, fibrosis, celiac disease and neurodegenerative diseases [7–15].

Various inhibitors of TG2 have been developed [16], amongst them a class of 6-diazo-5-oxo-L-norleucine (DON) containing small peptides. The DON residue, being a glutamine analogue, is able to enter the enzyme's active site, resulting in irreversible binding to the active site cysteine residue, thereby inactivating the enzyme (Fig. 1A). Amongst the DON carrying TG2 inhibitors is **Z006** (Fig. 1B), a small selective and potent peptidic inhibitor [17] that has shown intracellular activity in cell culture [18].

With the increasing interest in TG2 as a therapeutic target, the demand for TG2 imaging agents also increases [19]. *In vitro*, open conformation TG2 can be discriminated from its closed conformation using a TG2 Föster Resonance Energy Transfer (FRET) biosensor [20] or fluorescent inhibitors that are selective for the open conformation [19]. As an alternative for inhibitors, fluorescent or radioactive amine substrates can be used to intercept transglutaminase formed intermediate thioesters [19,21]. This latter approach could, in theory, lead to multiple

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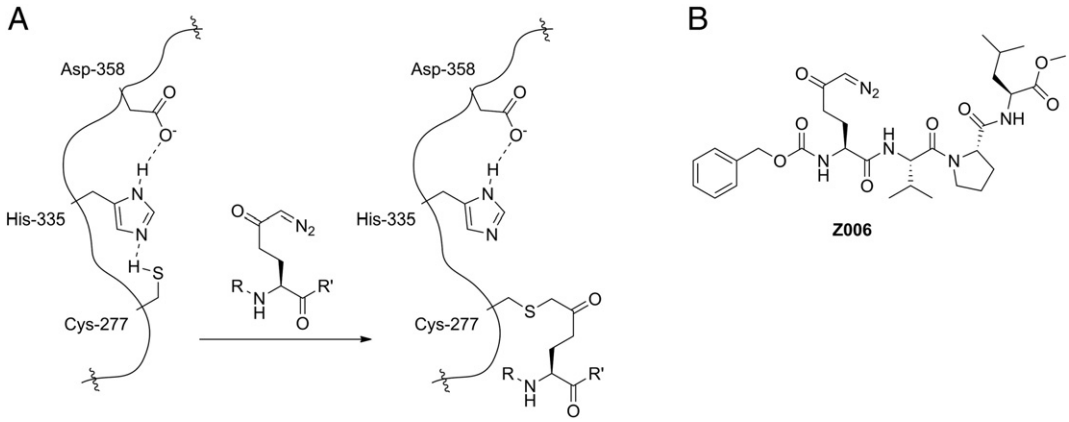


**Fig. 1.** A) General irreversible inhibition of human TG2 by DON inhibitor; B) structure of the well-known TG2 inhibitor **Z006**.

incorporations of detectable substrates per TG2 enzyme, resulting in signal magnification. Selectivity of such substrates is limited, however, as the intermediate thioesters react non-selectively with nucleophiles. Furthermore, processing of amines *in vivo* is not limited to TG2, thereby casting doubt on the specificity of this approach to detect TG2 activity *in vivo*. Nevertheless, as an amine acyl donor substrate, 5-biotinylamido pentylamine has been shown to accumulate in lung tissue in a pulmonary hypertensive rat model [10]. This accumulation could be prevented by treatment with the TG2 inhibitor **ERW1041E**. Clearly, the limitations of this approach include the need to sacrifice test animals, which hampers longitudinal studies. In addition, it is clear that translation to humans is not possible. As an alternative approach, radiolabeled compounds for non-invasive imaging of TG2 using positron emission tomography (PET) can be applied [21]. Carbon-11 labeled tracers for non-invasive imaging of TG2 *in vivo* have recently been developed [22]. Although these compounds demonstrated selective and specific binding to TG2 *in vitro*, they suffered from low metabolic stability *in vivo*, potentially limiting their value for *in vivo* imaging of active TG2.

PET is a non-invasive imaging technique that allows for quantitative *in vivo* imaging of biological processes, such as enzyme or transporter activity. To image a specific biological target (*e.g.* receptor, enzyme, transporter) PET depends on the incorporation of a positron emitter into a biologically active molecule, relevant for this specific target [23]. For radiolabeling of small organic molecules, commonly carbon-11 (half-life: 20.4 min) or fluorine-18 (half-life: 109.8 min) are used. Because of the low amount of tracer mass administered (typically several nanomoles), *in vivo* biology remains undisturbed, making PET a purely analytical imaging technique.

The main objective of the present study was to develop a PET tracer for imaging active TG2 *in vivo*. Because of its high potency and selectivity, **Z006** was used as a lead compound.

## 2. Materials and methods

### 2.1. General

All reagents were obtained from commercial sources (Sigma Aldrich, St. Louis, USA; Bachem, Bubendorf, Switzerland) and used without further purification. Human recombinant transglutaminases and assay reagents were obtained from Zedira GmbH (Darmstadt, Germany). Solvents were obtained from Biosolve (Valkenswaard, the Netherlands) and used as received unless stated otherwise. Dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) were dried over activated 3 Å molecular sieves. Tetrahydrofuran (THF) was first distilled from $LiAlH_4$ and then stored on activated 3 Å molecular sieves. Diazomethane solution in diethylether ($Et_2O$) was obtained using Diazald® as precursor by dropwise addition of Diazald in $Et_2O$ to a heated solution of KOH in EtOH/$H_2O$/carbitol and continuous distillation of generated diazomethane to a receiver flask at 0°C. Careful: Diazomethane is explosive and toxic! Reaction monitoring by thin layer chromatography was performed on pre-coated silica 60 F254 aluminum plates (Merck, Darmstadt, Germany). Spots were visualized by UV light, ninhydrin, vanilline or $Cl_2$/TDM. Evaporation of solvents was performed under reduced pressure at 40°C using a rotary evaporator. Flash column chromatography was performed manually on Silica gel 60 Å (Merck, Darmstadt, Germany). NMR spectroscopy was performed on a Bruker (Billerica, MA, USA) Avance 250 (250.13 MHz for $^1$H and 62.90 MHz for $^{13}$C) or a Bruker Avance 500 (500.23 MHz for $^1$H and 125.78 MHz for $^{13}$C) with chemical shifts ($\delta$) reported in parts per million (ppm) relative to the solvent ($CDCl_3$, $^1$H 7.26 ppm, $^{13}$C 77.16 ppm; dimethyl sulfoxide (DMSO)-$d_6$, $^1$H 2.50 ppm, $^{13}$C 39.52 ppm).

Electrospray ionization-high resolution mass spectrometry (ESI-HRMS) was carried out on positive or negative mode using a Bruker microTOF-Q instrument (capillary potential of 4500 V).

[$^{11}$C]$CO_2$ was produced by the $^{14}$N(p,$\alpha$)$^{11}$C nuclear reaction performed in a 0.5% $O_2$/$N_2$ gas mixture using an IBA Cyclone 18/9 cyclotron (IBA, Louvain-la-Neuve, Belgium). [$^{18}$F]Fluoride ion was produced by $^{18}$O(p,n)$^{18}$F nuclear reaction using an IBA Cyclone 18/9 cyclotron (Louvain-la-Neuve, Belgium). Radioactivity levels were measured using a Veenstra VDC-405 dose calibrator (Joure, The Netherlands). Radiochemistry was carried out in homemade, remotely controlled synthesis units [24].

Analytical HPLC was performed on a Jasco (Easton, MD, USA) PU-2089 pump station equipped with a Luna Phenyl-hexyl column (5 μm, 250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) using $H_2O$/MeCN/TFA (60:40:0.1; *v*/*v*/*v*; 'Method A') or a Grace Alltima C18 column ((5 μm, 250 mm × 4.6 mm, Columbia, USA) using either $H_2O$/MeCN/TFA (55:45:0.1; *v*/*v*/*v*; 'Method B′) or $H_2O$/MeCN/TFA (50:50:0.1; *v*/*v*/*v*; 'Method C′) as eluent at a flow rate of 1 mL·min$^{-1}$, with a Jasco UV-2075 UV detector ($\lambda$ = 254 nm) and a NaI radioactivity detector (Raytest, Straubenhardt, Germany). Chromatograms were acquired with Raytest GINA Star software (version 5.01).

Preparative HPLC for isolation of radiotracers was performed on a Jasco PU-2089 pump station equipped with a Grace Alltima C18 column (5 μm, 250 mm × 10 mm, Columbia, USA) using $H_2O$/MeCN (40:60; *v*/*v*; 'Method D') as eluent at a flow rate of 4 mL·min$^{-1}$, a Jasco UV-1575 Plus UV detector ($\lambda$ = 254 nm), a custom made radioactivity detector and Jasco ChromNAV CFR software (version 1.14.01) for data acquisition.

Analytical HPLC for analysis of the non-polar fraction of the metabolite experiments was performed on Dionex (Sunnyvale, CA, USA) UltiMate 3000 HPLC equipment with Chromeleon software (version 6.8) equipped with a Phenomenex Gemini C18 column (5 μm, 10 mm × 250 mm, Phenomenex, Torrance, CA, USA) with eluent MeCN (A) and 0.1% TFA in water (B) as eluent according to the following scheme ('Method E'): 0 min, 80% B at 0.25 mL·min$^{-1}$; 0.5 min, 80% B at 3.0 mL·min$^{-1}$; 6.0 min, 40% B at 3.0 mL·min$^{-1}$; 12.5 min, 80% B at