European Journal of Pharmacology 755 (2015) 16–26

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Identification and characterisation of a prototype for a new class of competitive PPARγ antagonists

Tilo Knape [a], Daniel Flesch [c], Laura Kuchler [b], Lisa K. Sha [b], Annika K. Giegerich [b], Sandra Labocha [d], Nerea Ferreirós [d], Tobias Schmid [b], Mario Wurglics [c], Manfred Schubert-Zsilavecz [c], Eugen Proschak [c], Bernhard Brüne [b], Michael J. Parnham [a], Andreas von Knethen [b,*]

[a] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Project Group Translational Medicine and Pharmacology TMP, Theodor-Stern-Kai 7, 60596 Frankfurt am Main, Germany
[b] Institute of Biochemistry I - Pathobiochemistry, Faculty of Medicine, Goethe-University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
[c] Institute of Pharmaceutical Chemistry, Department of Biochemistry, Chemistry and Pharmacy, Goethe-University Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany
[d] Pharmazentrum frankfurt/ZAFES, Institute of Clinical Pharmacology, Goethe-University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form
18 February 2015
Accepted 19 February 2015
Available online 5 March 2015

Keywords:
Competitive PPARγ antagonist
MTTB
PPARγ
PPARγ agonist
PPARγ antagonist
SPPARγMs

ABSTRACT

Understanding of the physiological role of peroxisome proliferator-activated receptor gamma (PPARγ) offers new opportunities for the treatment of cancers, immune disorders and inflammatory diseases. In contrast to PPARγ agonists, few PPARγ antagonists have been studied, though they do exert immunomodulatory effects. Currently, no therapeutically useful PPARγ antagonist is commercially available. The aim of this study was to identify and kinetically characterise a new competitive PPARγ antagonist for therapeutic use.

A PPARγ-dependent transactivation assay was used to kinetically characterise (E)-2-(5-((4-methoxy-2-(trifluoromethyl)quinolin-6-yl)methoxy)-2-((4-(trifluoromethyl)benzyl)oxy)-benzylidene)-hexanoic acid (MTTB) in kidney, T and monocytic cell lines. Cytotoxic effects were analysed and intracellular accumulation of MTTB was assessed by tandem mass spectrometry (LC-MS/MS). Potential interactions of MTTB with the PPARγ protein were suggested by molecular docking analysis.

In contrast to non-competitive, irreversible inhibition caused by 2-chloro-5-nitrobenzanilide (GW9662), MTTB exhibited competitive antagonism against rosiglitazone in HEK293T and Jurkat T cells, with IC_{50} values in HEK293T cells of 4.3 μM and 1.6 μM, using the PPARγ ligand binding domain (PPARγ-LBD) and the full PPARγ protein, respectively. In all cell lines used, however, MTTB showed much higher intracellular accumulation than GW9662. MTTB alone exhibited weak partial agonistic effects and low cytotoxicity. Molecular docking of MTTB with the PPARγ-LBD supported direct interaction with the nuclear receptor.

MTTB is a promising prototype for a new class of competitive PPARγ antagonists. It has weak partial agonistic and clear competitive antagonistic characteristics associated with rapid cellular uptake. Compared to commercially available PPARγ modulators, this offers the possibility of dose regulation of PPARγ and immune responses.

Abbreviations: ANGPTL4, angiopoietin-like 4; AP-1, activator protein 1; aP2, adipocyte protein 2; CD36, cluster of differentiation 36; DMEM, Dulbecco's Modified Eagle's Medium; FABP4, fatty acid binding protein 4; FCS, foetal calf serum; FMOC-L-leucine, N-(9-fluorenylmethoxycarbonyl)-L-leucine; GLUT4, glucose transporter 4; GW9662, 2-chloro-5-nitrobenzanilide; HEK293T, human embryonic kidney 293 cells stably expressing the large T antigen of *simian vacuolating virus 40*; HuT-78, human T cell-78; LBD, ligand binding domain; LPL, lipoprotein lipase; MCC-555, netoglitazone; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MTTB, (E)-2-(5-((4-methoxy-2-(trifluoromethyl)quinolin-6-yl)methoxy)-2-((4-(trifluoromethyl) benzyl)oxy)-benzylidene) hexanoic acid; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor "kappa-light-chain-enhancer" of activated B cells; OD, optical density; PBS, phosphate buffered saline; PDK4, pyruvate dehydrogenase kinase, isozyme 4; PPARγ, peroxisome proliferator-activated receptor gamma; PPREs, PPARγ response elements; RPMI, Roswell Park Memorial Institute; RT-qPCR, reverse transcriptase-quantitative real-time PCR; RXRα, retinoid X receptor alpha; S.E.M., standard error of the mean; SPPARγMs, selective PPARγ modulators; TZDs, thiazolidinediones

* Correspondence to: Institute of Biochemistry I - Pathobiochemistry, Faculty of Medicine, Goethe-University Frankfurt, Theodor-Stern-Kai 7, Building 74, 60590 Frankfurt am Main, Germany. Tel.: +49 69 6301 6989; fax: +49 69 6301 4203.
E-mail address: vonknethen@biochem.uni-frankfurt.de (A. von Knethen).

http://dx.doi.org/10.1016/j.ejphar.2015.02.034
0014-2999/

1. Introduction

The ligand-dependent, activated transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear hormone receptor superfamily (Issemann and Green, 1990). It is predominantly expressed in adipose tissue and cells of the immune system (Chawla et al., 1994; Tontonoz et al., 1994, 1995). As with other members of the nuclear hormone receptor superfamily, the tissue- and ligand-specific activation and transcriptional target gene regulation of PPARγ is a multistep process (Kliewer et al., 1994; Seargent et al., 2004). It involves specific binding of several natural and synthetic ligands (Forman et al., 1995; Kliewer et al., 1995), heterodimerisation with the retinoid X receptor alpha (RXRα), the recognition and interaction of sequence-specific PPARγ response elements (PPREs) in the promoter region of their target genes and finally the recruitment of cofactors and other nuclear coregulatory proteins (Chawla et al., 2001; Kliewer et al., 2001; Nolte et al., 1998; Spiegelman and Heinrich, 2004).

Because of its role during adipogenesis and in glucose metabolism, in which PPARγ predominantly induces gene expression of adipocyte protein 2 (aP2, also called fatty acid binding protein 4, FABP4), cluster of differentiation 36 (CD36), lipoprotein lipase (LPL) or glucose transporter 4 (GLUT4), for example, it has been studied intensively as a therapeutic target (Lehmann et al., 1995; Nakano et al., 2006; Tontonoz et al., 1995). The most prominent synthetic PPARγ agonists with high receptor affinity are the thiazolidinediones (TZDs). These are used in type-2 diabetes mellitus, in which they improve insulin sensitivity and cause a concomitant reduction of free glucose levels (Rangwala and Lazar, 2004; Staels and Fruchart, 2005). PPARγ can also bind directly to other proteins and inhibits signal transduction. This capability, called transrepression, is mainly mediated by direct protein–protein interactions between PPARγ and other transcription factors, such as the nuclear factor "kappa-light-chain-enhancer" of activated B cells (NFkB), nuclear factor of activated T cells (NFAT) or the activator protein 1 (AP-1) (Chen et al., 2003; Wang et al., 2001; Yang et al., 2000). In this way, PPARγ inhibits pro-inflammatory signalling and induces an anti-inflammatory response (Pascual and Glass, 2006; Ricote and Glass, 2007). In this context, activation of PPARγ by TZDs is effective in reducing inflammation. In contrast to the proven beneficial effects and widespread use of the TZDs *in vivo*, they are also associated with a number of deleterious side-effects, serious tolerability and safety issues, including significant weight gain, peripheral oedema, congestive heart failure and bone fracture (Nesto et al., 2003; Nissen and Wolski, 2007).

On the other hand, PPARγ also induces apoptosis and thereby exerts immunosuppressive activity. Recently, PPARγ antagonism, especially by the PPARγ antagonist 2-chloro-5-nitrobenzanilide (GW9662) (Leesnitzer et al., 2002), has guided the development of new drugs and therapeutic strategies for a wide range of cancer types, such as breast cancer (Burton et al., 2008; Seargent et al., 2004), for regulation of adiposity (Nakano et al., 2006) and modulation of immunity and inflammatory diseases (Schmidt et al., 2011). Because of the irreversible binding of GW9962 to the PPARγ protein, it is not suitable for therapeutic use. The increased understanding of the physiological role and clinical relevance of PPARγ has emphasised the critical need for the discovery, identification and characterisation of new PPARγ agonists, antagonists or selective PPARγ modulators (SPPARγMs), while avoiding the known side-effects.

In this report, the identification and characterisation of (E)-2-(5-((4-methoxy-2-(trifluoromethyl)quinolin-6-yl)methoxy)-2-((4-(trifluoromethyl) benzyl)oxy)-benzylidene) hexanoic acid (MTTB) as a prototype for a new class of competitive PPARγ antagonists is described. Compared to the reference compound, GW9662, MTTB showed properties of a competitive PPARγ antagonist, with weak partial agonism, high intracellular uptake and low cytotoxicity *in vitro*. Because of its apparently reversible binding to the PPARγ protein, compounds such as MTTB may be well suited for controlled therapeutic use in immune disorders.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of the highest grade of purity and if not indicated otherwise, commercially available from AppliChem GmbH (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The PPARγ antagonist, GW9662, the SPPARγMs, N-(9-fluorenylmethoxycarbonyl)-L-leucine (FMOC-L-leucine) and netoglitazone (MCC-555) were acquired from Cayman Chemical Company (Ann Arbor, USA) and the PPARγ agonist, rosiglitazone, from Enzo Life Sciences GmbH (Lörrach, Germany). Cell culture media and supplements were purchased from PAA Laboratories GmbH (Cölbe, Germany) and Sigma-Aldrich Chemie GmbH.

2.2. MTTB compound

MTTB was designed, synthesised and kindly provided for experimental analysis by Daniel Flesch and Dr. Mario Wurglics (Institute of Pharmaceutical Chemistry, Goethe-University Frankfurt, Frankfurt am Main, Germany). It was dissolved in dimethyl sulfoxide (DMSO). MTTB was tested by C^{13}- and H^{1}-nuclear magnetic resonance, electrospray ionisation-mass spectrometry and combustion analysis to confirm the compound identity and purity.

2.3. Cell culture

Human T cell-78 (HuT-78) cells (Gazdar et al., 1980), Jurkat T cells (Schneider et al., 1977), human embryonic kidney 293 cells, stably expressing the large T antigen of *simian vacuolating virus 40* (HEK293T cells) (Graham et al., 1977) and human monocytic MonoMac6 cells (Ziegler-Heitbrock et al., 1988) were obtained from LGC Standards GmbH (Wesel, Germany). HuT-78 cells, Jurkat T cells and MonoMac6 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and HEK293T cells in Dulbecco's Modified Eagle's Medium (DMEM) in a humidified 5% carbon dioxide atmosphere at 37 °C. Both media contained 10% (v/v) heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. Additionally, the RPMI 1640 medium of the MonoMac6 cells contained 1 × non-essential amino acids, 1 mM oxalacetic acid, 1 mM sodium pyruvate and 9 mg of bovine insulin per ml. The media were changed three times a week and the cells passaged before reaching confluency. When using DMSO, in all cases, the final concentration of DMSO did not exceed 0.1%, a concentration that was not cytotoxic to the cell lines used.

2.4. Transient transfection of cultured cell lines

For the PPARγ-dependent transactivation assay, 1×10^4 HEK293T cells per well were seeded in 96-well plates and cultured overnight, as described above, to allow attachment of the HEK293T cells. The next day, the HEK293T cells were transiently transfected using the JetPRIME™ transfection reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany), as described by the manufacturer. Transfection was carried out with 0.01 μg per well