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The development and validation of an immunoassay for the measurement of anti-thymidine phosphorylase antibodies in mouse and dog sera

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ABSTRACT

Erythrocyte encapsulated thymidine phosphorylase (EE-TP) is under development as an enzyme replacement therapy for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a fatal metabolic disorder resulting from an inherited deficiency of the enzyme thymidine phosphorylase. We report here the development and validation of a sensitive electrochemiluminescent (ECL) bridging immunoassay to support Good Laboratory Practice (GLP)-compliant preclinical safety studies of EE-TP in the mouse and dog. Affinity-purified rabbit anti-*E. coli* thymidine phosphorylase (TP) antibody was used as a calibrator standard with an effective working range of 2.5–7500 ng/mL. The minimum required dilution (MRD) for both mouse and dog sera was 1:10. The mean analytical recoveries for anti-TP antibodies spiked into serum at 70 ng/mL and 7000 ng/mL were 117.9% and 93.2%, respectively for mouse, and 112.0% and 104.3%, respectively for dog. The intra-assay precision (coefficient of variation, CV) ranged between 1.1% and 8.0% in mouse serum, and 1.9% and 2.5% in dog serum. Inter-assay precision ranged between −1.6% and 6.7% in mouse serum, and −13.0% and −2.5% in dog serum. Assay cut-point/screening cut-point correction factors were 201.37 and 44.4, respectively for mouse and dog sera. For future analysis of positive test samples, less than 37.12% (mouse) and 31.41% (dog) inhibition of the assay signal in the confirmation assay will confer anti-TP antibody specificity. Assay drift and hook effects (prozone) were not observed. The intra-assay and inter-assay accuracy for robustness were within ±20%.

1. Introduction

The development of enzyme replacement therapies has seen the successful treatment of a range of inherited metabolic diseases, including adenosine deaminase deficiency, Gaucher disease and other lysosomal storage diseases.

Erythrocyte encapsulated thymidine phosphorylase (EE-TP) is currently under development as an orphan designated enzyme replacement therapy for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a fatal autosomal recessive disorder caused by mutations in the nuclear gene encoding for the enzyme thymidine phosphorylase leading to a plasma and tissue accumulation of thymidine and deoxyuridine [1–3]. This impairs mitochondrial DNA (mtDNA) replication and repair, causing mtDNA depletion and ultimately mitochondrial failure and the clinical features, which include enteric neuromyopathy, peripheral polyneuropathy and progressive external ophthalmoplegia [4,5]. MNGIE is relentlessly progressive and invariably leads to death in the second and third decades of life. EE-TP aims to correct the molecular lesion in MNGIE by encapsulating the deficient enzyme within the patient's own (autologous) erythrocytes in vitro, and then returning these to the patient where the pathological thymidine and deoxyuridine freely diffuse across the erythrocyte membrane to undergo metabolism to the normal products by the encapsulated enzyme. It is proposed that regular intravenous administrations of EE-TP to patients with MNGIE will lead to a sustained reduction or elimination of plasma and cellular thymidine and deoxyuridine concentrations, resulting in an amelioration of the intracellular nucleotide imbalances, and translation into clinical improvement [1,3]. EE-TP therapy has the advantage of prolonging the circulatory half-life of the enzyme and maintaining therapeutic blood levels, reducing the dosage and frequency of therapeutic interventions, and potentially minimising the immunogenic reactions which are observed in

Abbreviations: Anti-TP, anti-thymidine phosphorylase; CF, correction factor; CV, coefficient of variation; ECL, electrochemiluminescent; EE-TP, erythrocyte encapsulated thymidine phosphorylase; EMA, European Medicines Agency; FDA, Food and Drug Administration; GLP, Good Laboratory Practice; IR, instrument response; LLOQ, lower limit of quantification; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; MRD, minimal required dilution; mtDNA, mitochondrial DNA; PBS, phosphate-buffered saline; QC, quality control; RE, relative error; SCP, screening cut-point; SD, standard deviation; TP, thymidine phosphorylase; ULOQ, upper limit of quantification; VCP, validation cut-point.

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enzyme replacement therapies administered by the conventional route [6–9].

Therapeutic proteins carry a risk of inducing unexpected and unpredictable unwanted immune responses. Although even human sequence proteins have also been shown to elicit immune responses in some patients, the expected risks of immunogenic reactions following treatment with non-human sequence proteins are particularly high. The potential to generate immune responses varies considerably and can be influenced by several factors, including the biophysical characteristics of the protein, the route of delivery, the degree of exposure, and the use of concomitant medication during administration. Host factors may also play a part, for example, the patient's age, immune status, and genetic predisposition to mounting an immune response [10]. The consequences of immune reactions range from a transient appearance of antibodies, without any clinical consequence, to a decrease in therapeutic efficacy, and to severe life threatening conditions. The evaluation of the immunological potential of a drug candidate is thus an essential component of its development, from both a safety and efficacy perspective. Typically the testing strategy starts during pre-clinical development where the appropriate bioanalytical method is developed and validated for the detection of anti-drug antibodies.

The aim of this study was to assess the performance, validate and define the acceptance criteria and cut-point of an immunoassay method for the measurement of anti-thymidine phosphorylase (anti-TP) antibodies in mouse and dog sera to support Good Laboratory Practice (GLP)-compliant pre-clinical safety studies of EE-TP in the Balb/c mouse and Beagle dog.

2. Materials and methods

Assays were validated according to Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines and the study was conducted in compliance with Good Laboratory Practice (GLP) standards [11–19].

2.1. Reagents

Recombinant *E. coli* thymidine phosphorylase (TP, 26.6 mg/mL) the therapeutic enzyme candidate produced for the GLP pre-clinical studies were employed in the development and validation of this immunoassay (Sigma–Aldrich, Israel). Affinity-purified rabbit anti-TP antibody (0.518 mg/mL) was custom produced (Open Biosystems, USA). The wash buffer was phosphate-buffered saline (PBS, Sigma–Aldrich, UK) with 0.05% Tween 20 (Sigma–Aldrich, UK). Blocker A solution consisted of 5% (w/v) Blocker A in MSD phosphate buffer (Meso Scale Discovery, USA). The assay buffer (PBS, 0.05% (v/v) Tween 20, 1% (w/v) Blocker A) consisted of 1 volume of 5% Blocker A solution and 4 volumes of wash buffer. The Read buffer T (4×) was supplied by Meso Scale Discovery, USA and was diluted 1 in 2 with ultra high purity grade water.

2.2. Preparation of biotinylated and sulfo-TAG TP conjugates

The bridging immunoassay format requires TP to be conjugated with biotin and conjugated with sulfo-TAG; biotinylated TP served as the capture antigen and the sulfo-TAG conjugated TP as the detection antigen. TP was conjugated with biotin using EZ-Link sulfo-NHS biotin kit (Pierce Biotechnology, USA). TP was first desalted using a Zebra desalt spin column (Pierce Biotechnology, USA) equilibrated with PBS, with centrifugation at 1000 × *g* for 2 min, and then diluted with PBS to form a secondary stock solution of 10 mg/mL. A calculated volume of EZ-Link sulfo-NHS biotin was added directly to 500 μL of TP secondary stock solution and incubated for 30 min, with mixing at room temperature. Biotin conjugated TP was desalted using a Zebra desalt spin column equilibrated with PBS, with centrifugation at 1000 × *g* for 2 min and stored at 4 °C until use.

Conjugation of TP with sulfo-TAG was performed using Sulfo-TAG NHS ester (Meso Scale Discovery, USA). TP was first desalted as described above and diluted with PBS to form a secondary stock solution of 10 mg/mL. A calculated volume of sulfo-TAG ester was added to 350 μL TP secondary stock solution and incubated with mixing for 2 h at room temperature. The sulfo-TAG TP conjugate was desalted as described above and stored at 4 °C until use.

2.3. Negative control sera pools

Individual dog ($n = 11$) and mouse ($n = 15$) sera samples were screened by analysis ($n = 1$ in duplicate) against positive control calibration curves for the presence of anti-TP antibodies. Blank sera from individual dogs and mice were pooled to produce negative control dog and mouse sera pools, respectively. These were stored at −20 °C until required for defining the cut-point and preparing quality control (QC) samples and calibration curves.

2.4. Positive control standards

Primary positive control standard stock of anti-TP antibodies (0.518 mg/mL) was diluted with negative control sera (dog or mouse as appropriate) to form a secondary positive control standard stock of 100 μg/mL and this was further diluted with negative control sera (dog or mouse as appropriate) to form a tertiary positive control standard stock of 25,000 ng/mL. The tertiary standard stock was diluted with negative control sera (dog or mouse as appropriate) to produce working standards over the range 2.50–7500 ng/mL. Prior to analysis the positive control standards were diluted 1 in 10 with assay buffer.

2.5. Validation QC samples

Pooled negative control sera from dog and mouse were spiked with anti-TP antibodies to provide three concentrations relative to the cut point: low (just above cut point), middle (mid assay dynamic range) and high (high assay dynamic range). The negative control sera were used as the negative control QC standards. All QC samples were prepared in 20 μL aliquots and stored at −70 °C. Prior to assay the QC samples were diluted 1 in 10 using 10 μL QC sample and 90 μL assay buffer.

2.6. Immunoassay procedure

Assays were performed using a bridging electrochemiluminescent (ECL) immunoassay. Briefly, 25 μL positive control standard, blank, negative control, QC samples and test samples (with or without thymidine phosphorylase as appropriate) were added to wells of a polypropylene 96-well plate (Fisher Scientific, UK) followed by 50 μL of conjugate mastermix (0.0625 μg/mL biotin TP/0.0313 μg/mL sulfo-TAG TP, using assay buffer as the diluent). The plates were covered and incubated at room temperature for 2 h on a microtitre plate shaker set at 600 rpm (Micromix, DPC Ltd, Wales). Following the start of this incubation, 150 μL Blocker A solution were added to each well of a multi-array 96-well standard streptavidin plate (Meso Scale Discovery, USA), which was then covered and incubated at room temperature for 2 h with shaking at 600 rpm. The multi-array plate was then washed three times with 200 μL wash buffer per well using a microtitre plate washer (Wellwash, Thermo Life Sciences Ltd, UK). The last wash was aspirated and the plate blotted dry by inversion over absorbant paper. Fifty microlitres from each well of the polypropylene 96-well plate were transferred to corresponding duplicate wells in the multi-array 96-well standard streptavidin plate. The multi-array plate was then