Therapy using erythrocyte-encapsulated enzyme has the advantage of prolonging the half-life of the enzyme and maintaining therapeutic blood levels, reducing the dose and frequency of therapeutic interventions, and preventing the need for expensive chemical modification. The therapeutic index can be strongly improved, especially by reducing immunogenic reactions, which are often observed in enzyme therapy administered by the conventional route. Two products are presented in this paper: erythrocyte-encapsulated L-asparaginase and erythrocyte-encapsulated thymidine phosphorylase. For the first, the native enzyme has been the mainstay in the treatment of lymphoblastic leukemia for decades. Improving the therapeutic index by utilizing this new formulation now offers new possibilities in cancer therapy. The second product is proposed for patients diagnosed with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disorder of nucleotide metabolism caused by mutations in the nuclear TYMP gene (thymidine phosphorylase deficiency). These two approaches illustrate how this new formulation works as an enzyme bioreactor in the treatment of cancer and in enzyme replacement therapy.

Key words: Erythrocytes – Asparaginase – Cancer therapy – MNGIE – Thymidine phosphorylase – Enzyme replacement therapy

ERYTHROCYTE-ENCAPSULATED L-ASPARAGINASE (GR-ASPA)

Background

Asparagine synthetase (ASNS)-deficient cancerous cells are dependent on a plasma source of asparagine for survival (Fig. 1). This is well established in the case of acute lymphoblastic leukemia (ALL), but can also be extended to other hematological or solid malignancies (1-3). Rapid depletion of asparagine results in the selective death of tumor cells, whereas normal cells are able themselves to synthesize asparagine. Asparaginase hydrolyzes asparagine to aspartate and ammonia. Three preparations of asparaginase are available: one derived from Escherichia coli, another derived from Erwinia carotovora and pegaspargase, the polyethylene glycol succinimidyl conjugate of E. coli L-asparaginase. However, several side effects (anaphylactic reactions, coagulopathy especially) are associated with these preparations, which can be life-threatening for some fragile patients (4). In addition, the half-lives for the free forms are very short (1.24 days for E. coli asparaginase and 0.65 days for Erwinia) (5).

Although the pegylated formulation extends the half-life of the enzyme to 5.73 days, several injections are still required to obtain therapeutic efficacy. There is therefore a need for a formulation of asparaginase with a longer enzyme half-life and a better safety profile, particularly for fragile patients. E. coli L-asparaginase entrapped inside homologous red blood cells (GR-ASPA), is a new pharmaceutical formulation of the enzyme that was developed to respond to these needs; GR-ASPA is a cell-based medicinal product presenting an improved safety profile and an enhancement of the pharmacokinetics and pharmacodynamics of the enzyme.
**Product description**

GR-ASPA consists of *E. coli* L-asparaginase loaded inside red blood cells (RBCs) preserved in SAG-mannitol (SAG-M) solution. L-Asparaginase is an enzyme which hydrolyzes free circulating asparagine. The cleavage of asparagine produces ammonia (NH₃) and aspartic acid. The activity of asparaginase is expressed as International Units (IU), and is defined as the quantity of ammonia (in μmol) produced per minute at 37 °C.

Packed homologous RBCs provided by the blood bank are employed. These are ABO typed to be compatible with the recipient (the patient). The volume of GR-ASPA in the final product bag is adapted according to the dose and the weight of the patient. GR-ASPA is presented in polyvinyl chloride (PVC) bags. This product was granted orphan drug designation by the EMA and the FDA.

**Mechanism of action**

As demonstrated by several teams performing different technologies of entrapment (6-11), L-asparaginase remains catalytically active entrapped inside the RBCs, while asparagine is physiologically and actively “pumped” through the membrane of the RBCs using the N⁺ channel system. Thus, plasma asparagine is transported through the RBC membrane to the intracellular compartment, where it is cleaved by the entrapped L-asparaginase (Fig. 2). L-Asparaginase-loaded RBCs thus perform as “cellular circulating bioreactors”. Due to the constraints of the RBC membrane, the enzyme is protected from reactions which would result from systemic exposure, thus reducing the occurrence of side effects. In fact, GR-ASPA concomitantly works with a “free” extracellular fraction of asparaginase contained within the GR-ASPA bag, as a consequence of the liberation of asparaginase during the natural hemolysis of the RBCs. However, the contribution of the free fraction of GR-ASPA in the depletion of plasma asparagine is very small. The constantly liberated asparaginase in the bloodstream due to intravascular hemolysis is also very low and is not able alone to deplete the plasma asparagine, but does contribute to the action of the cellular bioreactor. Pharmacokinetics of GR-ASPA (based on asparaginase activity) have demonstrated that asparaginase loaded in RBCs has a half-life of approximately 1 month (11).

**Product manufacturing**

GR-ASPA is a cell-based medicinal product manufactured as a personalized medicine (Fig. 3). Briefly, the physician prescribes GR-ASPA, and the hospital then places an order for the product with ERYTECH Pharma. The patient’s weight, ABO blood type and a valid irregular antibody screening (IAS) are attached to the order. The qualified person on the manufacturing site immediately places an order with the blood bank for a leukocyte-reduced packed RBC unit compatible with the patient. The product is manufactured under current Good Manufacturing Practices (cGMP) using a 3-hour automated process. A washing step removes the preservative solution from the packed RBCs. L-Asparaginase is mixed with the RBCs.
washed suspension and the osmotic fragility of the RBCs is measured rapidly to determine the parameters for the next step. The RBC suspension is dialyzed against a hypotonic solution and resealed. During the lysis step, water entering the erythrocyte via the membrane leads to the formation of pores within the cell membrane. Thus, the enzyme enters the erythrocyte through these pores. The resealing solution allows the recovering of isotonicity and closure of the pores. A final washing step allows product purification, eliminating non-entrapped drug and hemolyzed cells. Finally, the preservative solution is added. According to the prescribed dose (IU/kg), the volume of GR-ASPA is adjusted in the final product PVC bag. Indeed, the product release specifications are constant and reproducible from batch to batch, such as the corpuscular concentration of L-asparaginase (117 ± 19 IU/mL), extracellular hemoglobin (0.11 ± 0.03 g/dL), osmotic fragility (< 3.5 g/L of NaCl) and extracellular L-asparaginase (0.4 ± 0.2 IU/mL, i.e., < 1% of the total activity).

Based on the specifications outlined in Table I, the qualified person releases the product and ships it (kept at 2-8 °C) to the prescriber; the time between placing an order and product delivery is less than 2 days. Currently a 72-hour shelf-life for the final product is considered. SAG-M solution preserves GR-ASPA for up to D+72 hours between +2 °C and +8 °C and then at room temperature (between +20 °C and +25 °C) during 6 hours (Table II).

Upon receipt, the nurse checks the integrity of the bag and the storage temperature tracer. Under clinical trial conditions, the product arrives directly to the clinical department where the patient attends. However, in the future, the hospital pharmacy will be responsible for the ordering and receipt of the product. The product is injected intravenously within 15-30 minutes, with careful adherence to the guidelines of current transfusion practice. A traceability system assures the linkage between the blood bank and the patient’s hospital.

**Preclinical data**

The regulatory bodies agreed that it was not relevant to assess the toxicity of this human-based product in a xenogenic model, for example, the mouse. Preclinical studies were instead performed with mouse RBC-encapsulated asparaginase, manufactured using a similar process, but with some adaptations to obtain a similar and suitable product. For example, the addition of 6% bovine serum albumin (BSA) to SAG-M was required to stabilize the asparaginase-loaded mouse RBCs, whereas in human RBCs SAG-M alone was sufficient.

**Non-clinical pharmacokinetics/pharmacodynamics**

Mouse GR-ASPA (L-asparaginase entrapped in mouse RBCs) was injected to OF1 mice as a single dose of 100 IU/kg. Mice were sacrificed and blood was collected at several time points after injection. The half-life of asparaginase encapsulated in RBCs is 8.55 days. Total L-asparagine depletion is observed 15 minutes after injection of mouse GR-ASPA until 28 days. At 34 days, L-asparaginase activity is no longer detectable and plasma L-asparagine returned to normal values. Fifteen minutes after mouse GR-ASPA injection, L-aspartate
Table II. Stability data of batches of GR-ASPA.

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Storage time (h)</th>
<th>Encapsulated L-asparaginase (IU/mL)</th>
<th>Extracellular L-asparaginase (IU/mL)</th>
<th>Extracellular hemoglobin (g/dL)</th>
<th>Osmotic fragility (g/L NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
<td>53.1 ± 4.0</td>
<td>0.31 ± 0.04</td>
<td>0.1 ± 0.0</td>
<td>&lt; 3.5</td>
</tr>
<tr>
<td>2-8 °C</td>
<td>24</td>
<td>51.6 ± 3.6</td>
<td>2.30 ± 0.32</td>
<td>0.6 ± 0.1</td>
<td>&lt; 3.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>50.6 ± 1.8</td>
<td>2.98 ± 0.45</td>
<td>0.8 ± 0.1</td>
<td>&lt; 3.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>47.3 ± 3.0</td>
<td>3.28 ± 0.54</td>
<td>0.9 ± 0.1</td>
<td>&lt; 3.5</td>
</tr>
<tr>
<td>20-25 °C</td>
<td>(72) + 6</td>
<td>48.9 ± 3.5</td>
<td>3.50 ± 0.43</td>
<td>1.0 ± 0.1</td>
<td>&lt; 3.5</td>
</tr>
</tbody>
</table>

increased from 12.33 μM to > 25 μM and remained elevated for 28 days. L-Aspartate being the resulting product of the conversion of L-asparagine, its concentration concomitantly increased when the L-asparagine concentration decreased. The normal L-glutamine concentration in OF1 mice is around 450 μM. Fifteen minutes after mouse GR-ASPA injection, the concentration decreased to 260.81 μM but recovered to a normal value from day 1. This transitory change is due to the L-glutaminase activity of extracellular L-asparaginase present in the mouse GR-ASPA preparation.

Non-clinical toxicity

The potential toxicity of mouse GR-ASPA was evaluated in mice following one or two intravenous injections at 200 IU/kg at 14-day intervals. No toxicity was observed after the first injection. Adverse effects (piloerection, hypoactivity, dyspnea, deaths) were noted after the second injection but appeared both in mice treated with GR-ASPA and the vehicle. These effects were therefore considered to be compatible with immediate hypersensitivity to the BSA present in the preservative solution. Minimal, namely perivenous, inflammation was observed in GR-ASPA-treated animals at the injection sites, mainly in animals injected twice; however, it was considered to be non-adverse. No treatment-related disorders were noted, especially in the pancreas, liver, spleen, kidney, heart, brain, intestine or thymus. In conclusion, one injection of mouse GR-ASPA was well tolerated, since neither clinical signs, changes in body weight or treatment-related findings at hematological and blood biochemistry investigations were noted in treated mice that remained asparagine-depleted for more than 16 days. No definitive conclusion can be drawn following repeated injections, as adverse effects may have been altered/masked by the hypersensitivity reaction induced by the formulation necessary to stabilize the product.

ASNS expression is low in several human cancer types

In addition to the extended use of asparaginase to other leukemias (e.g., myeloid leukemia) (12), we assessed if other human cancers are also deficient in ASNS and thus could be potentially sensitive to L-asparaginase treatment (13). We determined ASNS expression in several human cancer types using different tissue microarrays (TMAs) representing a total of 1,590 tumors. ASNS was faintly detectable, with a large proportion of cases harboring low expression (e.g., ovarian, liver, renal, bladder, head and neck and colon carcinomas).

Table I. GR-ASPA specifications.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IDENTIFICATION</strong></td>
<td></td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td>Positive</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>TEST</strong></td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)</td>
<td>70-95 fL</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (MCHC)</td>
<td>23-35 g/dL</td>
</tr>
<tr>
<td>Erythrocyte count: number of cells injected</td>
<td>XXX M/μL (for information)</td>
</tr>
<tr>
<td>Extracellular hemoglobin</td>
<td>≤ 0.2 g/dL of suspension</td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>≤ 4.5 g/L of NaCl</td>
</tr>
<tr>
<td><strong>ASSAYS</strong></td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular L-asparaginase concentration</td>
<td>78-146 IU/L</td>
</tr>
<tr>
<td><strong>ACTIVITY</strong></td>
<td></td>
</tr>
<tr>
<td>Volume of suspension</td>
<td>XXX mL (for information)</td>
</tr>
<tr>
<td>Total L-asparaginase activity in IU per bag</td>
<td>XXX IU (doses prescribed)</td>
</tr>
<tr>
<td>Total L-asparaginase activity according to the prescription</td>
<td>± 10%</td>
</tr>
<tr>
<td>Extracellular L-asparaginase activity per bag</td>
<td>≤ 2%</td>
</tr>
<tr>
<td><strong>OTHERS</strong></td>
<td></td>
</tr>
<tr>
<td>Integrity of bag (watertight): no leaks</td>
<td>Complies</td>
</tr>
<tr>
<td>Three samples sealed to the bag for cross-matching</td>
<td>Complies</td>
</tr>
<tr>
<td>Conformity of the barcode between initial RBC donor and final product</td>
<td>Complies</td>
</tr>
<tr>
<td>Compliance of name, surname, birth date of the patient</td>
<td>Complies</td>
</tr>
<tr>
<td>Compliance of blood group donor/patient/medicinal product</td>
<td>Complies</td>
</tr>
<tr>
<td>Atypical antibodies on previsional injection date (valid for 72 hours)</td>
<td>Complies</td>
</tr>
</tbody>
</table>

RBC, red blood cell.
Regarding pancreatic carcinoma, it appeared that the proportions of ASNS measured using an intensity scoring system were similar to those previously observed with standard tissue slides: ASNS-negative and low ASNS tumors represented 70.9% of cases (versus 68.7% for standard slides), whereas 29.1% of the tumors were positive and highly positive for ASNS (versus 31.3%). As evaluations of TMA and whole tissue sections were performed by different pathologists, these concordant data strengthen the validity of the staining method. Overall, these data suggest that a large proportion of pancreatic and other tumors might be sensitive to L-asparaginase therapy.

**In vivo antitumor efficacy studies**

SW1990, a human pancreas adenocarcinoma cell line, has a low ASNS expression and is sensitive in vitro to L-asparaginase. This cell line was used to establish a mouse xenograft model to assess the antitumor effect of systemic asparagine depletion induced by GR-ASPA. The mice were treated with L-asparaginase-loaded RBCs, pRBCs (processed RBCs but without any L-asparaginase encapsulation) or vehicle (the preservative solution: SAG-M + 6% BSA). Treatments were initiated 7 days after subcutaneous cell inoculation, when tumor volumes were about 80-120 mm³, and tumor growth was measured twice a week. Tumor growth was clearly delayed when mice were treated with entrapped L-asparaginase when compared with pRBCs or vehicle. At day 35, 75% of mice given GR-ASPA showed a tumor volume of <1000 mm³, while only 42% and 50% of mice treated with pRBCs and vehicle, respectively, had a similar tumor volume.

**Clinical data**

To date, 178 batches of GR-ASPA have been administered to 94 patients enrolled in 4 clinical trials. The first study was published where GR-ASPA was used to treat ALL patients in relapse (11). The GRASPALL 2005-01 study, a multicenter, randomized, controlled phase I/II trial, investigated three doses of GR-ASPA on duration of asparagine depletion in patients with ALL in first relapse. Adults and children were allocated in two balanced strata. Between February 2006 and April 2008, 18 patients received GR-ASPA (50 IU/kg; n = 6; 100 IU/kg; n = 6; 150 IU/kg; n = 6) after randomization, and 6 patients were assigned to the E. coli native L-asparaginase (Kidrolase®) control group. GR-ASPA was shown to be effective in depleting L-asparagine in children and adults with relapsed ALL. A single injection of 150 IU/kg of GR-ASPA provided similar activity to eight lots of 10,000 IU/m² intravenous injections of free asparaginase given every 3 days. In this study it was observed that a dose of 150 IU/kg allowed depletion for approximately 18 days. The half-life of encapsulated asparaginase activity was extended to 40 days, while the free asparaginase half-life is about 1 day. The safety profile of GR-ASPA showed a reduction in the number of allergic reactions and severity, and a trend towards fewer coagulation disorders. Other expected adverse events were comparable to free asparaginase and there was no difference between the three doses of GR-ASPA in term of frequency of “expected” adverse effects (Table III).

The parameters used to define the adverse events were those of the Common Terminology Criteria for Adverse Events v3.0.

**Table III. Frequency of “expected” adverse events for free asparaginase and GR-ASPA groups during the complete follow-up (4 months) in the study GRASPALL 2005-01.**

<table>
<thead>
<tr>
<th>adverse event</th>
<th>Free ASPA (n = 6)</th>
<th>GR-ASPA (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>allergic reaction</td>
<td>3 (50%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Grade III &amp; IV</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Grade II</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Coagulation disorders (ATIII decrease)</td>
<td>4 (66%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>With clinical thrombosis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Without clinical signs</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>1 (17%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td>Hepatic disorders</td>
<td>5 (83%)</td>
<td>9 (50%)</td>
</tr>
<tr>
<td>Hypoalbuminemia/proteinemia</td>
<td>2 (33%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>ATIII, antithrombin III</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With regard to hepatotoxicity, elevated levels of bilirubin and/or transaminases were reported only for grades III and IV. For the hypoproteidemia/hypoalbuminemia and coagulation disorders, all the events (grade I-IV) were reported. GR-ASPA was associated with fewer coagulation disorders compared to free asparaginase, and particularly a reduced decrease in antithrombin III. It has been suggested that glutaminase activity of E. coli or E. carotovora asparaginases may be responsible for hepatic toxicities associated with a decrease in plasma antithrombin III activity and albumin (14). We suggest that the reduction in side effects, especially liver toxicity and coagulation disorders, could be related to the significantly reduced glutamine depletion observed with GR-ASPA compared with free asparaginase (data not shown). Although it has been reported that glutamine deamination by asparaginase may contribute to enhanced leukemic cell apoptosis (15), a glutaminase-free asparaginase preparation from Vibrio succinogenes (Welinlina) has shown potent anti-lymphoma activity without hepatotoxicity (16). Another explanation could be a direct interaction between L-asparaginase and plasma coagulation factors, which could increase their degradation (17). The dose of 150 IU/kg is currently being used in a phase II/III pivotal trial in children and adults with ALL relapse. The dose of 100 IU/kg is optimal (efficacy/tolerance) in newly diagnosed patients over 55 years. Indeed, this frail subpopulation of patients has difficulty with the current forms of L-asparaginase due to the known side effects. A phase I clinical trial in pancreatic carcinoma confirmed that the maximal tolerated dose is > 150 IU/kg (the highest tested dose in the trial) for this form of L-asparaginase, even in solid tumors, offering new perspectives in patients where asparagine synthetase in tumor cells is reduced.

**Conclusion**

GR-ASPA is a new formulation of L-asparaginase with a good safety profile and enhanced enzymatic activity. This permits new therapeutic perspectives, particularly in the treatment of fragile patients and...
cancers other than ALL, for example, other leukemias (12) or solid tumors with a low expression of ASNS.

**ERYTHROCYTE-ENCAPSULATED THYMIDINE PHOSPHORYLASE (EE-TP)**

**Background**

The last two decades have seen the introduction of enzyme replacement therapies for the successful treatment of rare, inherited metabolic diseases. The administration of the missing enzyme, usually by intravenous or subcutaneous injection, enables the elimination of the pathological substrates which accumulate in these metabolic disorders, leading to an attenuation of clinical symptoms and disease progression. Chemical modifications of the native enzyme are often employed in the manufacturing process to increase protein stability, decrease immunogenicity and enable targeting of enzyme to the appropriate cellular compartment. The development of recombinant DNA techniques and overexpressing cells has made it possible to produce quantities of pure enzyme on a commercial scale.

Erythrocyte-encapsulated enzyme replacement therapy is an alternative approach to enzyme replacement therapy and aims to correct the fundamental lesion in inherited metabolic diseases by encapsulating the deficient enzyme within the patient’s own (autologous) erythrocytes in vitro, and then returning these to the patient. This approach is applicable to disorders where the elevated plasma pathological metabolites are able to freely diffuse across the erythrocyte membrane into the cell to undergo metabolism to the normal product by the encapsulated enzyme. The product is then free to diffuse out of the cell into the blood plasma, where it undergoes further metabolism as normal. The rationale for the development of erythrocyte-mediated enzyme replacement therapy is based on: 1) prolonging the circulatory half-life of the enzyme and maintaining therapeutic blood levels; 2) reducing the dose and frequency of therapeutic interventions; 3) negating the need for expensive chemical modification of the native enzyme; and 4) minimizing the immunogenic reactions which are often observed in enzyme replacement therapies administered by the conventional route (18, 19). The efficacy and safety of long-term erythrocyte-mediated enzyme replacement therapy has been demonstrated in the treatment of a patient with severe combined immunodeficiency due to adenosine deaminase deficiency with erythrocyte-encapsulated adenosine deaminase (EE-ADA) for over 15 years (20-22).

More recently, this therapeutic approach has been applied to the treatment of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disorder of nucleotide metabolism caused by mutations in the nuclear TYMP gene. This gene encodes for thymidine phosphorylase (TP; EC 2.4.2.4), the enzyme required for the normal metabolism of the pyrimidine deoxynucleosides thymidine and deoxyuridine (23). Mutations in the TYMP gene result in a complete or partial absence of TP activity, leading to an accumulation of thymidine and deoxyuridine in tissues and body fluids (24-29). Elevated systemic concentrations of these deoxynucleosides are mirrored by elevated intracellular concentrations of their corresponding triphosphates. This perturbs the physiological equilibrium of the four deoxynucleoside triphosphates within the mitochondria, thereby interfering with the normal replication of mitochondrial DNA (mtDNA), leading to multiple deletions, somatic point mutations and depletion of mtDNA (24, 26, 30, 31), and ultimately, mitochondrial failure (24, 26, 28). mtDNA encodes for polypeptides, transfer RNA (tRNA) and ribosomal RNA (rRNA) required for the synthesis of enzymes involved in oxidative phosphorylation. The consequent failure of cellular energy production is believed to directly cause the cardinal central clinical manifestations through damage to the nervous and muscular systems. The disease is relentlessly progressive and degenerative, with an almost universally fatal outcome.

There are no evidence-based treatments for MNGIE and thus there is a critical requirement to develop a treatment that will prove beneficial. Allogeneic hematopoietic stem cell transplant (HSCT) offers the possibility of a permanent correction of TP deficiency, but is still highly experimental, carrying a 44% mortality risk. It is limited by the availability of a matched donor, and patients are often in a poor clinical condition with an impaired capacity to tolerate transplant-related problems. The administration of HSCT to these patients presents pharmacological challenges in terms of administering drugs with possible mitochondrial toxicity, and the requirement for parental administration due to disturbed gastrointestinal function and impairment of absorption. A published consensus proposal for standardizing an approach to allogeneic HSCT in MNGIE recommends restricting the recruitment of patients with an optimal donor to those without irreversible end-stage disease (32). Thus, for many patients there is no treatment option and clinical management is based on symptom relief and palliation. EE-TP therapy is proposed for patients diagnosed with MNGIE due to thymidine phosphorylase deficiency. In practice, therapy is likely to be sought for patients in whom the risk of mortality from allogeneic HSCT would be too high, and also for those in whom there is no matched donor. EE-TP would also be indicated as a rescue or maintenance therapy prior to the availability of a suitable HSCT donor.

**Product description**

The active drug substance is recombinant *E. coli* TP, an enzyme which catalyzes the reversible phosphorylation of the pyrimidine nucleosides thymidine and deoxyuridine to 2-deoxyribose 1-phosphate and their respective bases, thymine and uracil (Fig. 4). The enzyme is part of the pyrimidine nucleoside salvage metabolic pathway and allows pyrimidine bases to be recycled for nucleotide biosynthesis, while the pentose 1-phosphates are converted to intermediates of the pentose phosphate shunt and glycolysis. To formulate the drug product EE-TP, TP is encapsulated within the patient’s own autologous erythrocytes. Orphan drug designation for EE-TP was granted by the European Commission in 2011 and by the FDA in 2010.

**Mechanism of action**

The rationale for the development of EE-TP is based on thymidine and deoxyuridine being able to freely diffuse across the erythrocyte membrane via nucleoside transporters into the cell, where the encapsulated enzyme catalyzes its metabolism to the normal products (thymine and uracil). The products are then free to diffuse out of the cell into the blood plasma, where they will be further metabolized as normal. Figure 5 shows the mechanism of EE-TP action.
Figure 4. Reactions catalyzed by thymidine phosphorylase.

Figure 5. EE-TP mechanism of action. Thymidine and deoxyuridine freely diffuse across the erythrocyte membrane via nucleoside transporters into the cell, where the encapsulated enzyme catalyzes its metabolite to the normal product (thymine and uracil).
EE-TP delivers its efficacy through the sustained reduction or elimination of elevated plasma thymidine and deoxyuridine concentrations. This results in a withdrawal of these metabolites from the cellular compartments and ameliorates the intramitochondrial nucleotide imbalances. By preventing further damage to mtDNA, mitochondrial turnover will allow mitochondrial regeneration and thus the restoration of mitochondrial function, the derangement of which is believed to cause the tissue damage in the nervous and muscle systems. EE-TP aims to arrest and reverse the progression of the clinical disease, with consequent clinical improvement, by reversal of the mitochondrial dysfunction in MNGIE.

**Product manufacturing**

EE-TP is in the early stages of clinical development and is currently manufactured in accordance with the provisions of Schedule 1 of The Medicines for Human Use (Marketing Authorisations Etc.) Regulations SI 1994/3144, where Schedule 1 provides an exemption from the need for a marketing authorization for a relevant medicinal product which is supplied to fill a “special need”. EE-TP is prepared under a Manufacturer’s Specials Licence (MS) held by St. George’s National Health Service (NHS) Trust Pharmacy.

The manufacturing process takes 7 hours and is conducted within ducted, negative-pressure isolators within hospital radiopharmacy facilities. A predetermined volume of the patient’s erythrocytes is removed and subjected to a reversible hypo-osmotic dialysis process; under hypo-osmotic conditions, the erythrocytes swell due to an influx of water until, at a critical size, pores form in the membrane. While permeable, TP enters the erythrocytes by diffusion. The permeability is reversed by restoration of iso-osmotic conditions, encapsulating the enzyme within the erythrocytes, which are then returned to the patient (33). The activity of TP which is encapsulated is specific to the patient’s erythrocytes and prescribed dose (IU/kg) and ranges between 22 and 108 IU/1 x 10¹⁰ cells. For intravenous administration, the EE-TP is reconstituted with autologous plasma, which is removed prior to the enzyme encapsulation procedure. Erythrocytes which were excess to the requirements of the encapsulation procedure and lymphocytes removed before the encapsulation procedure are also added to this cell suspension.

Specifications for EE-TP manufactured under a special’s license are outlined in Table IV. It is anticipated that additional specifications will be added during clinical development.

In the clinical trial setting, it is intended that reinfusion will take place within 48 hours of venesection. Samples of preclinical batches of EE-TP stored at 4 °C and 22 °C up to 96 hours demonstrated that storage temperature and time had no significant effect on TP activity (see Table V). This study confirmed the stability of EE-TP over the proposed permitted maximal time delay to infusion (96 hours).

**Preclinical data**

**Non-clinical pharmacodynamics**

Human erythrocytes loaded with recombinant *E. coli* thymidine phosphorylase (38 IU/1 x 10¹⁰ cells) and incubated at a hematocrit of 25% in either phosphate-buffered saline (pH 7.4 containing 15 μmol/L thymidine) or in plasma taken from a patient with MNGIE were shown to metabolize 87% of the extracellular thymidine after 5 minutes, and by 40 minutes all the thymidine had been metabolized. TP activity was undetectable in the extracellular media. These in vitro studies provided evidence that extracellular thymidine is able to permeate the erythrocyte membrane and undergo metabolism by the encapsulated TP, and provided justification for in vivo studies of EE-TP in patients with MNGIE.

**Non-clinical toxicity**

Twice-weekly infusions of EE-TP, achieving an exposure approximately four times higher than the proposed clinical dose, were tolerated in the BALB/c mouse and beagle dog over a 4-week period. Three distinct safety findings were identified in these studies:

- The intermittent intravenous injection of EE-TP to BALB/c mice resulted in thrombi/emboli in the lungs and enlargement of the spleen. There was generally no relationship to the number of doses administered and the findings were also seen in the con-
prior to initiating EE-TP therapy, a chromium [51Cr]-labeling study of attends a morning clinic for blood harvesting, and then returns later established Clinical Research Ethics Committees or the NHS Novel Human Use (Marketing Authorisations Etc.) Regulations SI accordance with the provisions of Schedule 1 of The Medicines for a confirmed diagnosis of MNGIE (22, 34). The supply of EE-TP is in compassionate pilot clinical evaluation of EE-TP in two patients with study of EE-TP in a single patient diagnosed with MNGIE, and a Clinical experience with EE-TP is derived from a proof–of-concept Clinical data Y. Godfrin and B.E. BaxC

**Clinical data**

Clinical experience with EE-TP is derived from a proof–of-concept study of EE-TP in a single patient diagnosed with MNGIE, and a compassionate pilot clinical evaluation of EE-TP in two patients with a confirmed diagnosis of MNGIE (22, 34). The supply of EE-TP is in accordance with the provisions of Schedule 1 of The Medicines for Human Use (Marketing Authorisations Etc.) Regulations SI 1994/3144. The studies were approved by constitutionally established Clinical Research Ethics Committees or the NHS Novel Procedures Committee. In the current clinical setting, the patient attends a morning clinic for blood harvesting, and then returns later in the day for reinfusion of autologous enzyme-loaded erythrocytes. Prior to initiating EE-TP therapy, a chromium [51Cr]-labeling study of EE-TP was conducted to evaluate the in vivo survival characteristics of the patient’s enzyme-loaded erythrocytes. This demonstrated a normal circulating mean cell life and half-life of 108 and 32 days, respectively, thus demonstrating the viability of the erythrocyte as a vehicle for sustaining therapeutic blood levels of TP. Administration of EE-TP (6-50 IU/kg) was shown to be effective in reducing/elimi- nating the elevated plasma and urine concentrations of thymidine and deoxyuridine. Three months after initiating therapy, one patient reported a reduction in the number of nausea and vomiting attacks and gained 4 kg, and the second patient, after only two cycles of therapy, reported an improvement in distal sensation in his hands and fingers, with continuing improvements at 3 months, such that he was able to return to guitar playing.

**Immunogenicity of EE-TP**

The encapsulation of therapeutic enzymes within autologous erythrocytes is employed as a strategy to prevent the development of immune reactions against the native enzyme. Indeed, 15 years of experience with EE-ADA therapy in a patient with adenosine deam- inase deficiency who had previously developed neutralizing antibod- ies against the polyethylene glycol-conjugated enzyme Adagen®, has shown that encapsulated adenosine deaminase is protected from the development of antibodies and an enhanced plasma clear- ance (20, 21).

TP, the active drug substance in EE-TP, is a recombinant E. coli pro- tein sharing a 40% amino acid sequence homology with the human sequence (35). Although erythrocyte encapsulation would be predicted to reduce the immunogenicity of the native enzyme, a poten- tial intravascular release of TP from damaged erythrocytes would be expected to evoke an immunogenic reaction in humans. Clinical experience in the compassionate clinical evaluation of EE-TP in two patients has shown mild adverse reactions to EE-TP adminis- tration during infusion. These events were transient and generally occurred within the first 5-10 minutes of EE-TP infusion and were managed by refining the enzyme-loading procedure and by pre- medication with antihistamine, corticosteroid antiinflammatory and antiemetic drugs. No antibodies against TP were detected, as meas- ured by ELISA. It is noteworthy that the recent introduction of the highly purified enzyme employed in the above preclinical studies had a significant effect on eliminating adverse reactions (erythema of face, chest tightness), such as to allow a careful and gradual withdrawal of the premedication. These mild reactions were not unex- pected; the development of hypersensitivity-type reactions and ana- phylaxis to recombinant biologicals is well documented; Aldurazyme® (36), Fabrazyme® (37), Naglazyme® (38), Cerezyme® (39), Myozyme® (40) and Elaprase® (41) are all approved enzyme replacement therapies and have reported immunogenicity rates ranging between 15% and 98%.

**Conclusion**

The results of nonclinical studies and compassionate clinical studies have not revealed any potential serious toxicities that would preclude the use of EE-TP in patients with MNGIE, but caution should be taken for infusion-related reactions, which may be related to release of cytokines or other chemical mediators. Collectively, the available data support the acceptable safety profile for a phase II clinical trial, an application for which is in preparation.

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**DISCLOSURES**

Y. Godfrin is a shareholder and employee at ERYTECH Pharma. B. Bax states no conflicts of interest.

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