Diamyd, an alum-formulated recombinant human GAD65 for diabetes and the prevention of autoimmune diabetes
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Diamyd Medical AB is developing Diamyd (GAD-65), an alum formulation of a full-length recombinant human glutamic acid decarboxylase 65 for subcutaneous injection, for the potential prevention and treatment of type 1 diabetes (T1DM) or latent autoimmune diabetes (LADA) in adults. Phase II clinical trials suggested that Diamyd was safe and well tolerated in patients with T1DM or LADA. Diamyd is currently in phase II/III and III clinical trials for T1DM.

Introduction
Glutamic acid decarboxylase (GAD) enzymes catalyze the formation of GABA, the well known inhibitory neurotransmitter [915882]. The two predominant forms of GAD are GAD65 and GAD67, encoded by separate genes on human chromosomes 10 and 2, respectively [915883], [915885]. Expression of GAD enzymes appears to be limited to neuroendocrine cell lineages. Only GAD65 is expressed in human pancreatic islets, while most mammals express both isoforms abundantly in the brain and pancreatic tissues [915883], [915885]. Among the endocrine cell types of the islets of Langerhans in humans, GAD65 colocalizes with insulin, glucagon and somatostatin immunopositive cells [915882], [915886], [915887].

There is a strong association between the presence of antiGAD65 autoantibodies (GADAs) and the relatively rare autoimmune disease stiff man syndrome (SMS), as well as the more common forms of autoimmune diabetes mellitus [915893]. Type 1 diabetes mellitus (T1DM) is typified by the complete autoimmune destruction of pancreatic β-cells, requiring insulin injections to maintain blood glucose within the normal range. In T1DM, autoantibodies to insulin, GAD65 and/or tyrosine phosphatase-like protein (IA-2) are present; GADAs are detected in 50 to 80% of patients with T1DM and can be used as a predictor of disease progression [915970]. GADAs are also detected in approximately 10% of patients initially diagnosed with type 2 diabetes mellitus (T2DM), where the hyperglycemic state is largely believed to be caused by insulin resistance. In this subset of GADA-positive patients with T2DM, the majority require insulin injections within 6 years of diagnosis because of a progressive autoimmune destruction of β-cells [915973]. Subsequently, this subform of T2DM has been termed latent autoimmune diabetes in adults (LADA) or type 1.5 diabetes mellitus [915974] to properly distinguish it from T2DM. Patients with T2DM can be treated with diet and exercise, as well as a number of orally bioavailable pharmaceuticals (eg, metformin) acting to increase insulin secretion or insulin sensitivity. Patients with LADA can initially control glucose homeostasis by diet and exercise; however, as the disease progresses, like T1DM, no treatment exists other than daily insulin injections [915974].

Immunomodulation of autoreactive T-cells is one strategy to potentially prevent, halt and/or reverse the progression of autoimmune diseases. In T1DM, general immunosuppression with cyclosporin A at the time of diagnosis prolonged β-cell function; however, the severe side effects of this treatment were undesirable and benefits did not last beyond the treatment period [915979]. Investigations of immunomodulation have been directed toward selectively targeting effector T-cells using mAbs directed against activated T-cell markers, antigen recognition, cellular activation and pancreatic homing signals, or alternatively immunoneutralizing cytokines from Th1 helper T-cells [915980]. A mechanistically different strategy of immunomodulation is to ‘tolerize’ the immune cells to autoantigens; it is thought that Th2 regulatory T-cells exposed to autoantigens may be able suppress
the Th1 response [915984]. In this regard, using either full-length or fragments of insulin or GAD65 should be capable of protecting against development of autoimmune diabetes.

Diamyd Medical AB (formerly BioSyn Holding AB), a Swedish based company, was granted exclusive license rights to GAD technology developed at the University of California, Los Angeles (UCLA), in 1997 [241317]. Diamyd, the company’s lead compound, is an alkum formulation of full-length recombinant human GAD65 (rhGAD65) [744043]. At the time of publication, phase II clinical trials had been completed in patients with LADA [744043] and T1DM [916055], and phase II/III and III clinical trials for subcutaneous immunization of GAD65 in T1DM were ongoing [832400], [862959], [868645]. Additionally, this formulation was investigated in a collagen-induced model of rheumatoid arthritis and in myelin oligodendrocyte glycoprotein- and myelin basic protein-induced encephalomyelitis models of multiple sclerosis; Diamyd was not effective in either indication [459942]. A novel intravenous formulation of rhGAD65 was also being investigated for movement disorders including SMS [641096].

Synthesis and SAR
Diamyd is a recombinant 65 kDa protein corresponding to human GAD65 [932195]. Unmodified rhGAD65 is formulated with aluminum hydroxide for subcutaneous administration [744043]. For clinical trials, Diamyd was produced by the Protein Sciences Corporation using a baculovirus expression system in Sf9 insect cells (ovarian cell from Spodoptera frugiperda) using the human complimentary (c)DNA for GAD65 [641891], [744043]. No further details were available (for a review of this production method see [939181]).

Preclinical development
As Diamyd Medical had not reported preclinical efficacy data at the time of publication, the author has provided a summary of a selection of the literature most relevant to the development of Diamyd. The majority of these studies were published by researchers working at the three universities which have granted Diamyd Medical licenses to use their patented technology and information (see Patent summary below).

Two main models of autoimmune diabetes have been characterized for preclinical studies: the non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat. In the NOD mouse, intraperitoneal, intrathymic, intravenous and oral administration of GAD65 (full-length or specific epitope fragments) prevented the progression of the autoimmune destruction of insulin containing β-cells [463631]. In contrast, intravenous rhGAD65 administered to BB rats had no effect on diabetes progression in these animals [916035], [916043]. Species differences exist in β-cell expression of GAD65 among mouse, rat and human [915883], [915885]; hence, caution must be used when comparing preclinical data to potential outcomes in patients.

Untreated NOD mice were examined for T-cell reactivity to insulin, GAD65, carboxypeptidase H and Hsp65 during their first 7 months of life. Sequential T-cell proliferative responses were observed to these antigens, beginning with GAD65 arising at approximately 4 weeks of age, which was concurrent with the onset of insulitis. Endogenously primed Th1 antiGAD T-cells recognized specific epitopes of GAD65: GAD65[236-256], GAD65[300-328] and GAD65[24-543]. Female 3-week-old NOD mice were administered intravenous injections of purified GAD65 (50 µg) and examined 9 weeks later for insulitis and GAD65-reactive T-cells. Of eight mice receiving GAD65, six showed a complete absence of islet mononuclear cell infiltration and the remaining two mice showed limited peri-insulitis. An approximate 5-fold reduction in T-cell proliferation was observed in GAD65-tolerized animals compared with control animals in response to full-length GAD65 or the three characterized GAD65 epitopes. In addition, a study monitoring glycaemia of GAD65-tolerized or control NOD mice found that 70% of control mice developed hyperglycemia by 19 weeks of age; in contrast, all GAD65-tolerized mice remained normoglycemic for over 37 weeks [916045].

In a similar study, T-cell proliferation in response to potential murine β-cell autoantigens (GAD65, GAD67, peripherin, carboxypeptidase H and Hsp60) was examined in untreated NOD mice. Significant T-cell proliferation recognizing GAD65 and GAD67 was observed in NOD mice beginning at 4 weeks of age; this was followed by sequential recognition of the other islet antigens. AntiCD4 antibodies blocked the proliferative effect of islet antigens on NOD mouse T-cells, suggesting that the measured proliferative responses were CD4+ T-cells. Intrathymic injection of of GAD65 (10 µg) in 3-week old NOD mice was observed to profoundly reduce islet insulitis (72% of islets in GAD65-treated animals were deemed to be free of islets compared with 5% of control islets), as well as significantly reduce the frequency and delay the onset of diabetes in these animals [916048].

Follow-up studies aimed to clarify if diabetes progression could be halted at a later stage of development and to identify the type of T-cell response occurring in GAD65-tolerized mice [453813], [916051]. In contrast to initial reports of diabetes prevention by GAD65 immunization at 3 weeks of age [916045], [916048], four intravenous injections of rhGAD65 (200 µg) were administered over a 12-day period to 12-week-old NOD mice, a time point where islet autoantibodies, T-cell reactivity and insulitis are present, but prior to overt hyperglycemia [453813]. This protocol significantly reduced progression of the mice toward a diabetic phenotype, with approximately 15% of GAD65-immunized mice developing overt diabetes by 25 weeks of age compared with approximately 70% of control (ovalbumin) mice. Vaccination using other β-cell antigens, including carboxypeptidase H and peripherin, were similar to ovalbumin control. GAD65-immunized mice showed significantly reduced T-cell proliferative responses, yet no reduction in GADAs [453813]. Similar
conclusions were drawn from experiments in which GAD65 (100 µg) in incomplete Freund's adjuvant was administered to 8-week-old NOD mice by intraperitoneal injection. This latter study also demonstrated syngeneic islet graft survival in diabetic NOD-recipient mice immunized with GAD65 [916051].

Using adoptive transfer, a technique to transfer immune cells from one animal to another, which can either induce diabetes or protect from diabetes progression depending on the type and reactivity of T-cells, the development of diabetes was inhibited in NOD mice receiving splenocytes from GAD65-vaccinated (100 µg) NOD donor mice. Only 10% of mice receiving cells from GAD65-vaccinated mice developed diabetes compared with 90% of mice receiving cells from a β-galactosidase-vaccinated control [916051]. Additional studies confirmed that CD4+ T-cells were responsible for the suppression of the diabeticogenic response in recipient NOD mice. IFNγ and IL-4 secretion from purified CD4+ T-cells from 35-week-old NOD mice that did not develop diabetes following injection (at 12 weeks of age) of ovalbumin (control) or GAD65 showed reciprocal results. CD4+ T-cells from control mice secreted high IFNγ and low IL-4, indicative of a Th1 immune response, while CD4+ T-cells from GAD65-treated animals showed the opposite, suggesting a Th2 immune response [453813]. Earlier research supported the hypothesis of Th2-mediated protective responses, as suggested by cytokine secretion from splenic cultures, as well as preferential induction of IgG1 versus IgG2a antibody production in GAD65-immunized animals relative to controls [916051]. Studies using IL-4-deficient NOD mice have shown that the ability to suppress the Th1 autoimmune response and progression to hyperglycemia via GAD65 administration is mediated by IL-4 [744041].

As T-cells recognize specific epitopes of antigens to elicit immune responses, epitopes of GAD65 were examined to identify which specific regions of the molecule are required to elicit the protective Th2 immune response in NOD mice [744041]. Single or multiple synthetic fragments of GAD65 (GAD65217-236, GAD65247-266, GAD65290-309, and GAD65500-585) were used to immunize NOD mice prior to insulitis (4 weeks) or post-insulitis (12 weeks), and mice were assessed for development of diabetes and histological analysis of insulitis. When early immunizations were administered, single epitopes (GAD65217-236 or GAD65290-309) or a combination of epitopes (GAD65247-266 plus GAD65500-585) were able to prevent progression to hyperglycemia and block monocyte infiltration in 75 to 90% of islets. In contrast, when similar epitope injections were administered to 12-week-old NOD mice, single GAD65 epitopes were largely ineffective and only co-injection of GAD65217-236 plus GAD65290-309 had similar efficacy to preventative immunizations administered at 4 weeks of age. It was hypothesized that the underlying cause of the differential recognition of GAD65 epitopes results from the frequency of peripheral T-cell clonotypes [744041]. Similar data showed that T-cell responses to GAD65 epitopes in NOD mice vary with age and disease state, and can be altered if immunized with GAD proteins [916052].

Gene transfer to ameliorate autoimmune diabetes by immunотolerizing animals to GAD65 has also been examined using plasmid vaccination and viral gene delivery. Repeated intramuscular injection of plasmid DNA (50 µg) encoding a modified form of GAD65 with an added secretory sequence was ineffective in preventing of diabetes progression in NOD mice, regardless of early (4-week-old) or late (12-week-old) immunization. However, co-injection of the GAD65 plasmid with an expression plasmid encoding IL-4 prevented approximately 90% of intra-islet insulitis when administered to 4-week-old NOD mice, but only approximately 40% of islets were free of insulitis when the two plasmids were injected into 12-week-old animals. In this study, T-cell cytokine secretion (IFNγ, IL-4 and IL-5) was similar between the two age-groups and followed the anticipated pattern, given the results for diabetes progression and insulitis. The IL-4 and -5 response to GAD65 was significantly greater in nondiabetic animals co-immunized with GAD65 and IL-4 plasmids compared with untreated and singly-immunized diabetic and nondiabetic animals. Conversely, the IFNγ response to GAD65 was significantly lower in nondiabetic co-immunized animals. In diabetic animals, these differences between treatment groups were not observed [744039]. These results were supported by a study using local in vivo electroporation of intramuscularly injected GAD65 plasmid modified with the signal sequence from IL-4 (100 µg). Development of diabetes in NOD mice was more persistently delayed using this modified GAD65 vaccination compared with unmodified GAD65 and no vaccination, although significance was not achieved. Multiple vaccinations (five injections at 4-week intervals) improved efficacy of the modified GAD65 vaccine, but not the unmodified vaccine [916053].

Another gene transfer strategy was also studied. Vaccinia virus encoding full-length mouse GAD65 (5 × 10⁷ plaque forming units) was administered intraperitonealy to 3-week-old NOD mice. Only 7% of these developed diabetes compared with 80 to 90% of untreated and vector-control mice at 40 weeks of age; a similar reduction in islet insulitis was also observed. Vaccinia-GAD65 efficiently caused a Th2 immune response: reduced production of IFNγ, increased production of IL-4 and increased IgG1 antibodies [916054]. Similar results were described using a fragment of GAD65500-585 delivered to NOD mice using a recombinant adeno-associated virus [651914].

Two non-invasive methods to immunomodulate NOD mice to give a Th2 response to GAD65 have been reported in the literature. The first examined intranasal administration of four GAD65 epitope peptides (peptides 17, 34, 35 and 36; 50 µg each) to 2- to 3-week-old NOD mice. This route of delivery efficiently stimulated Th2-mediated tolerance to GAD65, but only reduced diabetes incidence from 90% in control mice to 40% in
treated mice [916062]. In addition, a transgenic plant-based method for induction of oral tolerance to GAD65 was investigated. Prediabetic NOD mice were fed homogenized GAD65/IL-4-transgenic tobacco leaves to deliver IL-4 (~ 1 to 2 µg) and GAD65 (~ 6 to 8 µg) orally per day, and compared with rodents receiving non-transgenic leaf matter and the individual transgenic leaf material. Glycemia, glycosuria and insulins of test and control animals showed that only animals that consumed both GAD65/IL-4 transgenic tobacco leaves were protected against the development of diabetes. T-cell IgG isotype and cytokine measurements suggested a preferential shift towards a Th2 immune response [916065].

Toxicity

Initial tolerance studies were conducted in rabbits, rats, mice and marmosets using rhGAD65 (non-alum formulation), but have only been reported in a company report [459942]. During dermis injection of rhGAD65 to rabbits, minor injection site inflammatory responses were observed. Subcutaneous and intravenous injection of rhGAD65 to rats and mice at doses several 100-fold greater than the anticipated human dose similarly resulted in injection site inflammation. General toxicity leading to rodent death was observed at very high doses, without identifiable organ failure. Additional studies with intravenous rhGAD65 demonstrated no behavioral abnormalities or effects on cardiovascular parameters. Anaphylaxis was not observed in a 28-day subcutaneous rhGAD65 study in rats. During this study, rhGAD65 antibodies were detected in the blood, with no histological changes in pancreatic tissue or morphological changes in motor-end plates in muscle and skin. A similar study in marmosets utilizing doses intended for clinical trials did not demonstrate any changes in body weight or food consumption. ECGs, hematology, clinical chemistry and histology were unaffected [459942].

A buffer used in the manufacturing process of Diamyd, which remains in the final product at low concentrations, was analyzed for mutagenic potential in an Ames test, a mouse lymphoma assay able to detect point and chromosomal mutations, and an in vivo mouse micronucleus test. All experiments suggested that the buffer did not have mutagenic potential [459942].

Alum-formulated rhGAD65 (ie, Diamyd) toxicity studies were conducted in rats and mice, and were also reported in the same company document [459942]. Immunogenicity of alum-based Diamyd was greater than that of rhGAD65 in mice. To compare the toxicity results between rhGAD65 and Diamyd, rats were injected on five occasions over a 28-day period with either rhGAD65 or Diamyd; no adverse reactions were observed during the following 28-day period. Blood tests and microscopy of nerve tissue did not suggest development of autoimmune disease or any loss of tissue integrity. Repeated injections with Diamyd did not affect the normal immune response and recovery of mice infected with a murine-adapted strain of Influenza A. Additionally, Diamyd did not alter other rodent models of autoimmune or allergy conditions [459942].

A comparative study of the immunological effects of several diabetes-prone or non-diabetes-prone mice immunized with GAD65 suggested no ill effects in non-diabetic prone animals. RhGAD65 in incomplete Freund’s adjuvant (75 or 100 µg) was injected subcutaneously once or twice into non-diabetes-prone (BALB/c, C57Bl/6, NMR1 and SJL/J) or diabetes-prone (NOD) mice, and T-cell cytokine production, GAD antibodies, insulitis and glycemia were monitored. Despite high antibody titers and robust cytokine responses in several strains, no induction of insulitis or hyperglycemia was observed in non-diabetes-prone animals, whereas rhGAD65 treated NOD mice showed reduced insulitis [916069].

Anaphylaxis and death of NOD mice was reported upon intraperitoneal injection of peptide fragments corresponding to immunodominant GAD65 epitopes. GAD206-226, GAD217-236 and GAD286-300 (200 µg each) in incomplete Freund’s adjuvant were administered in three weekly injections. A fourth injection of the peptides in adjuvant was administered 4 weeks later to the mice and core temperature was measured until death; all mice showed anaphylactic reaction resulting in 86% mortality [916076].

Metabolism and pharmacokinetics

At the time of publication, pharmacokinetic data regarding alum-formulated Diamyd in humans or animals had not been reported. However, the elimination half-life of intravenous rhGAD65 (produced by Diamyd Medical using the SF9 production method) in rats was approximately 2.9 h following administration of rhGAD65 (100 or 330 ng) and was consistent with a single compartment model and first-order process [916078].

Clinical development

Phase I

In a phase I, randomized, double-blind, placebo-controlled, dose-escalation clinical trial, rhGAD65 (non-alum formulation) was administered subcutaneously to healthy volunteers (n = 24). Volunteers were genotyped to exclude diabetes-susceptible HLA haplotypes (HLA DR3/DQ2 and HLA DR4/DQ8) and confirmed to be islet autoantibody (GAD65, IA-2 or insulin) negative. Separate escalating doses of rhGAD65 (20 to 500 µg) were administered; the treatment did not induce autoantibodies to GAD65, IA-2 or insulin [353988], [459942].

Phase II

In a phase II, randomized, double-blind, placebo-controlled, dose-escalation clinical trial, Diamyd (4, 20, 100 or 500 µg) was subcutaneously administered twice (4 weeks apart) to patients (n = 47; n = 8 or 9 per treatment group; n = 13 for placebo) with LADA. Patients were GAD65-antibody positive and had been diagnosed with T2DM within 5 years previous to dosing, but had not been
treated with insulin (treatment with modulation of diet or oral antidiabetic agents was accepted). Patients in the 500-µg dose group were permitted two additional administrations depending on the GADA response (patients with < 2-fold increase in GADA titer were permitted an additional booster dose; if no increase was observed in the following 4 weeks, a second additional dose was permitted) [492752], [541501], [744043], [804957], [916084]. In the 500-µg dose group, an increase in GADA was observed in five patients during the first 4 weeks; the remaining three patients demonstrated increases in GADA following additional booster dosing. These patients had significantly (p < 0.001) increased log GADA levels after 24 weeks. Fasting plasma glucose levels were also assessed. Levels increased (by 1.2 to 1.3 mmol/l) in the placebo and 4-µg dose groups but decreased (by 0.3 to 0.9 mmol/l) in the 20-, 100- and 500-µg dose groups at 24 weeks, suggesting a treatment effect (p = 0.038). Similarly, HbA1c levels increased in the placebo and 4-µg dose group and decreased in the higher dose groups (p = 0.029) [744043]. Fasting and stimulated C-peptide levels increased in the 20-µg dose group only. Significance was achieved in fasting C-peptide levels relative to placebo (p = 0.0015) and in both fasting and stimulated levels relative to baseline (p = 0.0081 and 0.0236, respectively). Significant decreases in stimulated C-peptide levels were observed in the 4- and 500-µg dose groups relative to baseline (p = 0.003 and 0.0084, respectively), which was also different to placebo (p = 0.0117 and 0.0047, respectively). The CD4+CD25− to CD4+CD25+ cell ratio was increased at 24 weeks relative to baseline (p = 0.0128) in the 20-µg dose group; measurements at 4 and 8 weeks also showed significance (p > 0.05). This trend was not observed in any of the other dose groups. Additionally, a positive association between the change in fasting C-peptide and the CD4+CD25− to CD4+CD25+ cell ratio was observed in the 20-, 100- and 500-µg dose groups [541501], [744043]. Results published later suggested that the beneficial effects of the 20-µg Diamyd dose were not the result of shifting GAD65 epitope recognition by GADAs [804957]. Data from a 2-year follow-up of patients suggested fed and fasting C-peptide levels in the 20-µg dose group remained significantly elevated. HbA1c levels in the 20-µg group continued to decrease relative to placebo control patients [916084]. Data from a 5-year follow-up of patients supported these findings, and demonstrated that in patients who received the 20-µg dose, improvements observed in β cell function at six months were maintained for 5 years [933764].

A larger phase II randomized, double-blind, placebo-controlled clinical trial involving patients with LADA (n = 160; similar inclusion and exclusion criteria as above) initiated in 2004 aimed to evaluate the effects of subcutaneous Diamyd (20 µg; administered twice 30 days apart) on HbA1c. Safety parameters, progression of diabetes and changes in plasma C-peptide levels were also to be monitored [563610], [628605], [806791], [806792]. However, Diamyd Medical opted to invalidate this clinical trial because of uninterpretable results and the findings of an independent formal audit. Safety data were still evaluated and are included below [806791], [806792].

A phase II randomized, double-blind, multicenter, placebo-controlled clinical trial in recently diagnosed (within 18 months) patients (n = 70) with juvenile T1DM assessed the effects of subcutaneous Diamyd (20 µg; administered twice 4 weeks apart) on the preservation of residual insulin/C-peptide secretion over 15 months [877600], [916055], [916056]. At 9 and 15 month following the first dose, Diamyd-treated patients retained significantly more C-peptide than placebo control; stratification of data suggested Diamyd treatment was more effective if administered sooner after diagnosis of T1DM. Additionally, patients with T1DM receiving Diamyd immunization required a smaller degree of insulin dosage increase over the subsequent 15 months [877600]. Levels of cells expressing regulatory T-cell markers were significantly (p = 0.01) increased in the placebo group compared with the Diamyd-treated group and a reference group of healthy juveniles (n = 12) [916055]. In vitro stimulation with GAD65 of PBMCs collected 15 months after initial dose induced significantly (p < 0.0001) higher secretion of Th1- (IFN-γ), Th2- (IL-5 and -13), Th3- (IL-10) and Th17- (IL-17) associated cytokines in treated patients compared with placebo controls. Furthermore, induced levels of inflammatory cytokines (IL-6 and TNFα) and chemokines (IP-10, MIP-1α and MIP-1β) and FOXP3 mRNA expression were significantly higher (p < 0.05) in treated patients compared with placebo controls. In fact, GAD65-induced secretion of IL-5, -13 and -17, TNFα and FOXP3 mRNA were all higher in treated patients compared with the reference group of healthy juveniles (p < 0.0001) [916056].

**Phase III**

At the time of publication, data from phase III clinical trials had not been reported; however, three clinical trials were ongoing. A phase II/III randomized, double-blind, placebo-controlled, parallel-assignment clinical trial would evaluate Diamyd in patients (estimated n = 126) with new-onset T1DM [832400], [862985]. Two phase III randomized, double-blind, multicenter, placebo-controlled clinical trials would evaluate Diamyd each in patients (n = 300) with recent-onset T1DM. Patients would receive Diamyd (20 µg) on study days 1 and 30 to confirm earlier phase II clinical data, Diamyd (20 µg) on study days 1, 30, 90 and 270 or placebo on this second dosing schedule. One clinical trial would be based at centers in the US [862959], [886377] and the other at centers in Europe [868645], [887845], [931798]. One clinical trial would be based at centers in the US [862959], [886377] and the other at centers in Europe [868645], [887845], [931798].

**Side effects and contraindications**

The phase I clinical trial reported no significant treatment-related adverse effects at unformulated rhGAD65 (20 to 500 µg sc) [353988], [459942]. In the phase II clinical trial
in patients (n = 47) with LADA, 68% of patients reported at least one adverse event with the majority experiencing flu-like symptoms, particularly nasopharyngitis. Only three adverse events were deemed likely to be related to treatment prior to unblinding. These were vitiligo (later found to be in the placebo group), mild leukocytosis (100-µg group) and inflammation of the injection site (500-µg group). The patient with vitiligo was withdrawn from the trial after the first dose, but both the leukocytosis and inflammation were resolved without treatment. Hematology and biochemistry measurements were within normal limits in each treatment group at most visits; one patient in the 20-µg exhibited a transient increase in liver enzyme levels compared with placebo [744043]. Data collected from the invalidated phase II clinical trial in patients (n = 160) with LADA were reportedly good with no drug-related serious adverse events observed [872521]. The phase II clinical trial in patients with T1DM found no difference in adverse effects between placebo and treatment groups; there were no treatment-related serious adverse events although one patient in the placebo group was withdrawn following an undisclosed serious adverse event after the first injection [877600].

**Patent summary**

In 1994, as Synectics Medical was setting up its subsidiary Synectics Biotechnology AB (later Biosyn and now Diamyd Medical) to develop a vaccine for autoimmune diabetes, the company in-licensed rights to diabetes therapy using GAD from the University of California [932491]. Corresponding patents on recombinant GAD65 polypeptides have since been granted as US-05475086, US-05674978 and EP-00519469. Several equivalent patents (based on the same initial filings) claim nucleic acid sequences and host cells for producing such polypeptides. The lead inventor from the University of California has also claimed the use of GAD65 for prolonging survival of pancreatic tissue transplants in US-06207159.

By 2000, Diamyd Medical had also in-licensed GAD65 vaccine technology from the University of Florida (likely US-05891435 and EP-00693933) and the University of Washington (likely US-05792620 and US-06025176, both co-assigned to ZymoGenetics Inc).

Diamyd Medical has filed claims on GAD65 antigen formulations in WO-200435084, WO-200594877 and WO-2005102374, all of which were pending grant in the US and Europe at the time of publication. In addition, Diamyd Medical also claims modified GAD65 for use in T1DM in WO-09712034; at the time of publication, this had been granted in Europe (EP-00852619) but not in the US.

**Current opinion**

Currently there are no available therapies designed to prevent, halt or reverse autoimmune diabetes; however, Diamyd is one of three main immunotherapies (excluding mAbs) aimed at improving patient outcomes in autoimmune diabetes. In the US, approximately 1 in 400 to 600 children and adolescents have T1DM – an estimated 0.22% of individuals under the age of 20 years. T2DM is the more prevalent form of diabetes and is thought to affect 7% of the US population; however, only two-thirds of these patients are diagnosed with the disease [916138]. Of the T2DM population, it has been suggested that approximately 10% may actually have LADA [915973]. Thus, immunotherapies for autoimmune diabetes could potentially be applied to just less than 1% of the general population, less the number of undiagnosed cases (~ 0.2%). Predictions suggest a 2-fold increase in the incidence of diabetes by 2030, largely because of the increase in T2DM incidence [880516], although it is not clear whether the incidence of LADA will continue to represent 10% of T2DM or if it will more closely follow population growth. Nevertheless, the potential future market for immunotherapies makes their development financially worthwhile and medically important.

Diamyd appears to be a good lead candidate therapy for immunomodulation of autoimmune diabetes. Preclinical studies have shown that immunotolerization to GAD65 can be very effective in preventing diabetes in animal models of the disease. Two similar strategies include immunotolerization to a 24 amino acid fragment of hsp60 (DiaPep277 [Andromeda Biotech Ltd]) [431104] and a modified peptide derived from amino acids 9 to 23 of the insulin B chain (NBI-6024 [Neurocrine Biosciences Inc]) [460240]. Both strategies were first validated in the NOD mouse model, similar to GAD65 [463631]. While immunotolerization to the insulin B chain fragment was abandoned because it failed to meet phase II clinical trial endpoints [689625], a published DiaPep277 phase II clinical trial in newly diagnosed patients with T1DM [431104], along with several unpublished phase II clinical trials, has provided sufficient data for a larger phase III clinical trial, which is due for completion in 2010 [877162].

Unlike the NOD mouse, where it has been conclusively established that GAD65 autoantibodies appear at the immediate onset of the disease, human autoimmune diabetes is commonly present for years prior to diagnosis. As such, the initiating mechanisms of the disease are largely unknown and it is not clear if GAD65 autoimmunity is an early event in the human condition. Frequently, at the time of T1DM diagnosis, over 70% of insulin secreting cells have already been lost [916085], making the potential effectiveness of immunomodulation uncertain at this late stage of disease progression. The greater effectiveness of Diamyd administration in patients more recently diagnosed with T1DM implies that some residual function can be preserved from the remaining β-cell population [744043], but highlights the time-limited window that the drug has to be administered in order to be effective. Improvements in early diagnosis of T1DM and T1DM-susceptible individuals may lengthen the treatment time frame [916089]. In patients with LADA, there is a broader window between identification of a type 2-like diabetic phenotype and insulin dependence, provided patients...
are tested for the presence of GADAs, allowing a greater therapeutic window during which immunomodulation with Diamyd or alternative therapies may be effective.

Preclinical data have demonstrated that GAD65 immunization in NOD mice promotes a Th2 immune response, which promotes tolerance to the antigen. In humans, only total GADA and GADA epitope specificity has been examined during the LADA phase II clinical trial, and a significant increase in CD4+CD25+ cells was observed in patients treated with 20 µg Diamyd [744043], [804957]. From these trials, the mechanism of action of the GAD65 vaccine has not been firmly demonstrated. An immune mechanism might be inferred by the elevation of CD4+CD25+ cells; however, neither the specificity of these immune cells nor their production of immunomodulatory cytokines has been examined. Data regarding CD4+CD25+ cells in the placebo control group were not presented, raising questions as to the specificity of this response.

The use of full-length rhGAD65 in Diamyd is beneficial as any change in the epitope specificity of GADA during disease progression or during treatment is unlikely to affect the general specificity towards GAD65. However, there are a number of uncertainties regarding this therapy. The lack of a clear dose-response relationship is a confounding issue. Preclinical studies suggested that the effectiveness of GAD65 vaccination might be affected by the abundance of peripheral regulatory T-cells capable of recognizing the antigen, and generally inclusion of an adjuvant improved immunogenic effectiveness. However, in clinical trials with Diamyd, the 20-µg dose appeared to be the only effective dose with higher doses producing few effects. The mechanism behind this is not immediately evident and may cause problems in future clinical use of this agent with respect to optimal dosing. More extensive preclinical and clinical examination is required to clarify effective dosing regimens.

At the time of publication, data demonstrating the effectiveness of Diamyd in humans was scarce and based entirely on 47 and 70 patients with LADA and T1DM, respectively, in phase II clinical trials. This number of patients is too small to give great confidence in the effectiveness of Diamyd; however, the results are promising with an apparent persistent positive effect for 15 to 24 months. In the phase II LADA clinical trial, patients taking oral antidiabetic agents (eg, insulin releasers and insulin sensitizers) were not excluded from the trial; as these agents can affect determinations of HbA1c and fasting blood glucose, it is unclear what effect these may have had on the clinical outcomes of the trial. Future clinical trials of Diamyd in patients with LADA should either exclude patients receiving oral antidiabetic agents or the matched effect of combination therapy should be investigated. While the larger phase II clinical trial of Diamyd (20 µg) in patients with LADA was invalidated because of a potential mix-up of test and placebo at some point during the trial, it is worrisome that results were "inconclusive and contradictory" [806791]. Either test and placebo were indeed switched at some point during the trial or, similar to the case with insulin prevention trials, early positive results [211250], [916110] could not be duplicated in a larger multicenter trial [916111]. The true efficacy of this therapy will be provided with much more confidence following disclosure of phase III results.

Deals
In 1997, UCLA, who had originally developed the GAD gene technology, had granted an exclusive worldwide, royalty-bearing license to Diamyd [241317]; this agreement was modified in May 2001 for the exclusive use of the GAD gene in CNS diseases and for fewer royalties to be paid by Diamyd [459728].

In April 2005, Biovitrum AB adapted the production process to meet requirements for phase III trials and commercialization [593641]. Protein Sciences were contracted by Diamyd in December 2005 to produce the vaccine for phase III trials and prepare to file an IND [641891], [644016].

Diamyd sought to outlicense codevelopment rights to the vaccine in January 2002 [459682]; in April 2008, partnership negotiations were in progress [898419].

### Development status

<table>
<thead>
<tr>
<th>Developer</th>
<th>Country</th>
<th>Status</th>
<th>Indication</th>
<th>Date</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Diamyd Medical AB</td>
<td>Europe</td>
<td>Phase III Clinical</td>
<td>Insulin-dependent diabetes</td>
<td>21-APR-08</td>
<td>898419</td>
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<tr>
<td>Diamyd Medical AB</td>
<td>US</td>
<td>Phase III Clinical</td>
<td>Insulin-dependent diabetes</td>
<td>21-APR-08</td>
<td>898419</td>
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<tr>
<td>Diamyd Medical AB</td>
<td>Sweden</td>
<td>Phase II Clinical</td>
<td>Latent autoimmune adult diabetes</td>
<td>14-SEP-00</td>
<td>382244</td>
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### Literature classifications

**Chemistry**

<table>
<thead>
<tr>
<th>Study type</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production</td>
<td>Diamyd consists of unmodified rhGAD65 formulated with aluminum hydroxide. RhGAD65 is produced in a baculovirus expression system in Sf9 insect cells using the human cDNA for GAD65.</td>
<td>744043</td>
</tr>
</tbody>
</table>
### Biology

<table>
<thead>
<tr>
<th>Study type</th>
<th>Effect studied</th>
<th>Model used</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>Autoantigen recognition</td>
<td>NOD mice examined for T-cell reactivity to insulin, GAD65, carboxypeptidase H and Hsp65.</td>
<td>GAD65 was the initial autoantigen recognized in NOD mice at approximately 4 weeks of age, which was concurrent with the onset of insulitis.</td>
<td>916045</td>
</tr>
<tr>
<td>In vivo</td>
<td>Autoantigen recognition</td>
<td>NOD mice examined for T-cell reactivity to GAD65, GAD67, peripherin, carboxypeptidase H and Hsp60.</td>
<td>GAD65 and GAD67 were the initial autoantigens recognized in NOD mice at approximately 4 weeks of age. AntiCD4 antibodies blocked the proliferative effect of islet antigens on NOD mouse T-cells, suggesting that the measured proliferative responses were CD4+ T-cells.</td>
<td>916048</td>
</tr>
<tr>
<td>In vivo and ex vivo</td>
<td>Efficacy</td>
<td>Female 3-week-old NOD mice (n = 8) administered GAD65 (50 µg iv) and examined 9 weeks later for insulitis and GAD65-reactive T-cells.</td>
<td>Six mice showed complete absence of islet infiltration and two showed limited peri-insulitis. T-cell proliferation was reduced 5-fold in GAD65-tolerized animals compared with control animals in response to full-length GAD65 or GAD65 epitopes.</td>
<td>916045</td>
</tr>
<tr>
<td>In vivo and ex vivo</td>
<td>Efficacy</td>
<td>3-week-old NOD mice administered intrathymic GAD65 (10 µg).</td>
<td>Islets from treated mice showed reduced insulitis compared with control (72 versus 5%, respectively). The development of diabetes was delayed and the incidence reduced in treated mice.</td>
<td>916048</td>
</tr>
<tr>
<td>In vivo and ex vivo</td>
<td>Efficacy</td>
<td>12-week-old NOD mice administered rhGAD65 (200 µg iv) four times over 12 days.</td>
<td>15% of immunized mice developed diabetes by 25 weeks of age compared with 70% of control mice. GAD65-immunized mice showed significantly reduced T-cell proliferative responses, but no reduction in GADAs.</td>
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### Metabolism

<table>
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<tr>
<th>Study type</th>
<th>Effect studied</th>
<th>Model used</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>Plasma half-life of rhGAD65</td>
<td>Rats administered rhGAD65 (100 or 330 ng iv).</td>
<td>The elimination half-life was approximately 2.9 h and was consistent with a single compartment model and first-order process.</td>
<td>916078</td>
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### Clinical

<table>
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<th>Effect studied</th>
<th>Model used</th>
<th>Result</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Safety</td>
<td>Phase I randomized, double-blind, placebo-controlled, dose-escalation clinical trial of rhGAD65 (20 to 500 µg sc) in healthy volunteers (n = 24) genotyped to exclude diabetes-susceptible HLA haplotypes.</td>
<td>Treatment did not induce autoantibodies to GAD65, IA-2 or insulin. No significant treatment-related adverse effects were observed.</td>
<td>459942</td>
</tr>
<tr>
<td>Safety and efficacy</td>
<td>Phase II, randomized, double-blind, placebo-controlled, dose-escalation clinical trial of Diamyd (4, 20, 100 or 500 µg sc; administered twice 4 weeks apart) in patients with LADA (n = 47).</td>
<td>GADA levels increased in five of eight patients in the 500-µg dose group during the first 4 weeks. Fasting plasma glucose and HbA1c levels decreased in the 20-, 100- and 500-µg dose groups at 24 weeks. Relative to placebo and baseline, fasting and stimulated C-peptide levels increased in the 20-µg dose group only. The CD4+CD25+ to CD4+CD25− cell ratio was increased at 24 weeks in the 20-µg dose group only. Flu-like symptoms were the most common adverse events, although mild leukocytosis and inflammation of the injection site were also observed.</td>
<td>744043</td>
</tr>
<tr>
<td>Safety and preservation of residual insulin/ C-peptide secretion</td>
<td>Phase II randomized, double-blind, multicenter, placebo-controlled clinical trial of Diamyd (20 µg sc; administered twice 4 weeks apart) to patients with T1DM (n = 70).</td>
<td>Diamyd-treated patients retained significantly more C-peptide than placebo control. Diamyd treatment was more effective if administered sooner after diagnosis of TIDM. Additionally, patients with T1DM receiving Diamyd immunization required a smaller degree of insulin dosage increase over the subsequent 15 months. There were no differences in adverse effects between placebo and treatment groups.</td>
<td>877600</td>
</tr>
<tr>
<td>C-peptide secretion in vitro</td>
<td>Phase II randomized, double-blind, multicenter, placebo-controlled clinical trial of Diamyd (20 µg sc; administered twice 4 weeks apart) to patients with T1DM (n = 70).</td>
<td>Significantly higher secretion of Th1-, Th2-, Th3- and Th17-associated cytokines was observed in samples from treated patients compared with controls. Levels of inflammatory cytokines and chemokines and FOXP3 mRNA expression were also significantly elevated.</td>
<td>916056</td>
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</table>
Associated patent

Title: Cloning and expression of human islet glutamic acid decarboxylase autoantigen.
Assignee: The Board of Regents of the University of Washington; Zymogenetics Inc.
Inventors: Lernmark A, Karlsefne AE, Grubin CE, Hagopian W, O'Hara PJ, Foster DC.

References:

241317 BioSyn Holding has rights to UCLA's GAD technology. SCRIp 1997 2219/20 23
353988 Diamyd diabetes vaccine completes clinical trial. Diamyd Medical AB PRESS RELEASE 2000 January 31
382244 GAD-Diamyd – more effective than insulin? Diamyd Medical AB PRESS RELEASE 2000 September 13
459682 Preferential new share issue by Diamyd Medical AB realized. Diamyd Medical AB PRESS RELEASE 2002 January 09
459728 Diamyd Medical improves on license agreement with UCL. Diamyd Medical AB PRESS RELEASE 2001 May 14
459942 Quarterly report II – 99/00: 6 months report for Diamyd Medical AB (1 September 1999 – 29 February 2000). Diamyd Medical AB PRESS RELEASE 2000 May 03
463631 The NOD mouse model of type 1 diabetes: As good as it gets? Atkinson MA, Leher EH. NAT MED 1999 5 6 601-604
563610 Diamyd starts larger trial in type 2 diabetes patients in Sweden. Diamyd Medical AB PRESS RELEASE 2004 October 07
628605 Diamyd – a novel treatment for 10% of all type 2 diabetes patients - in a fully recruited phase II/III trial. Diamyd Medical AB PRESS RELEASE 2005 October 07
641096 Diamyd Medical demonstrates safety of new GAD formulation in pre-clinical study. Diamyd Medical AB PRESS RELEASE 2005 December 15
641891 Diamyd Medical appoints Protein Sciences 'USA' for production of its lead therapeutic diabetes vaccine for phase III trials and to submit an IND to the FDA. Diamyd Medical AB PRESS RELEASE 2005 December 19
644016 Protein Sciences raises $6 million. Protein Sciences Corp PRESS RELEASE 2006 January 09

689625 Neurobioscience: these results support a follow-up phase 2... Diamyd Medical AB PRESS RELEASE 2006 September 12
804957 GAD65 autoantibody epitopes in adult patients with latent autoimmune diabetes following GAD65 vaccination. Bekris LM, Jensen RA, Lagerquist E, Hall TR, Agardh CD, Cilio CM, Lethagen AL, Lemmark A, Robertson JA, Hamppe CS. DIABETIC MED 2007 24 5 521-526
806791 Diamyd diabetes study invalidated. Diamyd Medical AB PRESS RELEASE 2007 June 18
806792 Diamyd continues invalidated study. Diamyd Medical AB PRESS RELEASE 2007 June 19
832400 Diamyd Medical: US National Institutes of Health announces plans to sponsor a type 1 diabetes trial with the experimental Diamyd diabetes vaccine. Diamyd Medical AB PRESS RELEASE 2007 September 21
862950 Diamyd Medical: Diamyd files USIND for phase III trial with diabetes vaccine. Diamyd Medical AB PRESS RELEASE 2007 December 21
862985 NCT00293997: Effects of recombinant human glutamic acid decarboxylase... National Institute of Diabetes and Digestive and Kidney Diseases. CLINICALTRIALS.GOV: 2007
868645 Diamyd initiates European submission for phase III studies with diabetes vaccine. Diamyd Medical AB PRESS RELEASE 2008 January 18
886377 Diamyd Medical: Diamyd gets authorization to begin phase III study in the US. Diamyd Medical AB PRESS RELEASE 2008 March 14
887845 Diamyd diabetes vaccine receives approval to start phase III trials in Europe. Diamyd Medical AB PRESS RELEASE 2008 March 19
898419 Diamyd strengthens financial position and executes a fully subscribed direct placement. Diamyd Medical AB PRESS RELEASE 2008 April 21
915882 Structural and functional considerations of GABA in islets of Langerhans. β-cells and nerves. Sorenson RL, Garry DG, Brelje TC DIABETES 1991 40 11 1365-1374
Glutamic acid decarboxylase – Gene to antigen to disease. Lenman A J INTERN MED 1996 240 5 259-277


Prevention and prevention of type 1 diabetes: Progress, problems, and prospects. Skyler JS CLIN PHARMACOL THER 2007 81 5 768-771

Immunotherapy of insulin-dependent diabetes mellitus. Bach JF CURR OPIN IMMUNOL 2001 13 5 601-605

Tolerogenic strategies to halt or prevent type 1 diabetes. Cooke A, Phillips JM, Fairman NM NAT IMMUNOL 2001 2 9 810-815


GAD65 and insulin B chain peptide (9-23) are not primary autoantigens in the type 1 diabetes syndrome of the BB rat. Bieg S, Hanlon C, Hampe CS, Benjamion D, Mahoney CP AUTOIMMUNITY 1999 31 1 15-24


Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDavitt HO NATURE 1993 366 6450 72-75


Characterization of novel T-cell epitopes on 65 kDa and 67 kDa glutamic acid decarboxylase relevant in autoimmune responses in NOD mice. Zechel MA, Elliot JF, Atkinson MA, Singh B J AUTOIMMUN 1998 11 1 83-95

Regulatory cytokine production stimulated by DNA vaccination against an altered form of glutamic acid decarboxylase 65 in nonobese diabetic mice. Glinka Y, De Pooter R, Croze F, Prud‘homme GJ J MOL MED 2003 81 3 175-184

Prevention of autoimmune diabetes by immunogene therapy using recombinant vaccinia virus expressing glutamic acid decarboxylase. Jun HS, Chung YH, Han J, Kim A, Yoo SS, Sherwin RS, Yoon JW DIABETOLOGIA 2002 45 5 668-676

GAD65 vaccination significantly reduces insulin dependence at five years follow-up in a dose escalating study in adult-onset autoimmune diabetes patients. Agardh CD, Leathagen A, Cilo CM, Lynch K, Palmer M, Leslie DRG, Harris RA, Robertson JA, Lenman A DIABETES 2006 55 11 1753-1767


