Diabetes Research Institute[1], Miller School of Medicine, University of Miami, Miami, FL; Center for Drug Discovery[2], College of Pharmacy, University of Florida, Gainesville, FL, USA

Soft corticosteroids for local immunosuppression: exploring the possibility for the use of loteprednol etabonate for islet transplantation

N. Bocca,[1] A. Pileggi,[1] R. D. Molano,[1] S. Marzorati,[1] W. Wu,[2] N. Bodor,[2] C. Ricordi,[1] P. Buchwald[1]

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Peter Buchwald, Diabetes Research Institute, Miller School of Medicine, University of Miami, 1450 NW 10 Ave (R-134), Miami, FL 33136, USA
pbuchwald@med.miami.edu

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Transplantation of pancreatic islets into subcutaneous, neovascularized devices is one of the possibilities explored as part of our search for a cure of diabetes. We have recently reported that syngeneic transplantation in a subcutaneous prevascularized device can restore euglycemia and sustain long-term function in rats and that explanted grafts showed preserved islets and intense vascular networks. Because all of the transplanted tissue is localized within the device, if such a bioartificial pancreas approach is used, localized immunosuppression might provide sufficient protection against rejection to achieve long-term function, while also avoiding the serious systemic side effects and the susceptibility for opportunistic infections that are commonly associated with systemic immunosuppressive therapies as only much smaller and localized doses are needed. Soft steroids are obvious candidates because soft drugs are specifically designed to produce targeted local activity, but no systemic side effects due to prompt metabolic (preferably extrahepatic, e.g., hydrolytic) inactivation. However, local concentrations that are effective for immunosuppression, but non-toxic to insulin-producing β-cells have to be found, and nontrivial difficulties related to long-term local deliverability have to be addressed. Here, we report preliminary results obtained using *in vitro* studies with human islets used to establish a tentative therapeutic concentration range together with fully scaled three-dimensional finite element method (FEM)-based Comsol multiphysics computational models that were used to explore various possibilities to achieve and maintain these concentration levels within the device.

1. Introduction

1.1. Background

Transplantation of pancreatic islets (islets of Langerhans) can normalize metabolic control in patients with type 1 diabetes in a way that has been virtually impossible to achieve with exogenous insulin, but because of the lifelong immunosuppression required, the procedure is currently limited to the most severe forms of diabetes (Pileggi et al. 2006a, 2004; Ricordi 2003; Ricordi and Strom 2004). During recent years, various extravascular bioartificial pancreas approaches with encapsulated (immunoisolated) or non-encapsulated islets have been proposed as suitable sites for islet grafts; critical features common for all devices were ease of implantation, biopsy, and retrieval, while allowing for sustained graft function (Colton 1995; Kizilel et al. 2005; Knight et al. 2006; Narang and Mahato 2006; Pileggi et al. 2006b). Along these lines, islet transplantation into a subcutaneous, neovascularized device is one of the most promising therapeutic options we are currently pursuing as part of our search for a cure of diabetes (Pileggi et al. 2006a; Ricordi and Strom 2004).

1.2. Islet-containing biohybrid devices (rat model)

Recently, we have shown that reversal of diabetes and maintenance of normoglycemia is possible in chemically diabetic rat recipients by syngeneic islet transplantation in a subcutaneous, neovascularized device (Pileggi et al. 2006b). The biocompatible device consisted of a cylindrical stainless-steel mesh with removable polytetrafluoroethylene (PTFE) stoppers at each of its two ends and was implanted 40 days prior to islet transplantation to allow embedding by connective tissue and neovascularization. This approach consistently led to islet cell survival, early graft vascularization (10 days after implant), and supported long-term (> 5 months) survival and function of pancreatic islets, which are particularly susceptible to hypoxia at the unfavorable subcutaneous implantation site. After islet transplantation, nonfasting blood glucose levels returned to physiological ranges (< 200 mg/dL) and remained stable long-term similar to the control receiving intrahepatic islet grafts. Explanted grafts showed well-preserved islet structures embedded in connective tissue and intense vascular networks. Upon removal of the graft-bearing devices, hyperglycemia promptly resumed in all animals, demonstrating that long-term glycemic control was due to the transplanted islets.

1.3. Feasibility of local immunosuppression with steroids

One of the possible, but, as of today, yet unexplored advantages of a bioartificial pancreas is that the confinement of transplanted tissue within the device allows the implementation of localized immunosuppression, which might provide sufficient protection against rejection and allow long-time maintenance of function, while also avoiding the serious systemic side effects commonly associated with systemic immunosuppressive therapies (i.e., increased susceptibility to opportunistic infections). If a locally active therapeutic agent or a combination of therapeutic agents can be found, therapeutically active concentration levels need to be maintained only within the device and its surrounding (through some form of local delivery). This would make possible the use of much smaller doses than those required in a corresponding systemic administration, thus reducing or preventing the untoward side effects associated with long-term systemic immunosuppression.

The feasibility of local immunosuppression, including local immunosuppression with corticosteroids, has been shown in a number of cases. For example, local budesonide showed beneficial effects in rat models of liver (Weber et al. 1997) and intestinal transplantation (Ozcay et al. 1997). Selective 'local delivery' to the graft in these studies was achieved through the large first-pass effect of orally administered budesonide (Ozcay et al. 1997; Weber et al. 1997). Furthermore, in a rat model of intrahepatic islet allotransplantation, intraportal delivery of budesonide or cyclosporin did not prolong graft survival; however, intraportal delivery of tacrolimus did so and was more effective than intravenous delivery (Wang et al 1995). Topical corticosteroids in combination with cyclosporine allowed long-term survival in rodent skin allografts (Shirbacheh et al. 1998). The efficacy of local immunosuppression has also been shown in various rat models with budesonide, cyclosporine, sirolimus, tacrolimus, 16,16-dimethyl prostaglandin E_2 (PGE_2), anti-T-cell monoclonal antibody, and 15-deoxyspergualin being infused directly into the transplanted organ for up to two weeks via an Alzet® osmotic minipump implanted in the abdominal cavity (Shirbacheh et al. 1998). As a related note, even the immune-privileged status of the eye for cell transplantation has been attributed to the elevated free hydrocortisone (cortisol) levels observed in the eye ($\sim$10 ng/mL = $\sim$30 nM in the aqueous humor) due to low corticosteroid-binding globulin (CBG) concentrations here. These cortisol concentrations appeared adequate to suppress immune responses being sufficient to inhibit one-way mixed lymphocyte reactions (MLR) as well as antigen presentation (Knisely et al. 1994).

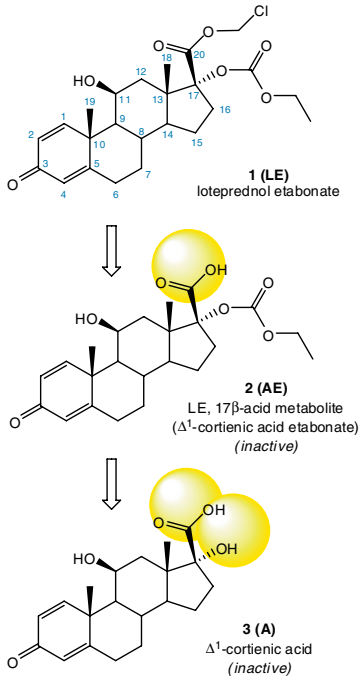
With local delivery, the highest drug concentrations are likely to be within the device; therefore, islets are directly exposed to the potential diabetogenic effects of some of these agents. Impaired glucose metabolism (Fernandez et al. 1999) and post-transplant diabetes mellitus (PTDM) (First 2003) are common in patients receiving systemically chronic therapy with calcineurin inhibitors (CNI; cyclosporine and tacrolimus). The deleterious effects of systemic administration of CNIs on islet graft function have been recognized (Alejandro et al. 1989, 1988; Ricordi et al. 1992). Administration of systemic glucocorticoids in the peri-transplant period has been associated with the development of islet graft dysfunction (Rilo et al. 1994). Also, systemic immunosuppression with sirolimus has been associated with impaired islet function and islet cell toxicity (Bell et al. 2003; Marcelli-Tourvieille et al. 2007; Zhang et al. 2006). Interestingly, mycophenolate mofetil and some of the emerging compounds such as fingolimod and leflunomide appear to have less of an effect on glucose metabolism (Egidi 2005). Steroids are also known to produce whole-body insulin resistance when administered systemically for longer periods and to exacerbate diabetes (Qi and Rodrigues 2007). However, this should not be a problem with local delivery as much lower levels are available systemically, especially if insulin resistance is mainly due to suppression of glucose transport (Pagano et al. 1983; Sakoda et al. 2000). Therefore, for such an approach to be effective, not only locally active agents have to be identified, but the nontrivial problems of delivering them locally and maintaining them at concentration levels that are immunosuppressive, but non β-cell toxic have to be solved.

1.4. Soft corticosteroids

Accordingly, soft steroids such as loteprednol etabonate (LE) are obvious candidates for local immunosuppressive agents. Corticosteroids are the most widely used class of immunosuppressive agents because of their broad spectrum of activity. They exert their main effect by binding to glucocorticoid receptors (GRs), a member of the steroid-thyroid-retinoid receptor super-family (Lu et al. 2006), but they can exert non-genomic effects as well (Maier et al. 2005; Norman et al. 2004), and there is increasing evidence for a physical and functional interaction between the GR and the T-cell receptor complex underlying the nongenomic glucocorticoid-induced immunosuppression in T cells (Löwenberg et al. 2007). Unfortunately, their long-term systemic use results in a distinctive cluster of side effects (e.g., Cushing's syndrome) (Buchman 2001; Schimmer and Parker 1996), a main reason why steroid-sparing agents are often employed in clinical practice. Soft steroids are, therefore, particularly promising as they, as any soft drug, are specifically designed to produce targeted local activity, but no systemic side effects due to prompt metabolic (preferably extrahepatic, e.g., hydrolytic) inactivation (Bodor and Buchwald 2006; Buchwald and Bodor 2004). Soft drugs are new, active therapeutic agents (often isosteric-isoelectronic analogues of a lead compound) with a chemical structure specifically designed to allow for predictable metabolism into inactive metabolites after exerting the desired therapeutic effect(s) (Bodor 1984; Bodor and Buchwald 2000, 2003). Therefore, in most cases, they produce pharmacological activity locally, but their distribution away from the site results in a prompt metabolic deactivation that prevents any kind of undesired pharmacological activity or toxicity. Furthermore, soft drugs should preferably rely on inactivation by hydrolytic enzymes because rapid metabolism can be more reliably carried out by esterases (which are ubiquitously distributed) rather than by oxygenases (which are located mostly in the liver and are subject to inhibition and induction).

We selected loteprednol etabonate (LE, **1**) as our first candidate for our application because it lacks serious side effects and it has been approved by the Food and Drug Administration (FDA) as the active ingredient of three ophthalmic preparations for clinical use (Lotemax®, Alrex®, Zylet®) (Bodor and Buchwald 2002; Noble and Goa 1998). Studies in rabbits (Bodor et al. 1992a) and rats (Bodor et al. 1992b) have demonstrated that, consistent with its design, LE is indeed active, it is metabolized into its predicted acidic metabolites AE (**2**) and A (**3**, Scheme), and these metabolites are inactive (Druzgala

Scheme



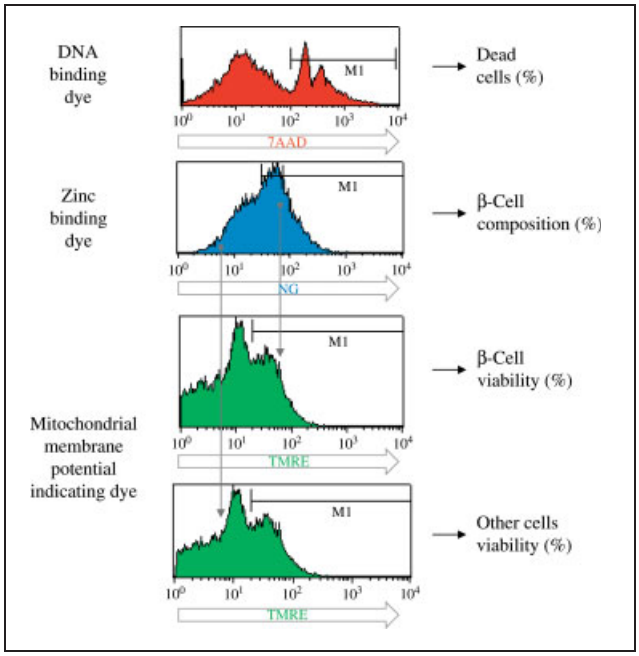


Fig. 1: Summary of the analytical methodology of Ichii and co-workers (Ichii et al. 2005a) utilized here to assess the viability and apoptosis of β-cells (shown with a set of cell data obtained here)

et al. 1991). Here, preliminary results are reported for *in vitro* studies with human islets that were used to establish a tentative therapeutic concentration range and for fully scaled three-dimensional finite element method (FEM)-based Comsol multiphysics computational models that were used to explore various possibilities to achieve and maintain these concentration levels within the device.

2. Investigations, results, and discussion

2.1. In vitro effects of LE on human islets

To establish potentially non-β-cell toxic local concentration levels for LE, *in vitro* pilot studies were performed with human islets to assess their viability and functionality (glucose-stimulated insulin release) following incubation with increasing LE concentrations. Islets were isolated with the automated method followed by purification on density gradients at the Human Cell Processing Facility of the University of Miami's Miller School of Medicine, as described before (Ichii et al. 2005b; Ricordi et al. 1988). Following one day recovery period in culture, they were incubated at 37 °C for three days in culture media (CMRL-supplemented Miami Media-1, MM1: Mediatech) with LE at initial concentrations of 0 (control), 10, 40, 160, and 640 nM. At the end of the incubation period, the β-cell content and viability of the islets was assessed with a recently introduced method that is based on cellular composition analysis coupled with identification of β-cell-specific apoptosis at the mitochondrial level through flow cytometry analysis (Ichii et al. 2005a). The analytical procedure used (see Experimental section for details) is summarized in Fig. 1 using a set of data obtained here, and the results obtained are summarized in Fig. 2. They indicate no detrimental effect of LE on viability for the tested concentrations ($\leq$ 640 nM); in fact, they even indicate a slight protective effect, as the percent of live β-cells seemed to increase somewhat with increasing LE concentrations. Because LE is susceptible to hydrolytic degradation in biological media, evaluatory HPLC assays have also been performed to assess the LE concentration change in the islet-containing media during the 72 h incubation period. According to these, LE concentrations decrease with a half-life of approximately $t_{1/2} = 36$ h in the incubation media as estimated from comparing the initial (day 0) and final (day 3) concentrations.

Because corticosteroids (e.g., dexamethasone) might directly inhibit insulin release by islet β-cells (Lambillotte et al. 1997; Pierluissi et al. 1986), the effects of LE on islet functionality were assessed by perifusion experiments consisting of dynamic glucose-stimulated insulin release (GSIR) tests that can be used to evaluate insulin secretion in response to stimulation by high glucose as well as total insulin content (as released in response to stimulation by KCl) (Cabrera et al. 2006). Results of a preliminary evaluation are shown in Fig. 3. A trend for a dose-related inhibition is noticeably present: the two lower doses (10 and 40 nM) showed no significant inhibition, whereas, the two higher doses (160 and 640 nM) showed some inhibition of the glucose-stimulated insulin release. Our preliminary results seem to be in agreement with a recent report showing that a 3-hour preincubaton with dexamethasone at the rela-

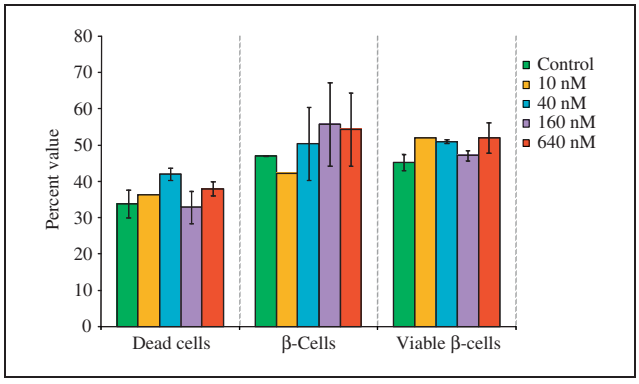


Fig. 2: Assessment of the effect of 72 h incubation with LE at various concentrations on the viability of pancreatic cells in human islets

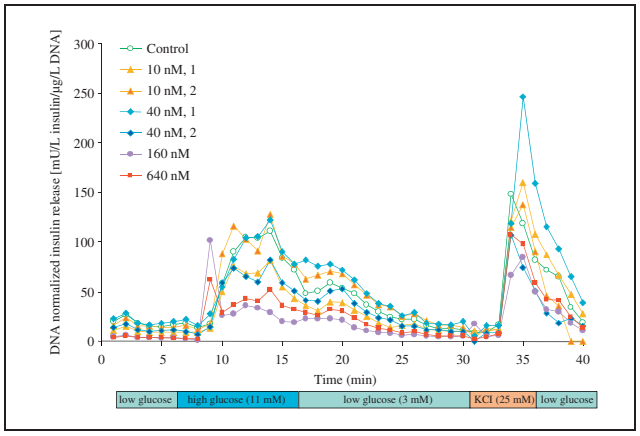


Fig. 3: A first, preliminary evaluation of the effect of 72 h incubation with LE at various concentrations on the insulin secretion of human islets as determined in a dynamic perifusion assay. Concentrations denote initial values estimated from the dilution ratios (LE showed some hydrolytic degradation over time in the media, see text), and results are shown without correcting for the dead space in the perifusion apparatus

tively high concentration of 1,000 nM resulted in reduction of GSIR from perifused rat islets by 60–70% (Zawalich et al. 2006). Considering the activity of LE (and dexamethasone), they are expected to produce effective immunosuppression at considerably lower levels (see below), so that a sufficiently wide local therapeutic concentration-range still exists.

The dissociation constant, K_D, of LE for the glucocorticoid receptor (GR) is in the 5 nM range; therefore, its main GR-mediated effects are expected to take place in this concentration range. With this K_D value, LE is about 1.5, 8, and 15 fold more potent than dexamethasone, prednisolone, and hydrocortisone, respectively (Bodor and Buchwald 2006; Buchwald 2005). This would suggest an immunosuppressive IC_{50} of about 6.0–6.5 nM for its immunosuppressive ability as measured by the whole blood lymphocyte proliferation (WBLP) assay on the basis of the data of Mager et al. (2003). LE has been shown to have anti-inflammatory activity similar to dexamethasone or beclomethasone dipropionate; for example, its IC_{50} values for inhibition of endotoxin (lipopolysaccharide, LPS)-induced release of tumor necrosis factor-α (TNFα) in 1 : 5 diluted human blood and for inhibition of anti-IgE-induced granulocyte-macrophage colony stimulating factor (GM-CSF) release in dispersed nasal polyp cells are 150 nM and 18.2 nM, respectively (Szelenyi et al. 2000). Accordingly, LE concentration levels of 5–500 nM (2–250 ng/mL) can serve as a first estimate of a therapeutic range that could be immunosuppressive, but not significantly β-cell toxic.

2.2. *Computational drug distribution models*

Using this range as a tentative target, fully scaled, three-dimensional finite element method (FEM)-based Comsol multiphysics computational models that incorporate both diffusion/convection and incompressible Navier-Stokes fluid mechanics application modes were used to explore various local delivery possibilities that could be used to achieve and maintain these concentration levels within a model device. As a device suitable for implantation in small rodents, we considered a small cylinder of r = 2.150 mm internal radius and h = 8 mm height. To have a computationally treatable model, certain strong simplifying conditions had to be introduced: we assumed no physical movement (resting fluid phase), no active transport due to blood flow within the chamber (where, at least in the first few days critical for survival, there is no significant vasculature), and sink conditions (c = 0) along the tissue-embedded walls (where the existing vasculature can effectively remove any drug that reaches there). With time, connective tissue and vessels would grow inside the device; this will affect both diffusion and flow, and consequently the distribution of the drug.

The resulting model of essentially aqueous liquid in a cylindrical device could be studied at full 3D scale by using a finite element method (FEM)-based multiphysics model (Comsol Multiphysics 3.3). Such FEM approaches are designed to solve coupled physics phenomena for problems based on partial differential equations (PDEs) by "discretization": the geometry is partitioned into small units of a simple shape (such as triangles or tetrahedrons for 2D or 3D problems, respectively) (Comsol 2006b). This, coupled with the multiphysics application mode, allows exploring not only the simplest, often investigated case of diffusive solute transport, but also coupled diffusive and convective transport processes (see Experimental section for a more detailed description of the pertaining physics models).

Three different possibilities were evaluated for their potential to achieve the targeted 5–500 nM therapeutic range inside the cylindrical bioartificial pancreas-type device: (1) zero-order (i.e., continuous rate) release from a single,

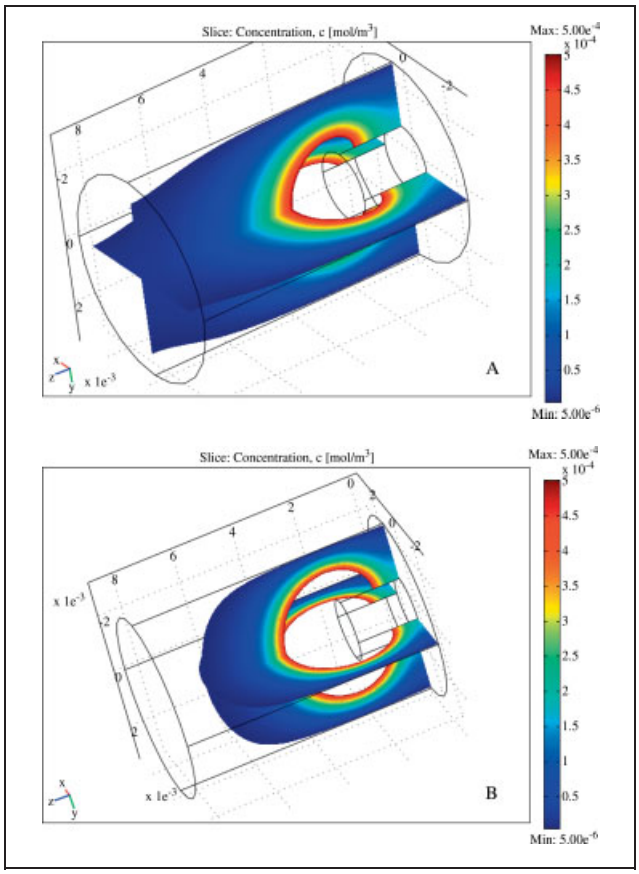


Fig. 4: Calculated drug concentrations for zero-order release from a single, membrane-covered slow-release cylinder (e.g., from a device similar to Retisert®). Only the approximate therapeutic range of 5–500 nM is colored in the figures with blue corresponding to lower and red to higher concentrations (color coding is shown in the legend on the right on a mol · m^{-3} scale). Drug release rates are 0.9 (A) and 9 nmol/day (B), but in the second case (B), a local first rate metabolic degradation with a half-life of 10 min was also assumed

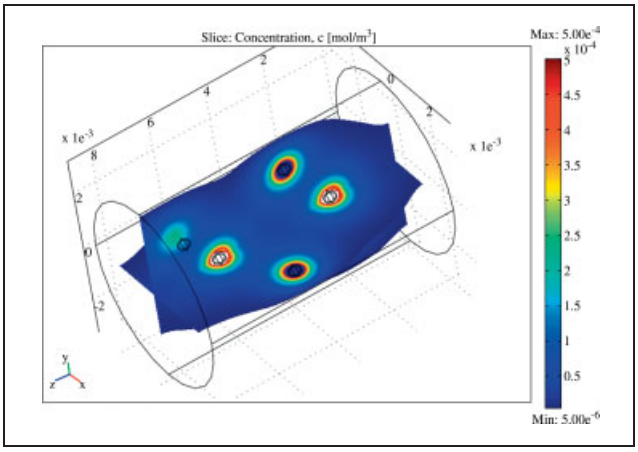


Fig. 5: Calculated drug concentrations for zero-order release from multiple, randomly distributed, slow-release spherical beads with a total drug release rate of 0.9 nmol/day (0.4 μg/day)

membrane-covered slow-release tablet (e.g., an implant similar to Retisert®, which can be used for the prolonged 2–3 year ocular release of fluocinolone acetonide) (Fig. 4); (2) zero-order release from multiple, randomly distributed, slow-release spherical beads (Fig. 5); and (3) a continuous liquid infusion through an orifice at one of the ends of the device (Fig. 6). Preliminary calculations suggested that if no local metabolic degradation is assumed, a drug delivery rate of approximately 1 nmol/day (~0.5 μg/day) can provide adequate coverage inside the cylindrical chamber for

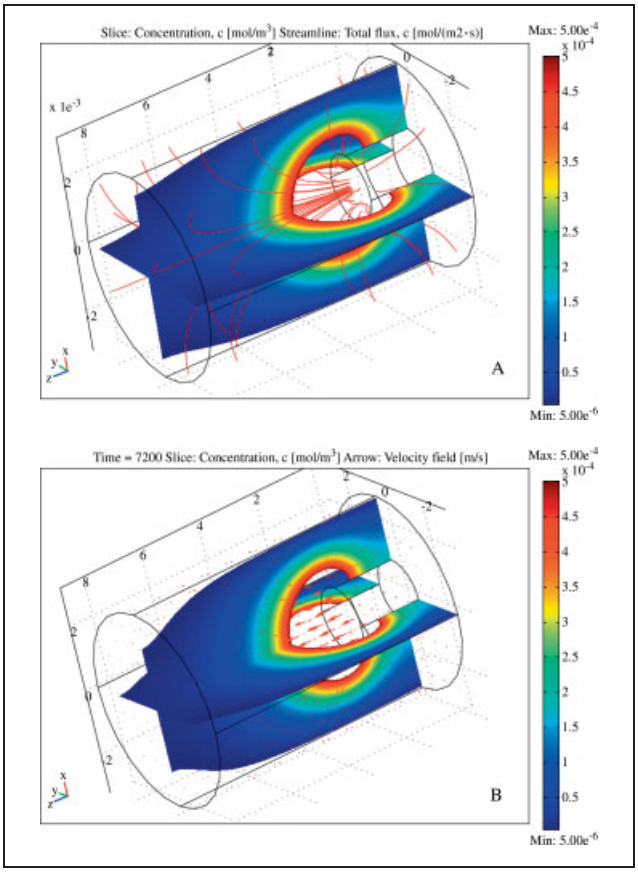


Fig. 6: Calculated drug concentrations for a continuous liquid infusion with a drug delivery rate of 0.9 nmol/day (infusion of a solution with a $c = 37$ μM concentration through $r = 1.0$ mm hole with a rate of 1 μL/h rate; no degradation assumed). In addition to the concentration distribution, the total flux streamlines (A) and the magnitude-scaled velocity field vectors (B) are also shown

each of these possibilities. All configurations can cover an acceptable percentage of the device-chamber (i.e., > 67% of the volume) as indicated by the corresponding figures (Figs. 4, 5, 6) and as confirmed quantitatively by the corresponding subdomain integrals (data not shown). The multiple spherical bead approach might provide the most uniform coverage, but only if the beads can be sufficiently uniformly distributed and maintained (Fig. 5). In all of these figures, only the estimated therapeutic range of 5–500 nM has been colored (with blue corresponding to lower and red to higher drug concentrations). The case of the infusion (Fig. 6) represents a true multiphysics application: here the diffusion model had to be coupled to the fluid dynamics model to calculate the velocity field **u** that results from the convection. Delivering a solution with a $c = 37$ μM concentration through $r = 1.0$ mm hole with a continuous rate of 1 μL/h is an entirely realistic possibility and will result in the needed ~1 nmol/day rate providing adequate coverage. Finally, in one case (Fig. 4B), for exploratory purposes, local metabolism has been also built into the model by allowing a first order, extrahepatic hydrolytic degradation with a rate corresponding to a half-life, $t_{1/2}$, of 10 min [a value observed for the hydrolysis of LE in rat blood (Bodor et al. 1992b)]. This requires an increased rate of drug delivery and reduces the percentage of covered volume (Fig. 4B).

In conclusion, preliminary studies to evaluate the feasibility of localized immunosuppression with the soft steroid loteprednol etabonate have been performed. *In vitro* tests with human islets suggest that there might be a sufficiently wide therapeutic window of local concentrations that are effective for immunosuppression, but not yet toxic even to the sensitive β-cells of the pancreatic islets. FEM-based multiphysics computer simulations suggest that these levels can be achieved and maintained by various realistic methods of continuous local delivery over a sufficiently large percentage of small cylindrical devices suitable for implantation in rodents. Animal studies to confirm these findings and to establish an adequate dose-range will be performed.

3. Experimental

3.1. In vitro experiments

3.1.1. Human islet isolation

Islets were isolated at the Human Cell Processing Facility of the University of Miami's Miller School of Medicine as described before (Ichii et al. 2005b; Pileggi et al. 2004; Ponte et al. 2007; Ricordi et al. 1988). Briefly, human pancreata were obtained from multi-organ cadaveric donors and processed with the automated Ricordi method (Ricordi et al. 1988) using different lots of the enzyme Liberase (Linetsky et al. 1997). The pancreas is "cannulated" through the duct to allow for the enzyme to distend the organ. Then, it is placed into the Ricordi chamber® and is continuously digested in order to obtain fragments of progressively decreasing sizes (Pileggi et al. 2004). The enzyme is activated by a heating circuit, and a continuous flow allows the islets to be collected (Ricordi et al. 1988). Islets are purified using a computerized semi-automated cell processor (COBE) at a temperature of 4 °C and a Ficol-based density gradient (Alejandro et al. 1990; Ichii et al. 2005b). The highly purified islets are generally contained in the top layer, while the less pure islets settle on the bottom layers of the gradient.

3.1.2. Islet incubation

Following isolation, islets were cultured at 37 °C in humidified mixed 95% air 5% CO_2 in non-tissue treated flasks with Miami-defined media 1 (MM1; Mediatech) containing 0.5% human serum albumin (Ichii et al. 2007). After 24 h of culture, islets were collected and divided in different culture conditions (1,500–2,000 IEQ per condition) for incubation with loteprednol etabonate (LE) at the following concentrations: 0 (control), 10, 40, 160, and 640 nM in MM1 culture media. LE was prepared as a stock solution of 0.5% (v/v) in ethanol and then further diluted with media to

obtain a 0.0005% solution, which was then used to prepare the predefined culture concentrations in each separate flask. After 72 h, islets were collected and processed separately for the two *in vitro* assessments: β-cell viability assessment and GSIR (dynamic perifusion stimulation).

3.1.3. Viability assessment

Viability assessment has been performed as described before (Ichii et al. 2005a). Briefly, aliquots of 1500 IEQ were dissociated by resuspending in 1 mL Accutase (Innovative Cell Technologies, Inc., San Diego, CA) in a 15 mL tube, incubating at 37 °C for 10 min, and vortexing gently at the end. For the assessment of apoptosis, single islet cell suspensions were incubated for 30 min with a Newport Green (NG) and tetramethyl-rhodamineethylester (TMRE) (Molecular Probes, Eugene, OR) mixture, 1 μM and 100 nM respectively. NG binds to zinc in the secretory granules of islet cells, allowing for the definition of cell subsets according to their zinc content, and thus labeling preferentially β-cells. TMRE selectively binds to mitochondrial membranes, allowing for the assessment of cells with functional mitochondria, and, therefore, can be used as a marker for cell viability; loss of staining is considered an early marker of apoptosis. After washing with phosphate buffered saline (PBS), cells were stained with 7-aminoactinomycin D (7-AAD; Molecular Probes, Eugene, OR) that binds to DNA after alteration of cell membrane permeability caused by cell death. Data presented here (Fig. 2) are average ± SD for two experiments. Analysis was performed using CellQuest software on a FACSCalibur flow cytometer (Becton & Dickinson Co., Mountain View, CA).

3.1.4. In vitro dynamic perifusion assay

The perifusion experiments consisted of a dynamic glucose-stimulated insulin release (GRIS) test performed using a custom built apparatus (BioRep Technologies, Inc., Miami, FL). Islets aliquots of 200 IEQ were loaded in Perspex microcolums, between two layers of acrylamide-based microbead slurry (Bio-Gel P-4, Bio-Rad Laboratories, Hercules, CA). Krebs buffer with selected glucose (low = 3 mM; high = 11 mM) or KCl (25 mM) concentrations was circulated through the columns at a rate of 100 μL/min. After 45 minutes of washing with the low glucose solution for stabilization, the islets were stimulated with the following sequence: 5 min of low glucose, 10 min of high glucose, 15 min of low glucose, 5 min of KCl, and 5 min of low glucose. Serial samples of 100 μL were collected every minute from the outflow tubing of the columns, and insulin concentrations were determined with a commercially available ELISA kit (Mercodia Inc., Wiston Salem, NC). At the end of the experiments, what was contained in the columns has been kept with 1 mL of T-per tissue protein extraction for DNA quantification (Quant-iT PicoGreen dsDNA Kit, Molecular Probes, Eugene, OR).

3.1.5. HPLC assay

High performance liquid chromatography (HPLC) has been used to assess LE concentrations and stability in the islet incubation media. Thawed samples (0.5 mL) were mixed with 0.01 mL of 1N HCl and 1 mL of a 4 : 6 (v/v) ethyl acetate/hexane mixture, vortexed for 1 min, and centrifuged. The upper organic layer (0.8 mL) was taken into a 1 mL glass tube (a). The remainings were re-extracted with another 1 mL of a 4 : 6 (v/v) ethyl acetate/hexane mixture, and the resulting upper organic layer (0.8 mL) (b) was taken and combined with (a). The combined samples (a + b) were evaporated in a vacuum centrifuge, and reconstituted in 0.1 mL of 6 : 4 (v/v) acetonitrile/water solution. For calibration standards, 0.01 mL of stock solutions (0, 1, 2.5, 5, 10 μg/mL in 60% acetonitrile/water) were added in 0.99 mL of medium and mixed to yield the standards of 0, 0.01, 0.025, 0.05, and 0.1 μg/mL. The resulting standards (0.5 mL) were taken, and prepared by the same procedures as for the samples. The HPLC system used consisted of a SpectraPhysics (San Jose, CA) SP 8810 isocratic pump with SP 8450 UV/Vis detector and SP 4290 integrator and was used with a Supelco Discovery C8 column, a mobile phase of acetonitrile/water/acetic acid at 60 : 40 : 0.2 ratio, a flow rate of 1 mL/min, an injection volume of 50 μL, and UV detection at 254 nm. Obtained retention times were 8.2 min for LE (**1**), 4.8 min for AE (**2**), and 3.8 min for A (**3**); the limit of detection for LE was 10 ng/mL.

3.2. Computational drug distribution model

Modeling was performed with a finite element method (FEM) (Comsol Multiphysics 3.3, Comsol AB, Stockholm, Sweden). Diffusion was assumed to be governed by the generic diffusion equation in its nonconservative formulation (incompressible fluid) (Comsol 2006a):

$$\frac{\partial c}{\partial t} + \nabla \cdot (-D\nabla c) = R - \mathbf{u} \cdot \nabla c \qquad (1)$$

where, c denotes the concentration of the species of interest [$mol \cdot m^{-3}$], D is the diffusion coefficient [$m^2 \cdot s^{-1}$], R is the reaction rate [$mol \cdot m^{-3} \cdot s^{-1}$], u is the velocity field [$m \cdot s^{-1}$], and ∇ is the standard del (nabla) operator (Riley et al. 1997):

$$\nabla \equiv \mathbf{i}\frac{\partial}{\partial x} + \mathbf{j}\frac{\partial}{\partial y} + \mathbf{k}\frac{\partial}{\partial z} \qquad (2)$$

As boundary conditions, a constant fixed concentration of $c = 0$ was used along the external, cylindrical sides (corresponding to sink conditions at the meshed, vascularized tissue boundaries) and insulation/symmetry, $\mathbf{n} \cdot (-D\nabla c) = 0$, was used along the two plastic-capped ends. Along the drug releasing surfaces, a constant flux, $-\mathbf{n} \cdot (-D\nabla c + c\mathbf{u}) = N_0$, was used as boundary condition with an inlet flux of 3.26×10^{-9} $mol \cdot m^{-2} \cdot s^{-1}$ (corresponding to a 0.9 nmol/day = 0.4 μg/day, total constant flux, i.e., zero-order release). In the diffusion only application, no convection is allowed; i.e., **u** is assumed to be 0. In all these models, a diffusion coefficient $D = 6 \times 10^{-10}$ $m^2 \cdot s^{-1}$ was assumed for LE, a reasonable value considering measured values for similar compounds; e.g. $\sim 7 \times 10^{-10}$ $m^2 \cdot s^{-1}$ for testosterone in phosphate buffer and 1.5% purified pig gastric mucin (Larhed et al. 1997) or 7×10^{-10} $m^2 \cdot s^{-1}$ for hydrocortisone and 4.9×10^{-10} $m^2 \cdot s^{-1}$ for betamethasone valerate in water (Seki et al. 2003). In most models no consumption, i.e., a reaction rate R of 0 was used, but in one case, degradation was allowed with a first-order consumption rate of $R = 0.00116c$ s^{-1}, corresponding to a half-life of 10 min, a value that has been observed for the hydrolysis of LE in rat blood (Bodor et al. 1992b).

In the more complex model needed for the case where the drug solution is continuously infused, this diffusion model had to be coupled to the fluid dynamics model to calculate the velocity field **u** that results from the convection. For fluid dynamics, the incompressible Navier-Stokes model for Newtonian flow (constant viscosity) was used (Comsol 2006a):

$$\rho\frac{\partial \mathbf{u}}{\partial t} - \eta\nabla^2\mathbf{u} + \rho(\mathbf{u} \cdot \nabla)\,\mathbf{u} + \nabla p = \mathbf{F}$$
$$\nabla \cdot \mathbf{u} = 0 \qquad (3)$$

Here, ρ is the density [$kg \cdot m^{-3}$], η is the viscosity [$kg \cdot m^{-1} \cdot s^{-1} = Pa \cdot s$], p is the pressure [$kg \cdot m^{-1} \cdot s^{-2} = Pa$], and **F** is the volume force [$kg \cdot m^{-2} \cdot s^{-2}$]. The first equation is the momentum balance, whereas, the second is simply the equation of continuity for incompressible fluids. As boundary conditions, outflow with zero pressure ($p = p_0 = 0$, $K = 0$) was used along the external cylindrical surface and no slip ($\mathbf{u} = 0$) was used along the capped ends. Along the drug releasing surface, a constant inflow/outflow velocity ($\mathbf{u} = \mathbf{u}_0$) was used as boundary condition with an inlet velocity of 8.85×10^{-8} $m \cdot s^{-1}$ (corresponding to an infusion rate of 1 μL/h and a total dose of 0.9 nmol/day = 0.4 μg/day with an assumed concentration of 37 μM). As a first estimate, an aqueous media at body temperature was assumed in all subdomains (e.g., $T_0 = 310.15$ K, $\rho = 993$ $kg \cdot m^{-3}$, $\eta = 0.7 \times 10^{-3}$ $Pa \cdot s$).

Fully scaled 3D geometries were built in Comsol (cylinder of r = 2.150 mm internal radius and h = 8 mm height, single slow-release cylinder of r = 1.0 mm radius, multiple slow-release spherical beads of r = 0.2 mm radius, and continuous infusion inlet of r = 1.0 mm radius). The geometry has been divided in fine mesh elements using Comsol's default setting, and the problem has been solved for stationary condition on a Dell Precision PC with a 3.2 GHz CPU running Linux. Computations were done with the Pardiso direct solver as the linear system solver as this seemed sufficiently stable and fast enough for all 2D and for most, not too complex 3D cases.

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References

Alejandro R, Feldman EC, Bloom AD, Kenyon NS (1989) Effects of cyclosporin on insulin and C-peptide secretion in healthy beagles. Diabetes 38: 698–703.

Alejandro R, Latif Z, Polonsky KS, Shienvold FL, Civantos F, Mint DH (1988) Natural history of multiple intrahepatic canine islet allografts during and following administration of cyclosporine. Transplantation 45: 1036–1044.

Alejandro R, Strasser S, Zucker PF, Mintz DH (1990) Isolation of pancreatic islets from dogs. Semiautomated purification on albumin gradients. Transplantation 50: 207–210.

Bell E, Cao X, Moibi JA et al. (2003) Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. Diabetes 52: 2731–2739.

Bodor N (1984) The soft drug approach. Chemtech 14 (1): 28–38.

Bodor N, Bodor N, Wu W-M (1992a) A comparison of intraocular pressure elevating activity of loteprednol etabonate and dexamethasone in rabbits. Curr Eye Res 11: 525–530.

Bodor N, Buchwald P (2000) Soft drug design: general principles and recent applications. Med Res Rev 20: 58–101.

Bodor N, Buchwald P (2002) Design and development of a soft corticosteroid, loteprednol etabonate. In: Schleimer RP, O'Byrne PM, Szefler SJ, Brattsand R (eds), Inhaled Steroids in Asthma. Optimizing Effects in the Airways. New York: Marcel Dekker, p 541–564.

Bodor N, Buchwald P (2003) Retrometabolism-based drug design and targeting. In: Abraham DJ (ed), Burger's Medicinal Chemistry and Drug Discovery. Vol. 2, Drug Discovery and Drug Development, 6th Edition, 6th ed, Vol 2. New York: Wiley, p 533–608.

Bodor N, Buchwald P (2006) Corticosteroid design for the treatment of asthma: structural insights and the therapeutic potential of soft corticosteroids. Curr Pharm Des 12: 3241–3260.

Bodor N, Loftsson T, Wu W-M (1992b) Metabolism, distribution, and transdermal permeability of a soft corticosteroid, loteprednol etabonate. Pharm Res 9: 1275–1278.

Buchman AL (2001) Side effects of corticosteroid therapy. J Clin Gastroenterol 33: 289–294.

Buchwald P (2005) General linearized biexponential model for QSAR data showing bilinear-type distribution. J Pharm Sci 94: 2355–2379.

Buchwald P, Bodor N (2004) Soft glucocorticoid design: structural elements and physicochemical parameters determining receptor-binding affinity. Pharmazie 59: 396–404.

Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci USA 103: 2334–2339.

Colton CK (1995) Implantable biohybrid artificial organs. Cell Transplant 4: 415–436.

Comsol AB (2006a) COMSOL Multiphysics Modeling Guide, 3.3 ed: COMSOL AB.

Comsol AB (2006b) COMSOL Multiphysics User's Guide, 3.3 ed: COMSOL AB.

Druzgala P, Hochhaus G, Bodor N (1991) Soft drugs. 10. Blanching activity and receptor binding affinity of a new type of glucocorticoid: loteprednol etabonate. J Steroid Biochem 38: 149–154.

Egidi FM (2005) Management of hyperglycaemia after pancreas transplantation: are new immunosuppressants the answer? Drugs 65: 153–166.

Fernandez LA, Lehmann R, Luzi L et al. (1999) The effects of maintenance doses of FK506 versus cyclosporin A on glucose and lipid metabolism after orthotopic liver transplantation. Transplantation 68: 1532–1541.

First MR (2003) Posttransplant diabetes mellitus. Transplantation 75: 1769.

Ichii H, Inverardi L, Pileggi A et al. (2005a) A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 5: 1635–1645.

Ichii H, Pileggi A, Molano RD et al. (2005b) Rescue purification maximizes the use of human islet preparations for transplantation. Am J Transplant 5: 21–30.

Ichii H, Sakuma Y, Pileggi A et al. (2007) Shipment of human islets for transplantation. Am J Transplant 7: 1010–1020.

Kizilel S, Garfinkel M, Opara E (2005) The bioartificial pancreas: progress and challenges. Diabetes Technol Ther 7: 968–985.

Knight KR, Uda Y, Findlay MW et al. (2006) Vascularized tissue-engineered chambers promote survival and function of transplanted islets and improve glycemic control. FASEB J 20: 565–567.

Knisely TL, Hosoi J, Nazareno R, Granstein RD (1994) The presence of biologically significant concentrations of glucocorticoids but little or no cortisol binding globulin within aqueous humor: relevance to immune privilege in the anterior chamber of the eye. Invest Ophthalmol Vis Sci 35: 3711–3723.

Lambillotte C, Gilon P, Henquin JC (1997) Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest 99: 414–423.

Larhed AW, Artursson P, Gråsjö J, Björk E (1997) Diffusion of drugs in native and purified gastrointestinal mucus. J Pharm Sci 86: 660–665.

Linetsky E, Bottino R, Lehmann R, Alejandro R, Inverardi L, Ricordi C (1997) Improved human islet isolation using a new enzyme blend, Liberase. Diabetes 46: 1120–1123.

Löwenberg M, Verhaar AP, van den Brink GR, Hommes DW (2007) Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends Mol Med 13: 158–163.

Lu NZ, Wardell SE, Burnstein KL et al. (2006) International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 58: 782–797.

Mager DE, Moledina N, Jusko WJ (2003) Relative immunosuppressive potency of therapeutic corticosteroids measured by whole blood lymphocyte proliferation. J Pharm Sci 92: 1521–1525.

Maier C, Runzler D, Schindelar J et al. (2005) G-protein-coupled glucocorticoid receptors on the pituitary cell membrane. J Cell Sci 118: 3353–3361.

Marcelli-Tourvieille S, Hubert T, Moerman E et al. (2007) In vivo and in vitro effect of sirolimus on insulin secretion. Transplantation 83: 532–538.

Narang AS, Mahato RI (2006) Biological and biomaterial approaches for improved islet transplantation. Pharmacol Rev 58: 194–243.

Noble S, Goa KL (1998) Loteprednol etabonate. Clinical potential in the management of ocular inflammation. BioDrugs 10: 329–339.

Norman AW, Mizwicki MT, Norman DPG (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov: 27–41.

Ozcay N, Fryer J, Grant D, Freeman D, Garcia B, Zhong R (1997) Budesonide, a locally acting steroid, prevents graft rejection in a rat model of intestinal transplantation. Transplantation 63: 1220–1225.

Pagano G, Cavallo-Perin P, Cassader M et al. (1983) An in vivo and in vitro study of the mechanism of prednisone-induced insulin resistance in healthy subjects. J Clin Invest 72: 1814–1820.

Pierluissi J, Navas FO, Ashcroft SJ (1986) Effect of adrenal steroids on insulin release from cultured rat islets of Langerhans. Diabetologia 29: 119–121.

Pileggi A, Alejandro R, Ricordi C (2006a) Islet transplantation: Steady progress and current challenges. Curr Opin Organ Transplant 11: 7–13.

Pileggi A, Molano RD, Ricordi C et al. (2006b) Reversal of diabetes by pancreatic islet transplantation into a subcutaneous, neovascularized device. Transplantation 81: 1318–1324.

Pileggi A, Ricordi C, Kenyon NS et al. (2004) Twenty years of clinical islet transplantation at the Diabetes Research Institute – University of Miami. Clin Transpl: 177–204.

Ponte G, Pileggi A, Messinger S et al. (2007) Toward maximizing the success rate of human islet isolation: Influence of donor and isolation factors. Cell Transplant: 16: 595–607.

Qi D, Rodrigues B (2007) Glucocorticoids produce whole body insulin resistance with changes in cardiac metabolism. Am J Physiol Endocrinol Metab 292: E654–E667.

Ricordi C (2003) Islet transplantation: a brave new world. Diabetes 52: 1595–1603.

Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW (1988) Automated method for isolation of human pancreatic islets. Diabetes 37: 413–420.

Ricordi C, Strom TB (2004) Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 4: 259–268.

Ricordi C, Zeng Y, Alejandro R et al. (1992) Effect of FK 506 on human pancreatic islets following renal subcapsular transplantation in diabetic nude mice. Transplant Proc 24: 1042.

Riley KF, Hobson MP, Bence SJ (1997) Mathematical Methods for Physics and Engineering. A Comprehensive Guide. Cambridge: Cambridge University Press.

Rilo HL, Carroll PB, Zeng YJ, Fontes P, Demetris J, Ricordi C (1994) Acceleration of chronic failure of intrahepatic canine islet autografts by a short course of prednisone. Transplantation 57: 181–187.

Sakoda H, Ogihara T, Anai M et al. (2000) Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. Diabetes 49: 1700–1708.

Schimmer BP, Parker KL (1996) Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Hardman JG, Limbird LE (eds), Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th ed. New York: McGraw-Hill, p 1459–1485.

Seki T, Mochida J, Okamoto M, Hosoya O, Juni K, Morimoto K (2003) Measurement of diffusion coefficients of parabens and steroids in water and 1-octanol. Chem Pharm Bull 51: 734–736.

Shirbacheh MV, Jones JW, Breidenbach WC, McCabe S, Barker JH, Gruber SA (1998) The case for local immunosuppression in composite tissue allotransplantation. Transplant Proc 30: 2739–2742.

Szelenyi I, Hochhaus G, Heer S et al. (2000) Loteprednol etabonate: a soft steroid for the treatment of allergic diseases of the airways. Drugs Today 36: 313–320.

Wang X, Alfrey EJ, Posselt A, Tafra L, Alak AM, Dafoe DC (1995) Intraportal delivery of immunosuppression to intrahepatic islet allograft recipients. Transpl Int 8: 268–272.

Weber T, Kalbhenn T, Herrmann G, Hanisch E (1997) Local immunosuppression with budesonide after liver transplantation in the rat: a preliminary histomorphological analysis. Transplantation 64: 705–708.

Zawalich WS, Tesz GJ, Yamazaki H, Zawalich KC, Philbrick W (2006) Dexamethasone suppresses phospholipase C activation and insulin secretion from isolated rat islets. Metabolism 55: 35–42.

Zhang N, Su D, Qu S et al. (2006) Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. Diabetes 55: 2429–2436.