Chapter 15

Screening for Antidiabetic Activities

Rima Caccetta and Hani Al Salami

Abstract

Screening extracts and drug entities for antidiabetic bioactivity is essentially limited to animal models as the processes leading to hyperglycemia and the complications of diabetes involve more than one organ. Further, in vitro results seldom translate into meaningful in vivo outcomes especially in a disease such as Diabetes Mellitus. In vivo studies on specialized animal models have allowed great progress in tailoring research questions towards individualized genetic and biochemical contributors and their effect on the pathogenesis of the disease process. Various disease models have been used either through genetic manipulation (transgenic models) or through chemical induction (diabetes-induced models). Although there is a surplus of animal models (spontaneous and induced) to study Type I and Type II diabetes, there is no ideal or standard model for studying the individualized effects of various classes of antidiabetic drugs. Rodents, most commonly rats and mice, have been used by researchers as animal models of the disease and both normoglycemic and diabetic animals are used to assess the antidiabetic activities of drugs or extracts under investigation. Screening for antidiabetic activities can be achieved by measuring a wide range of biomarkers and endpoints including blood glucose and insulin levels.

Key words: Diabetes mellitus, Drug discovery, Plant extracts, Type I, Type II

1 Introduction

Active constituents of many plant species are isolated for direct use as drugs or lead drug compounds. Historically different plant species have been used to treat diabetes mellitus and these medicinal plants are of prime interest as sources of drug molecules. Unfortunately, taken as plant concoctions, there are many drawbacks and its success in different patients relies on many factors including the type of diabetes they have. Continuous dosages are variable and commonly other interactive ingredients (which can be toxic) are also present.

Success in screening these extracts for potential antidiabetic activity relies on a number of factors including the extraction technique employed, the experimental design, and the biological model utilized. Most importantly, antidiabetic drugs must reduce blood glucose levels in order to be effective. Further, different animal
models can expand on the mechanism(s) of action and give an indication whether the drug mimics insulin or acts in some other means to enhance insulin release or its actions. Nonetheless, the initial screening needs to be promising enough to signal pursuit of isolating active constituents.

Diabetes mellitus (DM) is a chronic condition characterized by hyperglycemia (which can cause hypertension) with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. There are two major types of DM: Type I and Type II. Diabetics are genetically predisposed to either type of the disease but both types appear to rely on other, including environmental, factors to trigger or expose the disease. DM is on the rise with Type II diabetes increasing dramatically worldwide.

Type I diabetes (T1D) is believed to be an autoimmune disease of sudden onset usually during childhood. It is characterized by the lack of insulin release due to the destruction of pancreatic β-cells. This appears to be triggered (or accelerated) by an early fetal event such as blood group incompatibility or fetal viral infections or early exposure to cow’s milk proteins or nitroamines. Due to the lack of insulin, the body believes it is starved and thus breaks down biomolecules for the production of glucose and energy. The uncontrolled or poorly controlled diabetic exhibits elevated ketonemia.

Type II diabetes (T2D) is a metabolic disorder believed to be due to a genetic predisposition (involving a number of metabolic genes) which is in most cases subsequently triggered by becoming overweight with a sedentary lifestyle. The pre-diabetic state is characterized by insulin resistance leading to compensatory hyperinsulinemia. The disease is then manifested by impaired insulin secretion from the pancreas and insulin action in the peripheral tissue including skeletal muscle, and adipose tissue [2]. The reduced insulin action is attributed to the decline in the number and/or responsiveness of the insulin receptors. At the microscopic level, the pancreatic islets in T2D are characterized by an insufficiency in β-cell mass and increased β-cell apoptosis [3, 4] with the islets commonly containing large deposits of amyloid derived from islet amyloid polypeptide, also known as amylin (see Note 1) [5]. All these processes culminate in a deficit in glucose transport (internalization from the blood into peripheral tissues) which may in part be attributed to the decreased expression of the major transporters GLUT 4 (see Fig. 1); however, little or no reduction in GLUT-4 levels is observed in type II diabetic patients [6, 7]. Ultimately, uncontrolled T2D will develop to exhibit T1D symptoms and thus the patient in many cases eventually requires insulin injections.

There is a range of animal models available for screening for antidiabetic potential. Each model carries some of the described characteristics of the human disease but no one model alone will carry all the human manifestations and thus screening needs to incorporate at least a couple of different models to gain a better understanding of the effectiveness of the screened drug or mixture. It is important to use adequate controls (positive and negative controls, e.g., insulina and other drugs on the market) as comparisons but also to minimize the contribution to the noise which, in the case of glucose levels, is adrenaline. Further, it is advisable to visit a statistician in the planning stages of the study to ensure minimum animal use and that the numbers chosen will enable meaningful statistical analyses. This chapter presents a number of common rodent models used (see Table 1) for screening from natural sources and validating lead or new therapeutic drugs in vivo for antidiabetic activity. The choice of model is dependent on the assessment of the desired endpoints, including the sample size, but may also be budget driven.

1.1 Normoglycemia

Normoglycemic animals can be used for screening potential hypoglycemic agents. Many studies have used and continue to use normoglycemic controls and these have in some cases mirrored the effects in the diabetic test animals. However, the results have been variable mostly due to the lower effects seen on normoglycemics than diabetics. In an attempt to assess some peptides for insulin-mimetic potential, intravenous administration is necessary but one can use anesthetized normoglycemic rodents, e.g., Wistar rats. Such a model avoids the effects of adrenaline which can otherwise work to mask positive effects of the drug or extract screened.
There are several benefits of using normoglycemic animals which include the ease of caring for these animals and their cost. Further, normoglycemic models offer assessment with an intact pancreas which can provide valuable information on the mechanism of action when compared to a diabetic model (e.g., Streptozotocin induced, see Subheading 1.5). Using normoglycemic animals for initial screening is valid; however, one needs to remember that the effectiveness of the compound or extract screened might be diminished.

1.2 Genetically Modified

A good example of an animal model of T2D is Db/db mice. This model mimics T2D and represents an accurate estimation of many disturbed biomarkers including hyperglycemia, insulin resistance, and obesity, and show a marked decrease in skeletal muscle utilization without an accompanying decrease in GLUT 4 expression. This insulin resistant animal model evaluates improved glycemic control through overexpression of GLUT 4. Upregulation of GLUT4 expression has been shown to improve skeletal muscle glucose transport in these insulin-resistant db/db mice [8, 9].

Human transgenic rodents, Rip-HAT Mouse/RIP-HAT Rat, develop DM between 4 and 8 weeks and 5–10 months of age, respectively. These models exhibit selective β-cell apoptosis leading to the onset of type II diabetes in the rodent. Basically, rats and mice have similar islet amyloid polypeptide (IAPP) homology but differ from humans by substitution of proline residues in the amyloidogenic portion of IAPP, and therefore do not form amyloid fibrils nor spontaneously develop diabetes in midlife. Thus, insertion of a human transgene may provide a means to explore the role of the amyloid fibrils in β-cell destruction.

Rodent models that carry a spontaneous single gene mutation in either the leptin or the leptin receptor inbred in either a C57BL/6j or C57BL/6j mice (see Table 1) are widely used. However, there is concern that these genetic mutations are rare occurrences in humans. In fact, leptin administration is ineffective in human T2D patients, except for a rare population with mutations in their leptin and/or leptin receptor genes.

1.3 Selectively Bred

The Goto-Kakizaki (GK) rat is a non-obese Wistar sub-strain which develops T2D early in life. This model was developed in the 1970s by repeated selection of breeders with high blood glucose levels which lead to glucose intolerance after five generations.

1.4 High-Fat Diet Induced

Obesity is a well-established risk factor (~30%) for T2D and a high-fat diet is a major trigger of insulin resistance and thus the development of T2D in genetically predisposed individuals. Induction of type II DM has been successfully achieved in C57Bl6/J male mice and in Golden Syrian hamsters through the

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### Table 1

<table>
<thead>
<tr>
<th>Rodent</th>
<th>Strain Description</th>
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<tr>
<td>Mouse</td>
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<tr>
<td>GLUT4</td>
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<td>GSK38</td>
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<tr>
<td>Rip-HAT</td>
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<tr>
<td>DM</td>
<td>T2D</td>
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<td>Rat</td>
<td>RIP-HAT</td>
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<tr>
<td>Rat</td>
<td>DM</td>
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*Note: T2D refers to type 2 diabetes.*
administration of highly saturated Purina diet. The mouse develops T2D over 8–12 weeks exhibiting marked obesity, hyperinsulinemic, insulin resistance, glucose intolerance, and peripheral leptin resistance.

1.5 Streptozotocin (STZ) or Alloxan Induced

The induction of T1D through the administration of STZ [10] or alloxan [11] is a common practice. Both are cytotoxic and when administered in appropriate doses (25–50 mg/kg) target the β-pancreatic cells resulting in cell necrosis and eventual cessation of insulin production by the pancreas. Chronic administration of lower doses may result in the development of T2D model, while in higher doses both agents can become toxic and may result in severe tissue damage especially the liver which can result in internal hemorrhage and death of the animal. Some animals may not develop DM and so it is important to assess the animal condition before proceeding with drug experimentation.

2 Materials

2.1 Mice/Rats

Research animal models may be bought from local Animal Resource Centers or from overseas (e.g., Charles River Laboratories International). Animals may need to be housed for a period of time prior to experimentation for them to stabilize in their new environment. Commercial chow and water should be provided ad libitum and the bedding needs to be changed regularly. Frequent monitoring and handling of the animals is necessary and advisable. Animals may gain or lose weight which needs to be taken into consideration as it might affect the experimental procedures.

2.2 Anesthetics

1. Hypnorm®–Dormicum or ketamine–xylazine mixture (see refs. [12, 13]).
2. Dilute anesthetics in vehicle like 1× Phosphate Buffered Saline (1× PBS). Prepare a stock solution of 10× PBS: weigh 80 g of NaCl, 2 g of KCl, 14.4 g of Na2HPO4, 2.4 g of KH2PO4 and dissolve in Milli-Q water, make up to 1 L. This 10XPBS solution can be store on the shelf. To achieve 1XPBS, dilute a portion (as needed) of the 10× PBS tenfold and pH check the solution to be ~7.4 (see Note 2). The 1× PBS requires sterilization either by autoclaving or by filtration (through sterile 0.22 µm pore syringe filters) into sterile containers. The 1× PBS needs to be stored at 4 °C for up to 1 month.
3. Warming box.
4. 1 mL syringes.
5. 26–27 G needles.
6. Thick paper towels.

2.3 Controls

1. Insulin, e.g., Humulin R (100 U) (Diabetes Australia from Eli Lilly and Company).
2. Metformin, 1,1-dimethylbiguanide hydrochloride (Sigma–Aldrich).
3. PBS (see Subheading 2.2, item 2).

2.4 Glucose and Insulin Measurements

1. Accu-Chek Go Glucometer.
2. Accu-Chek Glucometer strips.
3. HTRF* Insulin assay (Cisbio bioassays) or insulin enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemicals).
4. Positive controls (see Note 3).
5. Plate reader.

2.5 Other Materials

1. pH meter.
2. Alloxan monohydrate dissolved in deionized water or STZ (see refs. [2, 3]).
3. Analytical balances.
4. Surgical lamp (optional).

3 Methods

Blood glucose and insulin levels are the key indicators for diagnosis of DM. Elevated serum triglycerides and abdominal obesity are commonly associated with insulin resistance and represent valuable clinical markers of the metabolic syndrome [14]. Further, since comorbidity of DM and dyslipidemia is common, cholesterol, triglycerides, inflammatory markers, and hypertension are usually measured in the same experiment. However, for the purposes of this chapter we shall focus on the measurements directly linked to DM, namely glucose and insulin. Blood glucose concentration can be measured using a one-touch meter (e.g., Accu-Chek, see Subheading 2.4, item 1). Insulin concentration in blood can be measured using by using ELISA kits (see Subheading 2.4, item 3) following the manufacturer’s protocols. Blood samples can be collected from the animal at different intervals before and after treatment and analyzed appropriately.

3.1 Experimental Design

1. Decide whether it is best to assess the acute or repeated use effects of the extract or compound you are screening (see Note 4).
2. Depending on the properties of the compound or extract to be assessed, you might decide on its mode of delivery to be ad libitum, oral gavage, intravenous, or intraperitoneal. Obviously, oral gavage studies cannot be carried out while animals are
under anesthesia. The route of administration you choose must take into consideration the limitations as well as the advantages in the assessment process. A no effect may be attributed to the mode of administration as the compound may require metabolism to be activated or metabolism may eradicate its activity.

3. Choose satisfactory positive and negative controls (see Subheading 2.3). The oral controls may be orally administered drugs like metformin while insulin is ideal for intraperitoneal or intravenous injections. Other marketed antidiabetic medications might also be useful especially to compare the activity and explore the possible mode of action of the drug under investigation.

4. For intravenous or intraperitoneal administration, choose a vehicle with buffering range similar to blood, e.g., PBS. Assess the pH of the samples before injecting as the drug might have modified the pH of the vehicle. If necessary adjust the pH to 7.3–7.4 before injecting into the animals.

5. Re-solubilized extracts might require filtration or centrifugation (see Note 5) following dissolution to remove undissolved debris before injecting the clear solutions. It is desirable that the volume to be administered is kept to a minimum (see Note 6).


7. Measure blood glucose and insulin levels using kits (see Subheading 2.4) following the manufacturers' protocols before injecting treatments to establish a baseline (see Note 7).

8. Measure blood glucose and insulin after injecting treatment samples at specific predetermined times; more frequently (e.g., every 10–30 min) in acute trials but less frequently (e.g., daily or weekly) for longer term trials.

3.2 Animal Housing

Following ethics approval, all animals should be maintained in an experimental animal facility and given standard diet and water ad libitum. Feed the rodents (unless in the case of diet induced diabetes) standard commercial laboratory chow, allow them free access to water and cage either individually or in pairs. House the animals under standard conditions and temperature (20 ± 1 °C), with a regular 12 h dark and 12 h light cycle. Monitor the animals for any signs of agitation, fighting, distress or abnormal drop in weight. Measure their body weights daily allowing frequent handling to reduce nervousness, induce calmness and reduce aggressiveness. Fasting the animals (18 h), food only, before experimentation may be necessary depending on the experimental design [12, 13]. In such cases animals need to be monitored for copenophagia and thus changing bedding when commencing fasting is recommended.

3.3 Animal Numbers and Randomization

The trial size and animal numbers will depend on many factors including the power of the study, the predicted average mean difference and intra and inter individual variation, as well as other factors.

1. If this is a pilot study then decide to do some preliminary analysis of the data after about eight animals to check if there is a significant difference. However, if you have preliminary data then do a power calculation to determine numbers per group. Numbers should be the same in each group including the control group.

2. Randomly allocate animals in their treatment groups.

3.4 Anesthesia

If the study is assessing the acute effects of the drug or extract (e.g., within the first 3–4 h), then the animals can be anesthetized and remain under anesthesia but monitored for normal breathing and for any signs of pain or distress. The choice of anesthesia is important as some can induce hyperglycemia. Also, since deep anesthesia can affect the glycemic levels of the animals a low dose to keep the animals sufficiently anesthetized is advisable. Anesthetics of choice are Hypnorn-Dormicum [12] or a combination of ketamine and xylazine adjusted to suit each animal according to body weight [13]. A successful technique includes the injection of an initial priming dose 30–45 min prior to test-substance administration followed by additional low doses, every 20–30 min, to keep the animal under [12, 13].

3.5 Routes of Administration

When considering the route of administration both the site of action and the solubility of the molecule or concoction under investigation are considered to be major determinants. Further, an effect might be missed depending on the route of administration as drug molecules might be activated or inactivated by metabolism. There are several routes; however for the purposes of this chapter only the most common systemic approaches are briefly outlined below as these are relevant to the end points focused on in this chapter.

1. Ad libitum.
   This is an option if the drug or extract is water soluble and does not have an offensive smell or taste which might deter the animals from drinking it. Usually the drug or extract (dry or lyophilized) to be tested is dissolved in the drinking water and the animals would drink it instead of pure water. A positive control such as metformin (see Subheading 3.1, step 3) can be used via this route of administration.

2. Gavage.
   Oral gavage is reserved to conditions where the drug may not be feasibly or accurately administered otherwise.
Many drugs require testing via the oral route but may not be easily soluble in water or may have a repulsive taste preventing accurate dosing. There are several techniques described in the literature and advised by animal resource centers. Feeding tubes are available commercially, specifically for mice or rats.

3. **Intravenous injection and intraperitoneal injection.**

Injection in the tail vein should be done at a high point (see Fig. 2). Intraperitoneal injection is generally used for fat-soluble drugs and slow release assessments. Both these routes are quite commonly used when assessing extracts which, depending on the extraction procedure, can have compounds that are water and/or lipid soluble [15].

### 3.6 Blood Sampling

Methods for blood collection include tail-nick, tail snip, saphenous vein, submandibular and retro-orbital bleeding. These methods have been previously outlined [16]. Tail vein sampling is the most effective way and one that will be outlined in this chapter.

1. A sampling schedule needs to be chosen.
2. The rodent can be held in position or if anesthetized, lying down.
3. The tail fur can be shaved slightly.

4. Depending on whether samples are taken daily, weekly or more frequently, incisions/sampling might require a procedure to avoid recently formed clots. The vein is located, and with multiple sampling in an acute study needle pricks (or small incisions with a scalpel) are started from bottom of the tail (see Fig. 2). Subsequent blood samples are taken above the initial incision and this continues in that general direction. Less frequent bleeds may not require such precautions.

#### 3.7 Glucose Measurement

Blood samples need to be analyzed immediately. Each sample (10 µL) can be analyzed by transferring onto Accu-Chek glucose strips (see Subheading 2.4). The readings are obtained from the Accu-Chek glucometer within 5 s. Blood glucose values can be obtained in mmol/L or mg/dL.

#### 3.8 Insulin Measurement

There are a number of kits (see Subheading 2.4, item 3) to measure serum and/or plasma insulin from blood collected via the tail vein. The protocols are provided with the kit. Such methods are quick and easy to perform and require small sample sizes (5–10 µL volumes are required for the HTRP® assay).

4. **Notes**

1. Amylin (or IAPP) is co-secreted with insulin by β-cells. Certain amylin isoforms can dimerize to form amyloid fibrils or oligomers which can initiate apoptosis [3, 4].
2. The 1x PBS should naturally be pH 7.4 and not require adjustment; however, if a small adjustment is necessary use HCl or NaOH.
3. Compounds that are well known to give significant hypoglycemic levels (commonly insulin) and vehicle to indicate the effects in the absence of a hypoglycemic agent. The route of administration needs to be considered when choosing the controls. The concentrations will need to be adjusted to give hypoglycemia but allow recovery not death of the animals.
4. If acute, anesthesia is recommended especially if you are doing multiple samplings. The animal needs to be kept warm by using a warming box although surgical light might also give heat.
5. Extracts using solvents can be dried under nitrogen or using a rotary evaporator. These extracts can be stored at -20 °C and reconstituted in suitable buffers (e.g., PBS) for injection. Reconstituted solutions, and also depending on the pH change, may have particulate undissolved residues which will require centrifugation or filtration to sediment and remove particulate matter before injection of the solutions in animal models.
6. The volume should be one that can be accurately measured and also one that easily and fully solubilizes the drug of interest.

7. This measurement might need to be done a few times before the blood glucose levels stabilize especially as the animal adrenergic levels drop with the animal relaxing.

References


Chapter 16

Screening for Antibacterial, Antifungal, and Anti quorum Sensing Activity

Elisa J. Hayhoe and Enzo A. Palombo

Abstract

The plate-dough diffusion assay is an invaluable screening tool to evaluate the antibacterial potential of natural products. It relies on the diffusion of test material from a cut well through agar seeded with bacteria. Samples that are capable of inhibiting bacterial growth will produce a clear zone surrounding their well. For the evaluation of antifungal activity of natural products, we describe the broth microdilution method. This assay is performed using a 96 well microtiter tray containing fungal inoculum, test medium and natural product material. Samples demonstrating antifungal activity will prevent any discernible growth as detected visually. A disk diffusion assay, utilizing the pigmented indicator strain Chromobacterium violaceum, is described here for the screening of natural products for anti quorum sensing activity. Inhibition of quorum sensing results in growth of non-pigmented bacteria.

Key words Antimicrobial, Antibacterial, Antifungal, Anti quorum sensing, Screening, Disk diffusion, Plate-dough diffusion, Broth microdilution, Natural product, Extract

1 Introduction

There are several methods reported in the literature to evaluate antibacterial activity of natural products. They involve either introducing the test compounds to a finite area of set agar (disk diffusion [1] and plate-dough diffusion assay [2]) or incorporating the test compounds into the molten agar or liquid media (broom [3] and agar dilution [4] assays). The advantage of using the diffusion methods for screening is that they require only a small volume of test material and the color or opaqueness of a sample will not interfere with interpretation of results. The plate-dough diffusion assay involves the inoculation of molten agar with test bacteria which is then set in a petri plate. Wells are cut into the agar which are then filled with the natural product material and suitable controls. Following incubation, zones of inhibited bacterial growth surround wells containing antibacterial compounds. The diameter of this inhibition zone is