Aldose Reductase Inhibitors and Nanodelivery of Diabetic Therapeutics

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Abstract: Nanotechnology is a rapidly emerging drug-delivery system that makes possible the controlled release of small molecules, and nanodelivery of therapeutic molecules using nanoparticles or nanogels represents a major improvement for more focused delivery of such therapeutic molecules. The delivery of insulin for the control of diabetes mellitus (DM) and aldose reductase inhibitor (ARI) for diabetic complications may provide better treatment of diabetes. A structural overview of aldose reductase including computational docking experiments with HAR-1, various ARIs, aldose-keto reductase, and nanodelivery of insulin, ARI's, and drug molecules are described.

Keywords: Aldose reductase, aldose reductase inhibitors, aldo-keto reductase, diabetes, insulin, nanodelivery, nanogels, tricyclic pyrone molecules.

I. INTRODUCTION

Diabetes mellitus (DM), commonly known as diabetes, is a worldwide health problem and a 2009 estimate has predicted DM to affect approximately 285 million people [1]. The occurrence of this disease is ever increasing, especially in developed countries and at its current rate of increase, it is predicted that within the next few decades DM will be one of the world's biggest health problems with an estimated 500 million cases [2]. The disease can be divided into two categories, type I, insulin-dependent diabetes mellitus (IDDM), and type II, non-insulin-dependent diabetes mellitus (NIDDM).

Type I involves an immunological mediated genetically programmed destruction of the β-cells, which produce insulin that facilitates the uptake of glucose in the cells of the organism. Type II derives from insulin dysfunction and insulin deficiency and resistance. Although insulin treatment improves the patients' standard of living, there is no cure for the disease so far. Because DM patients have a lower level of insulin in the blood, a higher level of glucose results which subsequently is reduced by aldose reductase (AR) to give an elevated level of cell-damaging sorbitol [3]. An inhibition of AR would diminish the level of sorbitol and ameliorate complications. Hence, aldose reductase inhibitors (ARI's) were discovered and developed as therapeutics for DM.

Hyperglycemia, or high blood sugar, is defined as an excessive amount of glucose presented in the blood plasma. During hyperglycemia experienced by people with DM, hexokinase (HK) becomes saturated, or overwhelmed by glucose, and AR shuttles a higher than normal amount of glucose through the polyol pathway (Fig. **1**) [4]. AR converts glucose into sorbitol, a polyol, through a NADPH dependant process. Sorbitol is further converted into fructose by sorbitol dehydrogenase (SDH) in a NAD^+ dependant reaction. It is widely accepted that the increase of flux of glucose through the polyol pathway under hyperglycemic conditions is a co-causative factor in the onset of diabetic complications including neuropathy, retinopathy, nephropathy and cataract.

Under normal glycemic conditions, glucose enters the glycolysis pathway and is converted into glucose 6-phosphate *via* hexokinase. Very little glucose is shuttled through the polyol pathway as glucose is not an ideal substrate for AR. It is under hyperglycemic conditions that the polyol pathway becomes significant leading to the increased formation of sorbitol and fructose. It is proposed that this could negatively impact cells in various ways such as, oxidative stress, hyperosmatic swelling, and higher occurrence of advanced glycation end products (AGE).

Firstly, an increase in AR activity depletes NADPH levels which can eventually lead to oxidative stress since glutathione reductase (GSR), an enzyme involved in the reduction of glutathione disulfide (GSSG) to GSH, an important cellular antioxidant, relies on NADPH as a cofactor. Secondly, polyols such as sorbitol, a polar molecule which does not diffuse out of cells, are osmolytes that poorly penetrate the hydrophobic lipid bilayer of cell membranes. The accumulation of sorbitol causes an influx of water into cells and a loss of cell integrity or increase in cellular osmolarity resulting in deleterious hyperosmotic swelling [5]. The increased production of fructose and its metabolites are said to pose another threat to the onset of diabetic complications since these species are more capable of undergoing non-enzymatic glycation reactions than glucose to form AGE. A hypothesis namely the metabolic flux hypothesis has come to light which attributes oxidative stress to be a significant pathogenic factor leading to diabetic complications when compared with increased tissue sorbitol and fructose levels [1]. The metabolic flux hypothesis merely associates the high turnover of glucose and NADPH through AR and subsequently sorbitol and NAD^+ through SDH to be the cause of high oxidative stress under hyperglycemic conditions [6]. It is generally agreed that inhibition of AR, the first enzyme of the polyol pathway

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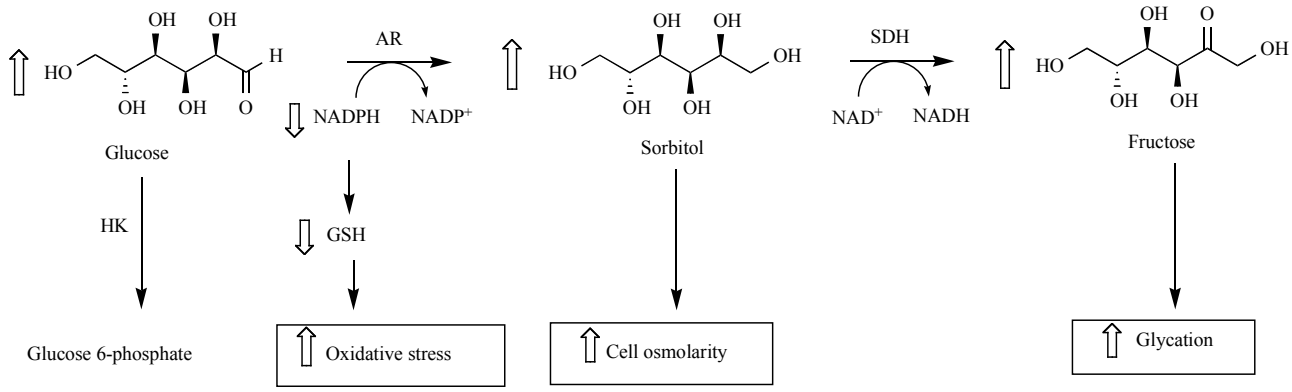

Fig. (1). A polyol pathway during hyperglycemic conditions. AR: aldose reductase; SDH: sorbitol dehydrogenase; HK: hexokinase; GSH: glutathione sulfhydryl (antioxidant). NAD^+: nicotinamide adenine dinucleotide. NADPH: nicotinamide adenine dinucleotide phosphate.

remains an attractive and effective drug target in treating diabetic complications.

Insulin, a 51-amino-acid peptide (MW 5.8 kDa), is produced by pancreatic β cells and counteracts the increase of blood-glucose levels after a meal [7]. To normalize blood glucose levels, insulin is administered by subcutaneous or intravenous injection, however, ~20% of insulin reaches the liver [8]. Alternative administrations of insulin *via* oral, nasal, rectal, pulmonary and ocular have been investigated [9]. The intestinal epithelium absorbs insulin, goes through the portal vein, and reaches liver inhibiting hepatic glucose output. However, proteolytic enzymes in the gastrointestinal tract quickly degrade insulin, and only ~5% of insulin is absorbed under physiological conditions. Hence, to improve absorption, various protections and deliveries of insulin from hydrolytic cleavage were studied [10], which include, formulation with antiproteases [11] and hydrogels [12], chemical modification with a cell-penetrating peptide, oligoarginine [13], enhancement of absorption with bile salts [14], surfactants [15], cyclodextrins [16], and delivery systems with liposomes [8] and lipid nano- or microparticles [17]. Nanodelivery has been at the forefront of investigations using nanoparticles to deliver insulin and/or DM drugs to targeted tissues.

II. ALDOSE REDUCTASE INHIBITORS

The search for effective ARI's (*in vitro* and *in vivo*) has been underway for more than 40 years and as a result many review articles have appeared [1, 4, 6, 18, 19]. Despite this ongoing research, currently only one drug (Epalrestat, Fig. **3**) is a marketed ARI, and in Japan only. Many of the early ARI's showed only weakly positive clinical trial data and could not be unequivocally proven to be effective against diabetic neuropathy [19]. Various ARI's showed promising *in vitro* efficacy but low efficacy *in vivo* or an unacceptably high incidence of hypersensitivity. Despite these frustrations, research toward the development of ARI's continued and was driven by a need of a drug to combat diabetic neuropathy and a developing solid base of encouraging pre-clinical data [6]. As a result of intensive research in this area, much structural information pertaining to AR has been discovered.

II.1. Structural Overview of AR

Aldose reductase enzyme (AR, ALR2, E.C. 1.1.1.21, alditol:$NADP^+$ oxidoreductase) is a cytosolic protein belonging to the superfamily of aldo-keto reductases. It is linked to the onset of diabetic complication due to the reduction of glucose to sorbitol using NADPH as the co-enzyme (Fig. **1**).

Its primary structure was first determined on rat lens AR (Demopoulos *et al*. 2005). Human AR is a small monomeric protein consisting of 325 amino acid residues (MW 36 kDa) and is expressed from gene AKR1B1 which is mapped at chromosome region 7q35 [3]. AR contains a TIM-barrel motif $[(\beta/\alpha)_8]$ and the core of AR contains eight parallel β strands with 8 peripheral α-helices running parallel to the β-sheet. Several X-ray crystal structures of AR have been published and all show variation. These include, a single-crystal X-ray analyses of AR [20], a human AR with a bound NADPH and zopolrestat, tolrestat, sorbinil [21-24], zenarestat [22], fidarestat [21], or sulfonyl-pyridazinone ARI-809 [23]. It has been postulated, with the help of molecular modeling studies that the active site of AR consists of two binding regions, polar region and non-polar region.

The polar region is comprised of amino acid residues Trp20, Tyr48 and His110 and the bound $NADP^+$ which can accommodate the polar carboxylate or cyclic imide functionality from ARI's. The non-polar region of the binding site is comprised of amino acid residues Trp111, Thr113, Tyr115 and Leu300, which can accommodate the hydrophobic portion of ARI's. It is well established that AR activity requires the co-factor NADPH, which must bind first followed by the incorporation of the aldehyde substrate. A hydride is then transferred from the C-4 atom of NADPH to the C-1 carbonyl carbon of the aldehyde substrate (such as glucose) with a concurrent proton transfer from Tyr48 *via* His110 to the substrate [6]. This step brings about the reduction of aldehyde to alcohol (such as sorbitol). The reduced substrate (alcohol) is then released, and AR undergoes a conformational change to dissociate $NADP^+$ [6].

II.2. Inhibitors

The first compound to be screened for treatment of diabetic complication, more specifically sugar cataract

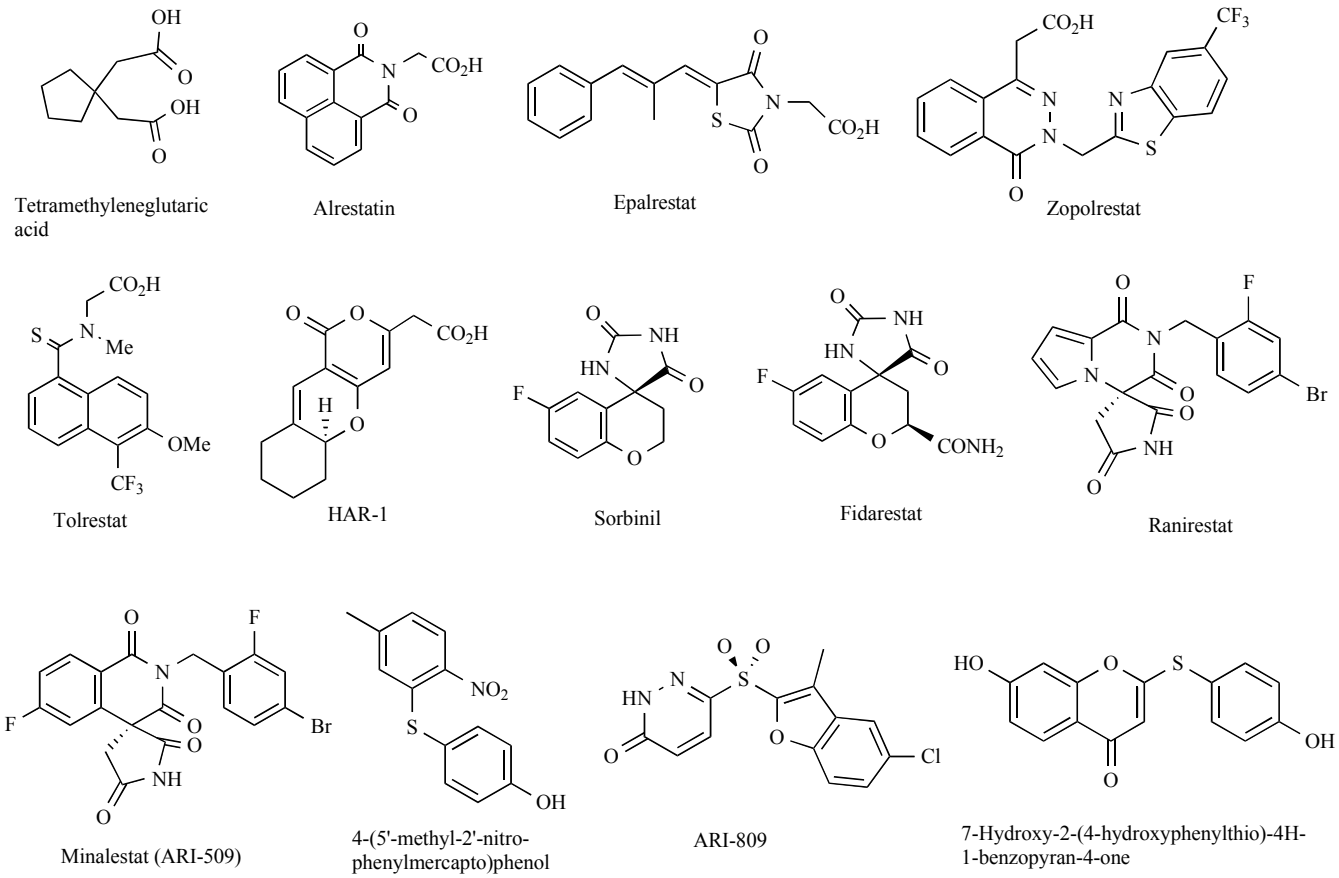

Fig. (2). Chemical structures of selected ARI's.

formation, was tetramethyleneglutaric acid (TMG) (Fig. **2**). The structure of TMG was designed so as to have a non-polar aliphatic region and a negative group such as a carboxylic acid which was thought to be necessary for inhibition of AR [5]. Treatment of galactose-exposed lens culture with TMG completely blocked the accumulation of galactitol suggesting an inhibition of AR. Treatment of TMG also completely prevented swelling of the galactose-exposed lens and vacuoles, suggesting protection from early stage cataract formation [5]. TMG treatment could maintain normal biochemical parameters such as myoinositol and amino acid levels as well as electrolyte composition [5]. TMG was not effective *in vivo* due to the two polar carboxylic acid groups that prevent the crossing of hydrophobic cell membranes. In a search for alternative ARI's, Alrestatin was reported in 1973 to have mediocre activity (IC_{50} value of ~ 10^{-5} M) and became the first ARI to be used *in vivo* and first carboxylic acid ARI to be extensively tested on AR [4]. Initial *in vivo* results obtained from Alrestatin were not convincing and it thus became a model compound for the development of alternative ARI's with carboxylic acid functionality. During this time a second class of ARI's having a cyclic imide functionality (mostly in the form of spirohydantoin) was developed, of which sorbinil was found to be particularly active [5]. The absence of a carboxylic acid moiety in sorbinil allowed for better diffusion through cellular membranes and thus provided better *in vivo* inhibitory activity of AR. These two classes of ARI's (carboxylic acid and cyclic imide) are considered to be the two main classes of ARI's which show *in vivo* activity [19].

Carboxylic Acid ARI's

Alrestatin

Alrestatin showed mediocre activity in pre-clinical trials and became the first ARI to be used *in vivo* [4]. Despite its initial disappointing *in vivo* activity, subsequent studies showed Alrestatin to have beneficial effects in diabetic patients suffering from diabetic polyneuropathy [4].

Epalrestat

Epalrestat was launched in 1992 by Ono Pharmaceutical Co., Ltd. Japan and is marketed for the treatment of diabetic neuropathy in Japan. Epalrestat is considered to be one of the most effective AR inhibitors for the treatment of diabetic neuropathy [14]. It has a relatively short half life in the body and therefore a dosing regimen of 50 mg three times a day is required. In Japan between 1997 and 2003, a 3-year randomized, open label, multicentre study was performed with Epalrestat (150 mg/d) and when compared with a control group was shown to significantly improve numbness of limbs, sensory abnormality and cramping in diabetic patients suffering from diabetic neuropathy [6]. Epalrestat was also shown to prevent deterioration of median motor neural conductive velocity (NCV). Since Epalrestat is a standard-of-care treatment for the treatment of diabetic

neuropathy it is tightly regulated. Epalrestat has still not been proven effective in randomized double-blind placebo-controlled trials [6].

Zopolrestat

Zopolrestat has been shown to dose-dependently protect NCV in diabetic rats with a ED_{50} value of 30 mg/kg/d and normalize elevated fructose and sorbitol levels in diabetic rat nerve trials with a ED_{50} value of 3 mg/kg/d [6]. A Zopolrestat study on diabetic patients was performed using a 12-week randomized, double-blinded, placebo-controlled multicentre Phase 2 trial. At a level of 1 g/d, Zopolrestat gave a 1 m/s NCV response which was said to be encouraging [6]. Unfortunately at this level (1 g/d) liver enzyme levels had increased about 7% which was unacceptable. Zopolrestat was subsequently tested in Phase 3 trials but at lower doses, 500 mg and 250 mg. Liver enzyme elevations were attenuated at these doses (0% at 250 mg and 2% at 500 mg) but NCV responses were undesirable (0.4 - 0.5 m/s) and thus trials using Zopolrestat were discontinued [6].

Tolrestat

Tolrestat has been shown to prevent sorbitol accumulation in diabetic rat lens and sciatic nerve and cataract formation in galactosemic rats [4]. Tolrestat was launched as an AR inhibitor in 1989 in several countries under the trade name Alredase, however it failed Phase 3 clinical trials in the U.S.A due to toxicity. It was later withdrawn in 1996 due to allergic reactions involving the liver as well as poor efficacy [25].

Tricyclic Pyrone Carboxylic Acid HAR

In our laboratories, we discovered a class of tricyclic pyrone molecules (code name HAR) possessing potent AR inhibitory activities. For instance, the IC_{50} value of HAR-1 (1H,7H-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-b][1]benzopyran-3-acetic acid; Fig. **2**) is 2 nM, and polyol accumulation in lens epithelial cells was reduced by 80% at 10 μM [26]. In galactose-fed rats without inhibitor, 43% and 56% of the combined GC/MS peaks were galactose and galactitol, respectively. However, in galactose-fed rats with HAR-1, 72% and 27% of the combined GC/MS peaks were galactose and galactitol, respectively. Hence, a clear inhibition of the formation of galactitol (a polyol) was observed. A decrease of protein kinase C [PKCγ, a PKC that is known to control gap junction (GJ) activity] and phosphorylation of serine residues of connexin 46 (Cx46; a protein that makes up the hemi-channel of connexon) in galactosemic rats (control animals) were found. Treatment of galactosemic rats with HAR-1 (100 mg/kg/d) resulted in normalization of PKCγ to normal level (as that of non-galactosemic rats) and without phosphorylation of serine residues of Cx46. Moreover, GJ activity in lens was normalized in HAR-1 treated galactosemic rats, while untreated rats showed a 62% decrease of GJ activity. When cells are exposed to high glucose or galactose, an increase of de novo synthesis of diacylglycerol resulted, and this leads to an early activation of PKC's. Phosphorylation of Cx46 at the serine residues causes a disassembly of connexon or a loss of GJ activity. On the other hand, treatment with Tolrestat, an ARI, under similar conditions did not normalize PKCγ level. Hence, beside the inhibition of AR, HAR-1 also normalizes GJ activity removing excess of glucose (or galactose) from cells through GJ. This dual mechanism of HAR-1 is distinctive.

To further study the efficacy of HAR-1 in the treatment of cataract, galactosemic dogs were examined. Galactosemia has been used as a rapid and humane model for DM in dogs. It allows drug testing without the severe pathology of late diabetes. Galactosemia is induced by the feeding of high galactose chow (40%) and causes structural changes in dog lens fiber sutures and accumulation of polyols within 1 month resembling early DM. Moreover, there is an unchanged level of sciatic nerve polyol at this early period allowing the sciatic nerve tissues to serve as controls. Similar to that in rats, a decrease of PKCγ level was observed in galactosemic dog lens, but synthesis of PKCγ was not affected from the measurement by quantitative real time polymerase chain reaction (qrt-PCR). A reduction of GJ activity in lens decreases the transport of small molecules such as glucose or galactose out of the lens, resulting in cataract. In HAR-1 treated galactosemic dogs (100 mg/kg/d for 6 weeks), the PKCγ levels in lens and peripheral sciatic nerve were normal (same levels as that of non-galactosemic dogs). Moreover, HAR-1 treatment also reduced polyol levels by 53% [27]. Optimization of HAR-1 dosages is needed.

HAR-1 and other structurally similar tricyclic pyrone compounds were found to have very low (or minimal) cytotoxicity *in vitro* and *in vivo* (rats and dogs) and were synthesized from a two-step sequence of reactions from commercially available 6-methyl-4-hydroxy-2-pyrone and cyclohexene-1-carboxaldehyde (Fig. **3**) in good yield [26, 28]. The ease of synthesizing HAR molecules along with their dual mechanisms make them the lead compounds for future investigation for human uses.

A computational docking study was performed to shed light onto the interaction of HAR-1 with the active site of

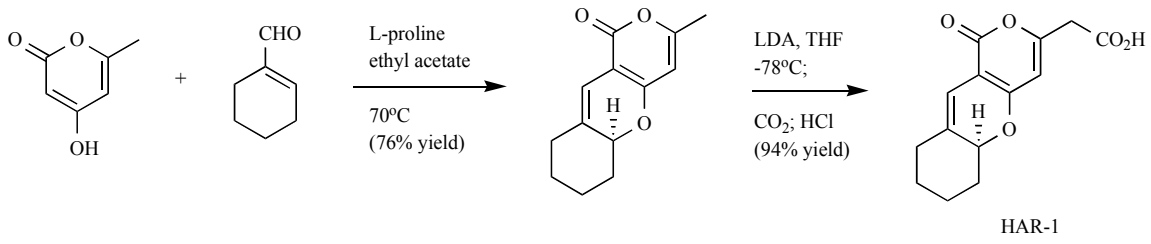

Fig. (3). Synthesis of HAR-1.

AR using Autodock 3 [29-31]. The docking was performed with pig AR enzyme (PDB entry 1ah0) without the ARI, Sorbinil, and solvent molecules were removed prior to docking. The $NADP^+$ cofactor was kept as part of the protein structure. HAR-1 was constructed having a chair conformation in its cyclohexane ring and was energy minimized before docking. A good pose was seen in one of the docked conformation whereby HAR-1 was positioned directly into the active site of AR (Fig. **4**), the same site occupied by Sorbinil in the native crystal structure. The pyrone ring of HAR-1 is positioned in the hydrophilic pocket of the active site and held into position by two hydrogen bonds. One hydrogen bond was found between the carboxyl hydrogen of HAR-1 and the carbonyl oxygen of nicotinamide moiety of $NADP^+$ (contact distance of 2.071 Å). The second hydrogen bond was present between the carboxyl oxygen of HAR-1 and the imidazole N-H of His110 residue (contact distance of 1.972 Å). The pyranyl carbonyl oxygen of HAR-1 shows a close contact with the methyl side chain of Val47 residue (contact distance of 2.827 Å). An overlay of the docked HAR-1 conformation and bound Sorbinil (from crystal structure) with the protein is illustrated in Fig. (**4**), right panel. The two molecules, HAR-1 and Sorbinil, are almost superimposable on each other. A close up view of the docked conformation (Fig. **5**) shows HAR-1 to be hydrogen bonded to the active site of AR and to be positioned directly above the C-4 carbon of $NADP^+$. Although HAR-1 contains a hydrophobic cyclohexane ring, it was not found to have any close contacts with any of the hydrophobic pocket residues Trp111, Thr113, Tyr115 and Leu300. The computational docking studies suggest that HAR-1 binds to the active site as other ARIs such as Sorbinil. Further studies on drug development of HAR-1 and its analogs are warranted.

Cyclic Imides

Sorbinil

Sorbinil was the first cyclic imide (spirohydantoin) to show AR inhibitory activity and was developed by a Pfizer research group in 1978 [5]. The inhibitory activity appears due to the C-4S enantiomer. The absence of a carboxylic acid moiety in Sorbinil allows for better diffusion through cellular membranes and thus provided better *in vivo* inhibitory activity of AR as compared with carboxylic acid derivatives. Sorbinil was shown to prevent progression of thermal and vibrational sensory deficits when compared with a placebo group during a 35 month non-blinded trial employing 20 patients on Sorbinil and 13 patients on a placebo [32]. Sorbinil unfortunately showed hypersensitivity in 10% of patients when administered doses of 250 mg/kg during *in vivo* trials, and subsequent studies were therefore halted.

Fidarestat

Fidarestat is a cyclic imide (spyrohydantoin) ARI related to sorbinil and was discovered by Sankyo Pharmaceutical Co. Ltd., Japan [6]. Early results have shown promise and Fidarestat has been employed in 6 Phase 2 clinical trials with doses ranging from 1 – 30 mg. It is believed that Fidarestat also proceeded to Phase 3 neuropathy clinical trials in Japan however in March 2006, Sankyo announced that "in order to concentrate its resources" it would not pursue European and U.S.A Phase 3 clinical trials [6]. Trials are ongoing.

Ranirestat

Ranirestat was discovered in 1998 and has been proven effective in randomized clinical trials. It is a cyclic imide (spirosuccinimide) and is being co-developed by three Japanese pharmaceutical companies. In a Phase 2 study, Ranirestat was shown to suppress the accumulation of nerve polyols as well as improve sural and median sensory NCV after 12 and 60 weeks of treatment at a dose of 20 mg/d [6]. Trials are ongoing.

Minalestat (ARI-509)

Minalestat is another cyclic imide (spirosuccinimide) that has shown to increase NCV and reduce nerve sorbitol and fructose levels when employed in pilot Phase 2 studies [32].

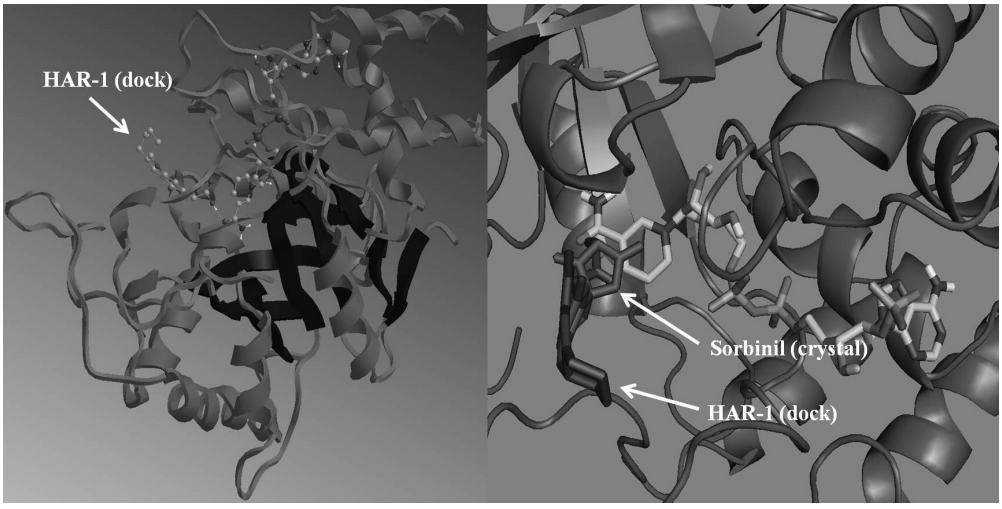

Fig. (4). Left panel: docking conformation showing HAR-1 within the active site of AR (PDB entry 1ah0, with Sorbinil ligand removed). AR contains $NADP^+$ as a co-factor. Right panel: close up of active site with the ligands HAR-1 and sorbinil superimposed. Red: docked conformation of HAR-1; magenta: crystal structure of Sorbinil bound AR (PDB entry 1ah0). The carbon skeleton of $NADP^+$ is shown in green color.

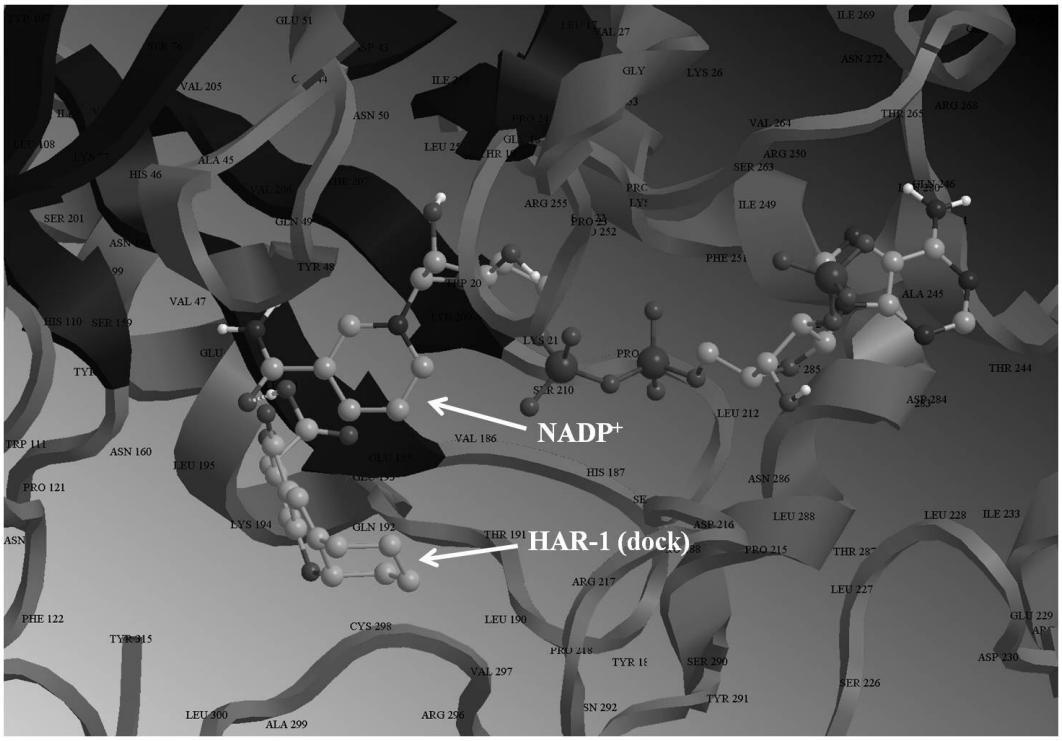

Fig. (5). Close up of the docked conformation showing HAR-1 (the carbon backbone is highlighted in green color) to be directly above the C-4 carbon of $NADP^+$ (the carbon backbone is highlighted in grey color).

Other ARI Inhibitors

More recently, alternative functionalities showing ARI inhibitory activity have come to light such as sulfoxide ARI-809, 7-hydroxy-2-(4-hydroxyphenylthio)-4H-1-benzopyran-4-one and 4-(5'-methyl-2'-nitrophenylmercapto)-phenol (Fig. **2**). The new drug ARI-809 has been tested in diabetic rats and proven highly efficacious in reversing and preventing neuroglial abnormalities [33]. Unfortunately, the compound absorbs UVA and UVB radiation causing spontaneous light induced retinal damage in albino rats during a 6 month dosing regimen and further trials were discontinued [34]. The compounds 7-hydroxy-2-(4-hydroxyphenylthio)-4H-1-benzopyran-4-one and 4-(5'-methyl-2'-nitro-phenylmercapto)phenol show promising *in vitro* AR inhibitory activities (EC_{50} values of ~ 1 μM) [18].

Encouraging results were obtained in the field of diabetic neuropathy as it opened up alternative avenues for drug discovery with the hope of finding more potent ARI's possessing higher *in vivo* activity and hopefully less pronounced hypersensitivity or toxicity. Nanodelivery of ARI (*vide infra*) may provide a more focused delivery of the drugs to specific target tissues especially for carboxylic acid ARI's.

III. ARI's AS POTENTIAL CANCER THERAPEUTIC AGENTS

Despite more than 40 years of research, currently only one drug, Epalrestat (Fig. **3**), is a marketed ARI, and in Japan only. As previously mentioned, many of the early ARI's showed only weakly positive clinical trial data and could not be unequivocally proven to be effective against diabetic neuropathy [19]. Various ARI's showed promising *in vitro* efficacy but low *in vivo* efficacy or an unacceptably high incidence of hypersensitivity. A probable reason for the adverse side effects of most ARI's can be ascribed to their low specificities to AR. For example, ARI's epalrestat, statil [35], zopolrestat [36] and tolrestat [37] have been shown to inhibit closely related enzymes of the aldo-keto reductase (AKR) family, aldehyde reductase (AKR1A1, EC 1.1.1.2) and aldo-keto reductase family 1 member B10 (AKR1B10), also known as aldose reductase-like-1 (ARL-1). AKR1B10 is a 316-amino-acid protein and shares 70% sequence identity with that of AR. It can reduce glyceraldehydes efficiently but not glucose.

Recently, AKR1B10 has been shown to be over expressed in liver and lung cancer cells and it is suggested that inhibitors which can regulate the activity of AKR1B10 will have benefits in cancer therapy [35]. AKR1B10, a monomeric 36 kDa homologue of AR, is capable of reducing a variety of aldehydes and ketones [35, 37]. It is found in normal human colon, small intestine and adrenal gland cells, and low levels in the liver, but it is up regulated in liver and lung cancer cells. Anti-cancer drugs containing carbonyl function such as daunorubicin (an anti-lung cancer drug) and oracin (a potential cytostatic drug) are inactivated due to the action of AKR1B10, which reduces them to their corresponding alcohols [36]. ARI, zopolrestat (20 μM), blocked AKR1B10 mediated reduction of daunorubicin by 43% [36]. It was found that AKR1B10 activity is important for cell survival through regulation of lipid synthesis, mitochondrial function, oxidative status as well as carbonyl levels, and a recent study showed that silencing of AKR1B10 gene resulted in caspase-3-mediated apoptosis [38]. Hence, ARI's may be used in combination with

carbonyl containing anti-cancer drugs, such as daunorubicin, to inhibit the degradation of the drug by AKR1B10.

IV. NANODELIVERY OF DIABETIC THERAPEUTICS

Nanoparticles are small particles with diameter of 10 to 1,000 nm and have great potential in drug delivery, however, they should be biocompatible and degraded (or digested) under physiological conditions. Since a number of reviews on nanoparticle-based deliveries including oral insulin delivery have appeared [39], we summarize the most recent studies on nanodelivery of insulin, peptides, and small molecules, possibly ARIs.

IV.1. Oral Delivery of Insulin Using Poly(ε-caprolactone)/Eudragit Nanoparticles

Poly(ε-caprolactone)/Eudragit nanoparticles for oral delivery of aspart-insulin were recently reported [10]. The nanoparticles were made up of a biodegradable polymer, poly(ε-caprolactone), and a nondegradable but biocompatible mucoadhesive polymer, Eudragit 1 RS, in a ratio of 50:50. Encapsulation of insulin and its derivative aspart-insulin have been performed using a multiple emulsion technique. Initially, an aqueous solution of insulin, or aspart-insulin, was emulsified by sonication in dichloromethane containing a mixture of the above two polymers, 1:1. The resulting emulsion was then poured into a polyvinyl alcohol aqueous solution (0.1%, m/v) and sonicated leading to the formation of a water-in-oil-in-water emulsion. After removal of dichloromethane, the nanoparticles were isolated by centrifugation at 45,000 X *g*. The amount of insulin encapsulated by the nanoparticles was determined by HPLC and the mean diameter of the nanoparticles was determined by photon correlation spectroscopy.

Nanoparticles so made were able to encapsulate 97.5% of insulin, and over a period of 24 hours released about 70% of the encapsulated insulin in neutral medium *in vitro*. Nanoparticles encapsulated with aspart-insulin were orally administered at a dose of 50 IU/kg in diabetic fasted rats and a significant reduction of glucose levels after 0.5 hr of delivery was observed when compared to controls receiving nanoparticles alone. The glycemia continued to decrease and a maximum decrease was observed after 6 - 8 hr. It appears that the aspart-insulin is stable and biologically active after oral administration when encapsulated in poly(ε-caprolactone)-Eudragit RS polymer. Aspart-insulin loaded nanoparticles showed more pronounced effect in reducing hyperglycemia than the insulin alone, 52% verse 24%, at a dosage of 50 IU/kg when administered orally. Aspart-insulin showed prolonged effect on oral glucose tolerance test in comparison to the insulin alone [10].

Due to hydrogen-bonding between the C-termini of B chains, insulin aggregates readily to form a dimer, which in the presence of zinc ions, oligomerizes to from a hexamer. In aspart-insulin, a proline residue on the C-terminal end of the B chain was replaced by an aspartic acid. The presence of aspartic acid increases the charge repulsion between the chains in insulin and maintaining insulin in monomeric form rather than the less absorbable dimer and hexamer units. Eudragit is a mucoadhesive polymer thus using it in the incorporation of nanoparticles is believed to increase absorption through the intestinal wall. The prolonged effect of aspart-insulin in comparison to insulin alone can be attributed to the stability of the nanoparticles and a slow degradation of these particles releasing insulin to the blood.

IV.2. Oral Delivery of Nanoencapsulated Insulin Using Zirconium Phosphate

Encapsulation of insulin into various biodegradable polymers and a controlled release of the encapsulated insulin is the most studied method of oral delivery. However, the encapsulation faces various problems including the resistance of degradation in the acidic environment of stomach and proteolytic cleavage by pepsin in the stomach and trypsin, chymotrypsin, and carboxypeptidases in the intestine. Moreover, the encapsulation process, involving various organic solvents and polymers, may modify the structure of proteins and result in a less effective protein. Thus a stable, robust, nontoxic, and viable carrier for insulin delivery is needed.

The hydrated form of zirconium phosphate (ZrP), known as the θ-ZrP [$Zr(HPO_4)_2 \cdot 6H_2O$], has been studied as a potential candidate for the oral delivery of insulin [40]. Inorganic layered nanomaterials have expandable interlayer space and they were used to encapsulate biomolecules to enhance their stability. A direct intercalation of insulin without the use of pre-intercalator and release of the loaded insulin under basic conditions *in vitro* were found. Circular dichroism studies showed insulin remains unchanged during intercalation and release. Insulin was found to be stable in the layered nanoparticle at room temperature. To determine the potential toxicity of ZrP nanoparticles to human cells, the cell viability of a human breast carcinoma cell line MCF-7 grown for 24 hr in the presence of different concentrations of nanoparticles ZrP was measured. The cell viability assay revealed an absence of toxicity to MCF-7 cells following exposure to the formulated ZrP for 24 hr.

The procedure for the preparation of ZrP nanoparticles involved addition of an aqueous solution of $ZrOCl_2 \cdot 8H_2O$ to a 35% H_3PO_4 solution. After refluxing for 2 days, the nanomaterial was filtered and washed with water to give a paste material. This material was characterized by X-ray powder diffraction. The intercalation process was performed by addition of insulin to a water suspension of θ-ZrP at different molar ratios, and the intercalated nanomaterial was obtained after 3 days. ZrP nanoparticle has an interlayer distance of 10.3 Å which can expand to accommodate and intercalate with insulin, and transmission electron microscopy images showed that a formation of insulin-intercalated ZrP phase consists of a 27-Å-interlayer distance. Experiments were performed to study the controlled release of insulin/ZrP at pH values between 8.2 and 7.4. The release of hormone from the layers was studied using pH stimuli and was monitored using a UV-visible spectrometer. The change in absorbance of the centrifuge aliquots of the suspensions for the characteristic band of insulin was observed at 280 nm. At pH 8.2, insulin was released from the layers at a fast pace in 5 min until reaching a plateau, and at pH 7.4, the release was slower, taking 30 min to reach a similar result. Between the pH range of 8.2 - 7.4 the six carboxylic acid

groups of insulin exist as deprotonated carboxylates because the isoelectric point of insulin is 5.4. Since this disrupts the attractive hydrogen bonding interactions between the phosphate containing layer and insulin, which was released into the solution.

Furthermore, ZrP resists an acidic environment, making it suitable to protect acid-sensitive biomolecules like insulin in the stomach, permit the absorption of the nanoparticles, and release of insulin from ZrP in the intestine once the pH becomes basic. ZrP has interlayer distances capable to intercalate sufficient insulin, is stable in the acidic environment and releases its content upon changing to basic pH [40]. Thus, ZrP nanoparticles have potential to be inorganic nanodelivery agents *in vitro*, however, *in vivo* efficacy remains to be demonstrated.

IV.3. Controlled Release of Encapsulated Insulin in Poly (D,L-lactic-co-glycolic acid) (PLGA) Microcapsules as Dry Powder Inhaler

There has been considerable interest in the pulmonary (lung) route for the delivery of insulin due to a number of reasons. These include, thin layered lung epithelium, high degree of vascularization, large surface area of the alveoli, and low proteolytic activity. Drugs administered through this route directly reach blood circulation, avoiding passage to the liver, which enhances the bioavailability. To lengthen the duration effect of insulin, a dry powder inhaler using PLGA-insulin formulation was developed [41]. Attributed to the proteolytic degradation in the lung, the duration effect of insulin nebulized inhaler was reported to be less than 10 hr. Although using protease inhibitors helps to prevent the proteolytic activity, potential toxicities by protease inhibitors to lung tissues can result. PLGA is a FDA-approved biocompatible and biodegradable polymer. The long-acting formulation of insulin was prepared by encapsulation of the protein into respirable and biodegradable microcapsules *via* an oil-in-oil emulsification/solvent evaporation method. In brief, insulin was dissolved in an aqueous hydrochloric acid solution and a PLGA solution in acetonitrile (1:5). The solution was dispersed into mineral oil in the presence of Span 80, a PEG-ylated sorbitan (a derivative of sorbitol) esterified with fatty acids. Microcapsules were collected by centrifugation and washing with hexane was performed to remove mineral oil. The particle size distribution had a volume mean diameter of 6 μm, which is suitable for pulmonary delivery of insulin.

Insulin-loaded PLGA microcapsules prepared as a dry powder inhaler were administered *via* a pulmonary route to diabetic rats, and serum insulin and glucose levels were monitored. Pharmacokinetic analysis indicated that insulin administered by PLGA microcapsules showed a sustained release profile resulting in a longer-mean-residence time; 4 and 5 folds longer than that administered pulmonary of respirable spray-dried insulin and subcutaneous (SC) injection, respectively. It appears that the nonporous nature of the microcapsules gradually become porous releasing insulin. The pharmacodynamic efficiency of PLGA microcapsules was significantly higher than that of SC administration [41]. The results imply that the biological activity of insulin is not affected by the encapsulation methodology. Tissue damages in the lung lead to an increase in the amount and activity of lactase dehydrogenase, and analysis of bronchoalveolar lavage fluid revealed that microcapsule administration did not increase the amount and activity of lactase dehydrogenase. It was therefore suggested that the formulation is nontoxic to lung tissues. However, a histological examination of lung tissues indicated a minor but detectable effect on the rat lung. The lung is immunologically active, and inhalation of insulin promotes the formation of antibody, which may lead to other complications. This along with the fact that a reduced amount of air is exhaled in the diabetic lung has decreased the enthusiasm of insulin delivery through the pulmonary route. Nonetheless, insulin loaded PLGA microcapsules showed acceptable size, uniformity, and good release property for the pulmonary delivery of insulin without serious damage to lung tissues. It was concluded that these microcapsules could be a pulmonary route delivery agent for insulin since *in vivo* studies demonstrated that over a period of 48 hr, baseline insulin concentrations could be achieved with one single puff of the dry powder inhaler.

IV.4. Liposomes and Iontophoresis for Noninvasive and Persistent Transfollicular Drug Delivery System

The delivery of drugs through skin has gathered attention because it avoids passing through the liver and reduces adverse effects. Transdermal administration of molecules is often performed by a process called iontophorosis, in which electrical driving force is applied to transport compounds across stratum corneum (the outermost layer of epidermis). However, the process is mainly useful for the transport of small, hydrophobic, and charged molecules. A combination of charged liposomes and iontophorosis was studied to deliver insulin *in vitro* and *in vivo* using diabetic rats [42]. Iontophorosis was carried out through hair follicles rather than the intercellular space in skin because of the size of liposomes, which is greater than 100 nm in diameter. The cationic liposomes were prepared from various combinations of 1,2-dioleoyl-3(trimethylammonium)propane (DOTAP) or cholesteryl hemisuccinate with cholesterol or/and other chemicals such as egg phosphatidylcholine or 1,2-dioleoylphosphatidylethanolamine in chloroform. After drying, the resulting thin lipid film was hydrated with HEPES buffer to pH 7.4 followed by sonication. Defined-size (100 – 150 nm) particles were obtained by filtration through polycarbonate membranes (a pore size of 100 nm), and encapsulation of insulin was conducted in a HEPES buffer solution. *In vitro* anodal iontophoresis of a 7:3 DOTAP/cholesterol cationic liposome achieved a maximum delivery distance into hair follicles, which is greater than that of anionic liposomes. The difference was ascribed to skin surface negative charge and electro-osmotic flow from anode to cathode during iontophoresis. Double fluorescent labeling studies (*in vitro* and *in vivo*) showed that the dye molecule, rhodamine, encapsulated in liposomes was delivered into hair follicles after iontophoresis.

On the other hand, iontophoresis of rhodamine alone showed no rhodamine in hair follicles, indicating that charged liposomes function as a carrier for transfollicular delivery *via* iontophoresis. Similarly, insulin alone was not delivered sufficiently into the hair follicles by anodal

iontophoresis. However, insulin could be successfully delivered into the epidermal region, the stratum corneum and marginally in or around the hair follicles when encapsulated in cationic liposomes.

Diabetic rats were administered with porcine insulin encapsulated in liposomes by iontophoresis, and its effect compared to that of insulin administered by intraperitoneal (i.p.) injection. The control i.p. injections showed that the lowest blood glucose level was attained after 4 hr, followed by a gradual increase in glucose levels thereafter. After 18 hr of iontophoresis of insulin loaded charged liposomes, the blood glucose levels had gradually decreased to approximately 20% of basal levels which was maintained for at least 24 hr after administration. This result was promising and indicated that iontophoresis of charged liposomes could become a diabetic therapy to bring about the delivery of insulin in an efficient and non-invasive approach [42].

When i.p. injection was performed on diabetic rats, the amount of insulin in blood plasma sharply increased within 1 hr and decreased near the basal value in 18 hr. However, iontophoresis of insulin-liposomes showed no such abrupt changes in the plasma insulin level and a significant amount of insulin was found even after 18 hr. The results suggest that insulin is released into the skin around hair follicles and transferred gradually to the blood *via* the capillaries, without significant decrease in the activities of insulin during the encapsulation and iontophorosis. The non-invasive and persistent drug delivery of insulin using charged liposome and iontophorosis may be a potential transfollicular route of insulin delivery.

IV.5. Delivery of Insulin Using Silica-Coated Liposome

A nanosphere, silica-coated phosphatidylcholine (PC; liposome), was made from the inclusion of tetraethyl orthosilicate and water into phosphatidylcholine and determined from confocal and electron microscopy [43]. The encapsulated insulin-silica-coated PC enhances the stability of insulin and reduces glucose levels *in vivo* (rats). Silica xerogels have been used as carriers for the controlled release of proteins and pharmaceutical substances [44]. The silica-coated liposome appears to have a protective action against lipid degradation in the gut lining, and the release of insulin from silica-coated PC occurs through diffusion and dissolution through pores present in the nanoparticles. Sol-gel derived silica appears to be biocompatible and biodegradable, hydrolyzes its siloxane bonds in human body, and excretes *via* kidneys [45].

IV.6. Delivery of Small Molecules and Proteins Using PEG-PEI Nanogels

Nanotechnology has rapidly emerged in the field of drug delivery. To specifically deliver various drugs including ARI, insulin, interferon, and anticancer drugs, we have modified polyethylene glycol-polyethylenimine (PEG-PEI), which has been previously reported [46]. Nanogels are nanosized particles of chemically cross-linked polymers that swell in a solvent and provide improved stability and bioavailability to drugs, bioactive peptides, and genes. The originally reported PEG-PEI nanogel is toxic to cells, and the toxicity may arrive from the amino function of PEI. Hence, modifications were made by further PEG-ylating the nanogel with activated PEG or acetylation with acetic anhydride. The extensive PEG-ylated PEG-PEI nanogel [47] and acetylated nanogel [48] have low cytotoxicity and are biocompatible. Extensive PEG-ylated PEG-PEI was used to encapsulate water-insoluble small organic molecules such as ARI and enter various cells including stem cells and Pan 02 cells. Acetylated nanogel (AcNg), a 200-nm-diameter polymer, does not enter cells but encapsulates interferon (IFN). AcNg improves the stability of IFN stored at $4^{o}C$, and the antiviral activity of IFN was significantly higher in IFN-AcNg complex compared to IFN alone. The results imply that insulin, a smaller size protein than IFN, can readily be encapsulated and administered. Recently, FOL-PEG-g-PEI-GAL, a PEG-PEI conjugated with folic acid and galactose (lactobionic acid) utilizing HOOC-PEG-NH_2 as the building block, was used to form a complex with DNA [49]. The encapsulation of a combination of ARI and insulin with nanogels is possible and potentially can be effective.

IV.7. Nano Delivery of Aldose Reductase Inhibitors

Unlike insulin, aldose reductase inhibitors are non peptidic compounds which do not pose problems related to proteolytic degradation. Proper formulation of nano-carriers can be useful in delivering ARI's *in vivo* with high efficiency; however there is no literature precedence for nano-delivery of ARI's in animal models.

Unger [50] prepared solid porous nano-particles by mixing surfactant like canola oil, olive oil, corn oil with active pharmaceutical ingredients in solvent (water) using roller mill containing zirconium oxide beads. Average size of the particle ranges from 100 nm - 2 μm. These particles are acoustically active thus the release of the active drug from the nano-particles can be triggered by ultrasound technology. Unger claims ARI's can be delivered effectively using this solid porous nano-particle as delivery vehicle.

Yoo [51] reported the preparation of a dissolvable nano-web porous film capable of incorporating ARI's. Nano-porous film contains nano-fibers having thickness of 10 - 50,000 nm. It is made up of nano-fiber forming polymer (different types of cellulose), gelling agent (gelatin and agar), plasticizer (sorbitol, mannitol) and the drug to be delivered. This nano-porous film has high surface activity and dissolvability ranging from 1 - 60 s in saliva, thus ARI's incorporated in the fiber can easily be absorbed through oral cavity and subsequently into the blood stream.

CONCLUSION

Diabetic complications such as neuropathy have been extensively studied and encouraging results using ARI's were found. Nanodelivery of ARI's may provide a focused delivery to target tissues. So far, oral-administration of insulin has not been approved by FDA, and Exubera, FDA-approved inhalation formulated insulin through pulmonary route, was withdrawn in 2007 due to poor sales, shorter effective duration, and safety concern [52] . Nanodelivery of insulin remains a challenge, and an effective oral delivery would eliminate daily injections. An effective oral-nanodelivery of insulin would require a biocompatible, biodegradable, and faster clearance nanoparticles, and a

longer duration of the drug of 10 – 12 hr would be appropriated. The current advancement and continuing research in the field of nanomaterials enable a better understanding of uptake mechanisms and nanotoxicity, which may lead to effective oral delivery of insulin in the near future.

CONFLICT OF INTEREST

Declared none.

ACKNOWLEDGEMENT

We gratefully acknowledge financial support by the National Institutes of Health (CA135599-01).

ABBREVIATIONS

Å = Ångström
AcNg = Acetylated nanogel
AGE = Advanced glycation end-products
AR = Aldose reductase
AKR = Aldo-keto reductase
ARI = Aldose reductase inhibitor
Aspart = Aspartic acid
d = Day
DM = Diabetes mellitus
DNA = Deoxyribonucleic acid
DOTAP = 1,2-Dioleoyl-3(trimethylammonium)propane
FDA = Food and Drug Administration
FOL = Folic acid
GAL = Galactose (lactobionic acid)
GC/MS = Gas chromatography/mass spectrometry
GJ = Gap junction
GSH = Glutathione sulfhydryl
GSR = Glutathione reductase
GSSG = Glutathione disulfide
HAR = code name for tricyclic pyrone molecule
HEPES = 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HK = Hexokinase
HPLC = High performance liquid chromatography
IDDM = Insulin-dependent diabetes mellitus
hr = hour
IFN = Interferon
i.p. = Intraperitoneal
IU = International unit
kDa = Kilodalton
kg = Kilogram
m/v = mass/volume
m = Meter
mg = Milligram
MW = Molecular weight
NAD^+ = Nicotinamide adenine dinucleotide
NADPH = Nicotinamide adenine dinucleotide phosphate
NCV = Neural conductive velocity
NIDDM = non-insulin-dependent diabetes mellitus
nm = Nanometer
nM = Nanomolar
PC = Phosphatidylcholine
PEG = Polyethylene glycol
PEI = Polyethylenimine
PKC = Protein kinase C
PLGA = Poly (D,L-lactic-co-glycolic acid)
qrt-PCR = Quantitative real time polymerase chain reaction
s = Second
SC = Subcutaneous
SDH = Sorbitol dehydrogenase
TMG = Tetramethyleneglutaric acid
U.S.A = United States of America
UV = Ultraviolet
X *g* = Times gravity
ZrP = Zirconium phosphate
µm = Micrometer
µM = micromolar

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Received: March 11, 2011 Revised: October 14, 2011 Accepted: October 25, 2011