THE INSULINOTROPIC EFFECT OF THIAZOLIDINEDIONE DERIVATIVES IN TYPE II DIABETIC MODEL, RIN-5F CELLS, AND ITS ACTION ON CULTURED HUMAN LUNG FIBROBLAST SHOWS OXYGEN FREE-RADICAL SCAVENGING ACTIVITY

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ABSTRACT

Objective: Diabetes mellitus is a persistent disorder caused by deficiency in insulin production. Oxidative stress plays an important role in physiopathology of diabetes. The present research determined the antidiabetic and antioxidant effects of 5-naphthalidin thiazolidinediones derivatives.

Methods: Both in vitro and in vivo experiments were carried out on diabetes induced male Swiss albino rats by single intramuscular injection of alloxan (0.15 mg/kg i.m) and IDDM-rats received 4b, 4c, or 4d (36 mg/kg, p.o). The derivatives were further subjected to insulin secretion by RIN-5F cells confirmed insulino- tropic effect. In vitro antioxidant activity was evaluated using enzymatic activities on cell lines. In addition, all the synthesized derivatives showed non-toxic effects against a diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96).

Results: The results revealed that 4b, 4c, and 4d showed promising results by substantially lowering the blood glucose levels on 21st day of post-administration. Serum insulin levels substantially rose, suggesting that derivatives exert insulino- tropic effects through different pathways. 4c showed oxygen free-radical scavenging activity.

Conclusion: Our study proves that oral administration of 5-naphthalidin-TZD twice a day is a valuable treatment for type II diabetes. It seems to be a good drug moiety endowed significant oxygen free-radical scavenging activity.

Keywords: Diabetes, Free-radical scavenging activity, RIN-5F cells, Diseased human lung fibroblast.

INTRODUCTION

Charaka and Sushruta, the two great men of Indian medicine of yore had described the disease as “Madhumeha,” a condition where large quantities of sugar are lost from the body in urine. A historical account reveals that as early as 700–200 B.C. Diabetes Mellitus (DM) was a well-recognized disorder in India and was even distinguished as two types; one is genetics and other one resulting from dietary indiscretion [1]. It is characterized by means of abnormally rise in blood glucose levels. The diabetic complications are diabetic neuropathy, retinopathy, nephropathy, and cardiovascular diseases [2].

Thiazolidinediones (TZDs; also called as “glitazones”) was introduced in late 1990’s as an oral addition therapy for type II diabetes mellitus and related diseases. They bind firmly to peroxisome proliferators activated receptor (PPARγ). This stimulates peripheral adiposities to promote adipogenesis and uptake of free fatty acids (in peripheral but not visceral fat), which leads to reduction in the fat storage [3].

An imbalance between reactive oxygen species (ROS) fabrication and antioxidant scavenging ability induces oxidative stress, which eventually leads to cellular and tissue damage in diabetic patients [4]. These effects can be minimized by neutralizing oxidants or scavenged by increasing antioxidant intake [5].

In this research, we investigated the insulino- tropic effect of 5-substituted TZD with standard reference rosiglitazone and on insulin secreting RIN-5F cells both in vitro and in vivo, which consist of a series of studies such as reduced or inhibited glucose absorption at intestinal level and marginal level, by increasing the entry of glucose into cells. The in vivo studies included the dose- and time-dependent stimulation of β pancreatic cells to release insulin in insulin-sensitive tissues and altering associated oxidative stress [6].

The antioxidative events were evaluated using catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) on cell lines, that is, diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96) [7].

METHODS

Drugs and chemicals

Alloxan procured from Sigma, St. Louis, MO, USA. Ascorbic Acid-Qualigens, Methanol-Qualigens was of the highest analytical grade available.

Rosiglitazone

Rosiglitazone is an oral hypoglycemic drug in the thiazolidinedione class of drugs. It acts as PPAR gamma receptors agonist [8].

Synthesis of 2-imino-4-thiazolidinedione

Synthesis of 2-imino-4-thiazolidinedione was carried out by taking equimolar quantities of 2-chloroethylacetate (104 Moles) with thiourea (96 Moles) and dissolved in ethanol and refluxed over a period of 3 h. The mixture was cooled to room temperature. The HCl salt which formed was added to water and neutralized with solution of sodium acetate, which precipitated 2-imino-4-thiazolidinedione on cooling. The product was filtered and dried at 60°C. TLC solvent system ethylacetate: methanol: 1:2.

IR showed absorption at (cm⁻¹): 3074, 3059(-NH, NH-), 1620(-C=O-), and 746(-C-S-C-). M.P = 186–188°C.
Synthesis of 2-4-thiazolidinedione

The 2-Imino-4-thiazolidinedione was hydrolyzed with 2N HCl in ethanol by refluxing for 15 h. The reaction mixture cooled was neutralized with saturated solution of NaHCO₃ (10%). The crude product 2-4-thiazolidinedione was separated as solid. This was recrystallized from ethanol: water (40:60) mixture. TLC solvent system ethylacetate: methanol (1:2).

Synthesis of 5-[(2-Hydroxy)-naphthylidene]-2,4-thiazolidinedione

Equimolar amounts of variously substituted aldehydes and 2,4-thiazolidinedione were refluxed in absolute ethanol (till the sample dissolves) for 4 h in the presence of piperidine. The mixture was cooled and poured on to crushed ice with stirring. The product obtained is washed with toluene to give 5-[(2-Hydroxy)- naphthylidene] - 2,4-thiazolidinedione. TLC solvent system used ethylacetate: methanol (1:2).

Synthesis of 5-[(2-Hydroxy)-naphthylidene]-3-(N-carbamate)-2,4-thiazolidinedione

5-[(2-Hydroxy)-naphthylidene]-2,4-Thiazolidinedione (0.1 mole) and triethylamine (0.15 mole) in DMF (150 ml) were added methyl chloroformate (0.15 mole) drop wise at 0–5°C. Then, the reaction mixture was refluxed for 15 h in an oil bath. The reaction was continuously monitored by TLC using toluene: ethylacetate (1:1). Later the reaction is poured into dil.HCl (160 ml, 50% v/v) with constant stirring. The crude product obtained was filtered, dried, and purified by crystallization using absolute alcohol.

Synthesis of 5-[(2-Hydroxy)-naphthylidene]-3-(N-semicarbazide)-2,4-thiazolidinedione

The mixture of 5-[(2-hydroxy)-naphthylidene]-3- (N- carbamate)-2,4-thiazolidinedione (0.1 mole) and hydrazine hydrate (0.43 mole) in alcohol (till the sample dissolves) was heated in an oil bath for 18 h. The reaction progress was monitored by TLC using toluene: ethylacetate (1:1). Cooled reaction gives yellow crystals. Re-crystallization by absolute alcohol is done.

Synthesis of 5-[(2-hydroxy)-naphthylidene]-3-(N-4'-arylmethyl semicarbazide)-2,4-thiazolidinedione

A mixture of 5-[(2-Hydroxy)-naphthylidene]-3-(N-semicarbazide)-2,4-thiazolidine dione (0.01 mole), substituted aldehydes (0.012 mole), and hydrochloric acid (0.18 ml) was refluxed for 4 h. Progress of the reaction was checked by TLC using toluene: ethylacetate (1:1) as eluent. The solid obtained was filtered, dried, and crystallized from absolute alcohol to give 5-[(2-Hydroxy)-naphthylidene]-3-(N-4'arylmethyl semi-carbazide)-2,4-thiazolidinedione.

General procedure for synthesis of substituted 5-[(2-hydroxy)-naphthylidene]-3-(N-4'arylmethyl semicarbazide)-2,4-thiazolidinedione (4a-g)

To a continuously stirred mixture of compound 4 (0.35g, 0.00110 mol) and appropriate arylidine compounds (0.3g, 0.00110 mol) in ethanol (8 mL), few drops of piperidine were added. The reaction mass was refluxed for 6–8 h. The progress of the reaction was continuously monitored by TLC. After cooling, the separated solid or residue was filtered, washed with hot ethanol. All the compounds were further
In vivo and in vitro screening for anti-diabetic activities

Animals [9-10]

Male albino rats weighing 150–200 g were used for this study. Rats were caged under controlled temperature of 20–24°C and maintained on 12 h light/dark cycle. They were fed with standard laboratory pellets and water ad libitum. For induction of diabetes, rats were kept on fasting before alloxan injection (Sigma-Aldrich) (150 mg/kg body weight in 0.9% NaCl, pH 4.5) by intraperitoneal route.

The animals were fed with commercially available rat pellet diet (Say Durga feeds and foods, Bangalore). Water was allowed ad libitum under strict hygienic conditions. The study protocols were duly approved by the Institutional Animal Ethics Committee (IAEC) of College of Pharmaceutical Sciences, Dayananda Sagar University (DSU/PhD/IAEC/09/2017–18), Bengaluru and studies were performed in accordance with the CPCSEA guidelines.

Experimental design [11-13]

In the present investigation, a total of 36 rats (30 diabetic surviving rats and six normal rats) were divided into six groups of six rats each. Control rats were treated with carboxymethyl cellulose (CMC) alone. The rats with a blood glucose level above 150 mg/dl were considered to be diabetic and used in the experiment for 15 day treatment.

- Group I: Normal control – Received 0.25% CMC p.o and sterile water for injection i.m.
- Group II: Alloxan control – Received 0.25% CMC p.o and alloxan 0.15 mg/kg i.m.
- Group III: Rosiglitazone treated – Received rosiglitazone 500 mg/kg in 0.25% CMC p.o and alloxan 0.15 mg/kg i.m.
- Group IV: Received 4b, 36, and 4d derivatives were added separately at the concentration of alloxan (0–10 mM) dissolved in citrate buffer, pH 4.4, and the medium is supplemented with 1% nonessential amino acids, L-lysine-coated 75 cm² flasks (~2.0–2.5×10⁶ cells/mL) in RPMI1640 medium.
- Group V: Received 4c, 36  mg/kg in 0.25% CMC p.o and alloxan 0.15 mg/kg i.m.
- Group VI: Received 4d, 36 mg/kg in 0.25% CMC p.o and alloxan 0.15 mg/kg i.m.

Treatment was continued for 15 days. On day 15, after overnight fasting, blood samples were collected by withdrawing a drop of blood from tail vein by tail tipping method. The blood was dropped on the reagent strip and inserted into the digital blood glucometer and the readings were noted.

In vitro studies using RIN-5F cells [14-17]

Rin-5F cells, that is, rat pancreatic beta cell line were grown in polyclonal tissue culture with 1% L-glutamine (Sigma-Aldrich, India), 2 mM penicillin, 100 units/mL of streptomycin, 100 mg/ml of phenoxyl methyl penicillin by Sigma-Aldrich, India, and 2 mM L-glutamine (Sigma-Aldrich, India).

In vitro screening for antioxidant activity [18,19]

Cell culture

Human lung fibroblast (COPD), HCC7231 (TACC CCL-96) cells were preserved at 37°C in an incubator with a humidified atmosphere of 5% CO₂/95% O₂. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, India) containing 5% fetal bovine serum (FBS, Sigma-Aldrich, India), 100 mg/ml of streptomycin, 100 unit/mL of phytoxy methyl penicillin by Sigma-Aldrich, India, and 2 mM L-glutamine (Sigma-Aldrich, India).

Cell viability [17]

Mitochondrial dehydrogenase (MTT) based cell viability test a colorimetric method used to evaluate the number of healthy cells in a sample. The Human lung fibroblast (COPD), HCC7231 (TACC CCL-96) was seeded in a 96 well plate at a concentration of 1.2±10⁤ cells/ml in a culture medium and 16 h after plating, cells were treated with 4b, 4c, and 4d derivatives were added separately at the concentration of 100 mg/ml and 1 h later 1 mM H₂O₂ was added to the culture and incubated for 24 h at 37°C, with 5% CO₂ in a humidified atmosphere and DMSO without sample is considered as control. The water insoluble formazan so formed can be solubilized using DMSO and the viable cells were quantitated at 570 nm on an ELISA reader.

% Cytotoxicity = (Acontrol – Asample) / Acontrol × 100

Where, Acontrol is the absorbance of sample and Asample is the absorbance of control.

Serum preparation [20]

The blood used for serum preparation were collected from all the animals through direct puncturing the retro orbital plexus with 21 G needle attached to 5 mL syringe, following mild ketamine anesthesia of the rats. The serum was prepared using standard method as described by Kohen. Blood was allowed to clot for 30 min and then centrifuged at 2500 rpm for 15 min and serum was collected.

Superoxide dismutase (SOD) activity [21]

Superoxide dismutase activity was assayed using 50 ml of xanthine oxidase (0.1 mg/ml) was used to generate a superoxide, which has a strong absorbance at 560 nm and nitroblue tetrazolium (NBT) was used as an indicator of superoxide production. SOD activity was then measured by the degree of inhibition of the reaction unit of enzyme providing 50% inhibition of NBT reduction. Results are expressed as U/mL.

 Catalase (CAT) activity [22]

The basic principle involved here is the disappearance of H₂O₂ in the presence of the enzyme source at 26°C. The reaction mixture contained 1.2 mM of 3% (v/v) H₂O₂ and 0.2 ml of serum (10 µL) in 0.05 M phosphate buffer (pH 7.0) at a final volume of 1.0 ml. Samples were allowed to stand for 25 min and the decomposition rate of hydrogen peroxide was measured at 240 nm for 5 min on a spectrophotometer. The change in absorbance is proportional to the breakdown of H₂O₂. A molar extinction coefficient of 0.041 mM⁻¹ cm⁻¹ was used to calculate the activity.

Glutathione peroxidase (GPx) activity [23]

The GPx activity in the serum was determined by the method used by Sarma et al. and one unit of GPx activity was defined as the amount of enzyme required to oxidize 1 µmol of NADPH per minute at 30°C at pH 7.0 and it is measured at 340 nm.

RESULTS

The body weights of normal and diabetic rats were significantly (p<0.001) decreased in the alloxan induced diabetic group with comparison to control. The data showed a progress in the body weight...
after 21 days of supplementation, the animals started to gain their normal weight back as shown in Fig. 1.

Administration of 5-naphthalidin-thiazolidinedione derivatives 4b, 4c, and 4d in alloxan induced diabetic rats produced a major reduction in serum glucose levels when compared with control are shown in Fig. 2.

5-naphthalidin-thiazolidinedione derivatives 4b and 4d produced a dose-dependent stimulatory effect on insulin secretion by RIN-5F cells proved to be having promising insulinotropic property as shown in Fig. 3.

The biological antioxidant activity and cell viability was carried on diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96) cell lines. This resulted showed a remarkable protection of cell lines from oxidative stress induced by free radicals as shown in Fig. 4.

The serum level of SOD, CAT, and GPx of experimental diabetic rats was carried out and the results are shown in Tables 1-4 and they protect organisms from increasing ROS and act as the main defense system.

DISCUSSION

Based on the literature survey Alloxan-induced diabetes mellitus has been described as an exploitable experimental model to study the activity of oral anti-diabetic agents. Diabetes is the major grooming health problem throughout the world, in the present paper, we have decided to synthesize some new 5-substituted thiazolidinone derivatives as shown in scheme and evaluate them for their possible insulinotropic effect. The structure of new compounds synthesized during present investigation has been authentically established by their UV, FTIR, H$^1$NMR, and C$^{13}$NMR.

As shown in the scheme cyclisation of 2-chloro ethyl acetate with thiourea in ethanol to give 2-imino-thiazolidinedione followed by hydrolysis with HCl to afford 2,4-Thiazolidinedione. On condensation with 2-hydroxynaphthaldehydes in the presence of piperidine to form 5-[2-(Hydroxy)-naphthylidene]-2,4-thiazolidinedione which on treatment with methyl chloroformate yielded 5-[2-(Hydroxy)-naphthylidene]-3-(N-Carbamate)-2,4-thiazolidinedione. This was followed by condensation with hydrazinehydrate to give 5-[(2-Hydroxy)-naphthylidene]-3-(N-semicarbazide)-2,4-thiazolidinedione. This semicarbazide was transformed into semicarbazones by condensation with substituted aromatic aldehydes to get 5-[(2-Hydroxy)-naphthylidene]-3-(N-4 ary methyl semicarbazide)-2,4-thiazolidinedione, using this as a parent compound derivatives 4a-e was synthesized [24].

The in vivo and in vitro oral hypoglycemic activity of 5-naphthylidene TZD derivatives was screened for the prominent action on insulin secretion. This could be due to the insulin secretagogue effect of antihyperglycemic constituents, C5 substituted groups in TZD, which are generally more active than thiazolidine analogs. The results
of this study also demonstrated that 4b, 4c, and 4d for 21 days had regenerative potential with increased insulin secretion from β-cells of islets of Langerhans of pancreas in alloxan-induced diabetic rats as shown in the Fig. 2. The reduction in serum glucose activity of derivatives was compared with standard reference rosiglitazone. From the results of the present study, it may be suggested that the mechanism of action of 5-naphthylidene TZD may be similar to rosiglitazone action.

Pancreatic β-cells play a key role in the production of insulin; therefore, we used RIN-5F cells [a rat islet cell line] to examine the levels of insulin. The direct exposure of 5-naphthylidene TZD derivatives to β-cells of Langerhans causes stimulation of cells to release insulin as shown in Fig. 3.

The MTT assay as shown in Fig. 4 revealed that exposure to 4b, 4c and 4d significantly reduced cell viability of diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96) cells line, compared to the normal cell line group. Enzymatic antioxidant activity of TZD derivatives 4b, 4c, and 4d was screened using endogenous enzymes (Gpx, CAT, and SOD) as shown in Tab-4.4c showed promising activity proving that 5-naphthylidene TZD derivatives emerged out as good anti-diabetic and promising antioxidant drug [25]. Derivatives induced a considerable decrease in the catalase activities (p < 0.05). A slight decrease in SOD and GPx activity was also obtained but was not statistically significant (p > 0.05). The decreased activities of the serum antioxidant enzymes play an important role in scavenging the toxic intermediates of incomplete oxidation.
Table 4: Changes in SOD, CAT, and Gpx of normal and diabetic rats after 21 days of treatment with 10a-b and 4c TZD derivative

| Experimental group | SOD (u/mg protein) | CAT (mm H2O2 decomposed/min) | Gpx (u/mg protein) |
|--------------------|---------------------|-------------------------------|-------------------|
| Normal control     | 2.24±0.01           | 21.17±1.32                    | 30.71±2.93        |
| Diabetic control   | 1.46±0.015          | 18.35±1.42                    | 35.30±2.37        |
| Diabetes+          | 1.77±0.02           | 17.42±1.55*                   | 40.6±4.23         |
| Rosiglitazone      | 2.03±0.003*         | 16.98±1.63                    | 54.59±1.78        |
| Diabetes+4b        | 2.20±0.048*         | 19.30±1.34*                   | 57.50±1.85*       |
| Diabetes+4c        | 1.98±0.04*          | 20.30±1.13                    | 29.50±1.94*       |

SOD: Superoxide dismutase; CAT: Catalase; Gpx: Glutathione peroxidase. *Each value is means±SEM (n=6); *p<0.01 when compared to the day 1 values of the same group.

CONCLUSION

Our research shows that po administration of 5-naphthylidene thiazolidin-4-ones derivatives as an alternative treatment for IDDM, through the rejuvenation of β cells of pancreas to stimulate insulin and other probable mechanism is they act as PPAR γ agonist. The phenolic moiety present endowed significant oxygen free-radical scavenging activity.

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AUTHORS’ CONTRIBUTIONS

All the authors have equal contribution for the manuscript preparation, and especially edited and the final copy was revised by Dr. Chandrashekhara and Dr. Asif.

CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

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