Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2016, Volume 4, Issue 1
ISSN: 2321–3647(online)

In Vitro Cytotoxicity Evaluation of Troglitazone, Rosiglitazone and Pioglitazone in HepG2 Cells

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ABSTRACT

The objective of this research work was comparative evaluation of in vitro cytotoxicity of troglitazone, rosiglitazone and pioglitazone in HepG2 cells. Briefly, the HepG2 cells were exposed to multiple concentrations (3.125–100 µM) of three drugs (troglitazone, rosiglitazone and pioglitazone) for 12 h, 24 h and 48 h. At the end of each treatment period, cytotoxicity was determined using multiple end points such as 3-[4,5-imethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, neutral red assay and lactate dehydrogenase leakage assay. Troglitazone showed time and concentration dependent cytotoxicity in all three end points with evident cytotoxic effects being observed at 50 and 100 µM concentrations. MTT assay showed higher cytotoxicity and early onset compared to neutral red and lactate dehydrogenase leakage assay at all time points. There was no cytotoxicity observed for rosiglitazone at all tested concentrations up to 100 µM. Similarly, pioglitazone also did not affect the viability of HepG2 cells up to the concentration of 50 µM however some cytotoxicity was noted at 100 µM concentration which could be partly attributed to the precipitation of test compound noted at 100 µM concentration after addition to the culture medium. The results from this research work indicated that among the three drugs tested in this study, only troglitazone induced time and concentration dependent toxicity in the HepG2 cells in all three end points with MTT assay being the most sensitive assay.

Keywords: Troglitazone, Rosiglitazone, Pioglitazone, HepG2, Cytotoxicity

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Received 11 December 2015, Accepted 06 January 2016

Please cite this article as: Sancheti PM *et al.*, In Vitro Cytotoxicity Evaluation of Troglitazone, Rosiglitazone and Pioglitazone in HEPG2 Cells. American Journal of Pharmacy & Health Research 2016.

INTRODUCTION

The development of a new drug is complex and needs integrated approach to evaluate efficacy and safety. Approximately, 30% of failures in the development of drugs are related to toxicity and safety issues[1]. Even though the failure of new drugs during the development or post marketing can be due to multiple reasons, hepatotoxicity remains one of the major safety concerns. There are many drugs which have either failed in the late stage clinical development or have been withdrawn from the market mainly due to the off-target or idiosyncratic toxicity. This poses an additional challenge faced by the pharmaceutical industry which could be due to inadequate in vitro screening design and partly due to poor clinical relevance of animal studies to human toxicity. In general, hepatotoxicity has the poorest correlation (about 50%) with regulatory animal studies[2-3]. The in-vivo animal studies do offer certain advantage over in vitro assays, but it is important to recognize that the animal tests are costly, low throughput and ethically debatable. Furthermore, only animal studies may not be adequate for the human hepatotoxicity potential assessment due to species specific differences. It is important to integrate the non-animal alternative test methods such as in vitro toxicity assays as a part of overall safety assessment plan for the drugs[4-5].

In vitro toxicity assays form an important part of overall integrated preclinical safety assessment. Since in vitro cytotoxicity is cell specific, choice of test system is very important[5]. Even though multiple cell lines or the primary cell cultures have been reported in literature, the human hepatoma cell line, HepG2, has been one of the most frequently employed test system for the assessment of hepatotoxicity as they are easy to maintain in culture, are stable, human derived and organ specific. Despite the inherent metabolic limitation, it is suggested that screening of multiple endpoints on HepG2 cells can predict hepatotoxicity with 80% sensitivity and 90% specificity[6]. While employing in vitro cytotoxicity assays for the safety evaluation, it is important to consider the choice of end points and design of an experiment as the concordance of these in vitro assays with human hepatotoxicity is poor due to various reasons such as short-duration of treatment, measurement of a single endpoint although complex and multiple mechanisms are involved in hepatotoxicity etc. It is important to have multiple time points and multiple end points for the assessment of hepatotoxicity.

The end points such as 3-[4,5-imethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, Neutral red (NR) uptake assay and Lactate Dehydrogenase (LDH) leakage assay are some of the most commonly employed endpoints in the in vitro cytotoxicity assays[7]. The MTT assay

very popular cell viability assay often used to determine cytotoxicity following exposure to toxic substances. MTT (3-[4,5- imethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. The neutral red assay is also used to measure cell viability, in which the living cells take up the neutral red, which is concentrated within the lysosomes of the health cells. The LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage[8].

The objective of this study was to evaluate comparative in vitro toxicity of troglitazone, rosiglitazone and pioglitazone in HepG2 cells at different time points using multiple end points. The drugs selected for this study belong to the thiazolidinediones (TZD) class of anti-diabetic drugs which target muscular insulin resistance and act as insulin sensitizers to improve blood glucose control in patients with type 2 diabetes mellitus. Drugs of this class act as ligands for the γ-subtype of the peroxisome proliferator-activated receptor (PPAR-γ), which is directly involved in the regulation of genes controlling glucose homeostasis and lipid metabolism9-10. There are multiple reports of hepatotoxicity associated with this class of drugs most notably troglitazone which was withdrawn from the market in the year 2000 due to serious idiosyncratic hepatotoxicity. This study was an attempt to investigate if the hepatotoxicity observed in clinic could have been better predicted by employing the in vitro cytotoxicity assay with multiple time points and using multiple biomarkers of cytotoxicity. The comparative evaluation of three drugs from the same class also provides an indication of the relative hepatotoxicity potential of these three drugs which have a very clear differentiation in the clinical settings.

MATERIALS AND METHOD

HepG2 cells were obtained from ATCC (American Type Culture Collection). Test compounds and reagents were procured from vendors as indicated: Troglitazone (Ramidus AB, Sweden), rosiglitazone (Tokyo chemical industry co. ltd, Japan), pioglitazone hydrochloride (Glenmark Pharmaceuticals ltd, Mumbai), LDH assay kit (Randox laboratories), MTT (Affymetrix Inc., USA), neutral red (Sigma Aldrich Inc., USA). The tissue culture media, fetal bovine serum, antibiotics and trypsin were purchased from Hyclone, Invitrogen, Gibco and Sigma respectively. All other chemicals and solvents were of analytical grade.

Cell Cultures and treatments

HepG2 cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere in 5% CO2 at 37°C. The passage number range for cell line was maintained between 12 and 15. The cells were cultured in 75 cm2 cell culture flasks. For experimental purposes, cells were cultured in 96-well plates at a cell density of 50000 cells/well (200 µl of cell suspension/well). Unattached cells were removed by changing the medium 24 h after seeding. The test drugs (troglitazone, rosiglitazone and pioglitazone) were dissolved in dimethyl sulfoxide to prepare the stock solutions and added to the culture medium to achieve the desired concentration in each well by making 200 fold dilutions. The final concentration of DMSO in the culture medium in each well was restricted to less than 0.5 %. The cells were treated with the test drugs (troglitazone, rosiglitazone and pioglitazone) in the concentrations range from 3.125 to 100 µM each (3.125, 6.25, 12.5, 25, 50 and 100 µM) for 12, 24 and 48 h. The treatment of cells was replicated in multiple plates so that each plate could be terminated at each time points. At the end of treatment, the multiple cytotoxicity end points were determined as described below. The visual inspection of the cells under microscope was also performed for qualitative evaluation of the cytotoxicity and cell damage.

MTT assay

The MTT assay was based on the protocol described by Mossmann[11] with some modifications. Briefly, at the end of the incubation time, the supernatant culture medium was removed from all the wells and replaced with 200 µl of fresh culture media. This was followed by addition of 20 µl of MTT solution (5 mg/mL in PBS) to each well and the cells were then incubated for 3 h in a humidified atmosphere in 5% CO2 at 37°C. At the end of 3 h incubation period, the supernatant from each well was carefully removed without taking out the formazan crystals inside the cells. Each well was then washed with PBS (200 µl) followed by addition of DMSO (100 µl). The plates were kept on shaker for 10 min for complete dissolution of formazan crystals. Aliquots of the resulting solutions were transferred to fresh 96-well plates and absorbance was recorded at 570 nm using microplate spectrophotometer system (FLOUstar Omega, BMG labtech). Results were analyzed by calculating % death relative to the control values.

Neutral red assay

The neutral red assay was based on the protocol described by Borenfreund and Puerner[12] with some modifications. This assay determines the accumulation of the neutral red dye in the

lysosomes of viable, uninjured cells. At the end of the incubation time with the test drugs, the supernatant culture medium was removed from all the wells and replaced with 200 µl of fresh culture media. This was followed by addition of 20 µl of NR solution (0.5 mg/mL in PBS) to each well and the cells were then incubated for 3 h in a humidified atmosphere in 5% CO2 at 37°C. At the end of 3 h incubation period, the supernatant from each well was carefully removed and each well was then washed with PBS (200 µl). This was followed by the addition of 100 µl of acid-alcohol solution (1% acetic acid in 50% ethanol) to each well and gentle shaking for 10 min to achieve the complete dissolution. Aliquots of the resulting solutions were transferred to fresh 96-well plates and absorbance was recorded at 540 nm using the microplate spectrophotometer system (FLOUstar Omega, BMG labtech). Results were analyzed by calculating the % death relative to the control values.

LDH leakage assay

Cytotoxicity induced by the test drugs was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure to the test drugs, the culture medium from each well was aspirated and transferred to separate tubes serially numbered followed by centrifugation at 5000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined using a commercially available kit from Randox laboratories ltd on Randox Daytona biochemistry analyzer. The assay is based on the conversion of pyruvate to lactate in the presence of LDH. The results were analyzed and are presented as percentage change from the control values.

Statistical analysis

All experiments were performed three times in duplicate and data was statistically analyzed using one-way ANOVA with Tukey's post test (multiple comparison test) using GraphPad Prism statistical software version 5.02 for Windows, GraphPad Software, San Diego California USA. A $p < 0.05$ was considered significant in all evaluations.

RESULTS AND DISCUSSION

Cytotoxicity of troglitazone

The concentration and time dependent effects of troglitazone on the viability of HepG2 cells as determined by three different assays are summarized in the Figure 1. In summary, troglitazone showed time and concentration dependent cytotoxicity in all three end points (MTT assay, NR assay and LDH leakage assay). No reduction in the cell viability was noted up to the concentrations of 12.5 µM even at 48 h treatment duration however at the concentration of 25

µM, very slight cytotoxicity was noted in MTT assay (only at 24h and 48h time points) and Neutral red and LDH assays (only at 48h time point). The cytotoxic effects at 50 and 100 µM concentrations were evident in all three assays which increased with increase in the treatment duration from 12h to 48h. The highest cytotoxicity was noted at 48h time point at 100 µM concentration. In general, MTT assay showed higher cytotoxicity compared to Neutral red assay at all the time points. The qualitative microscopic evaluation of the cells treated with different concentrations of troglitazone at various time points indicated morphologic changes in troglitazone treated cells compared to the control cells (Figure 4B).

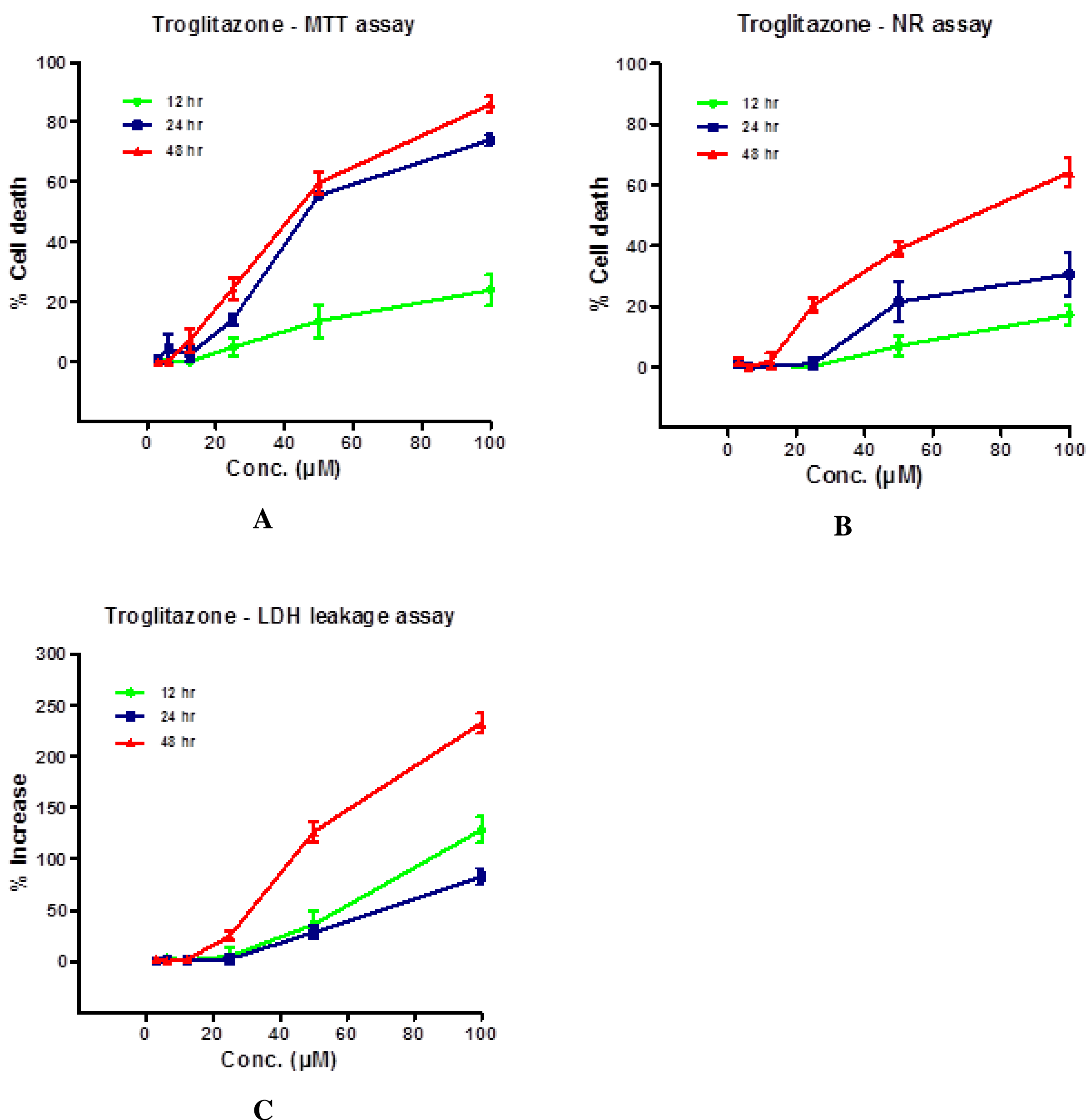

Figure 1: Concentration and time dependent effects of troglitazone on HepG2 cells viability

using different cytotoxicity end pints A) MTT assay B) Neutral red assay C) LDH leakage assay. Values represent Mean ± SD from 3 separate experiments.

Cytotoxicity of rosiglitazone

The concentration and time dependent effects of rosiglitazone on the viability of HepG2 cells determined by three different assays are summarized in the Figure 2. Rosiglitazone did not affect the viability of HepG2 cells at any of the tested concentrations and time points in all three cytotoxicity end points. The qualitative microscopic evaluation of the cells treated with different concentrations of rosiglitazone also did not indicate any morphologic changes or cell damage as compared to the control cells (Figure 4C).

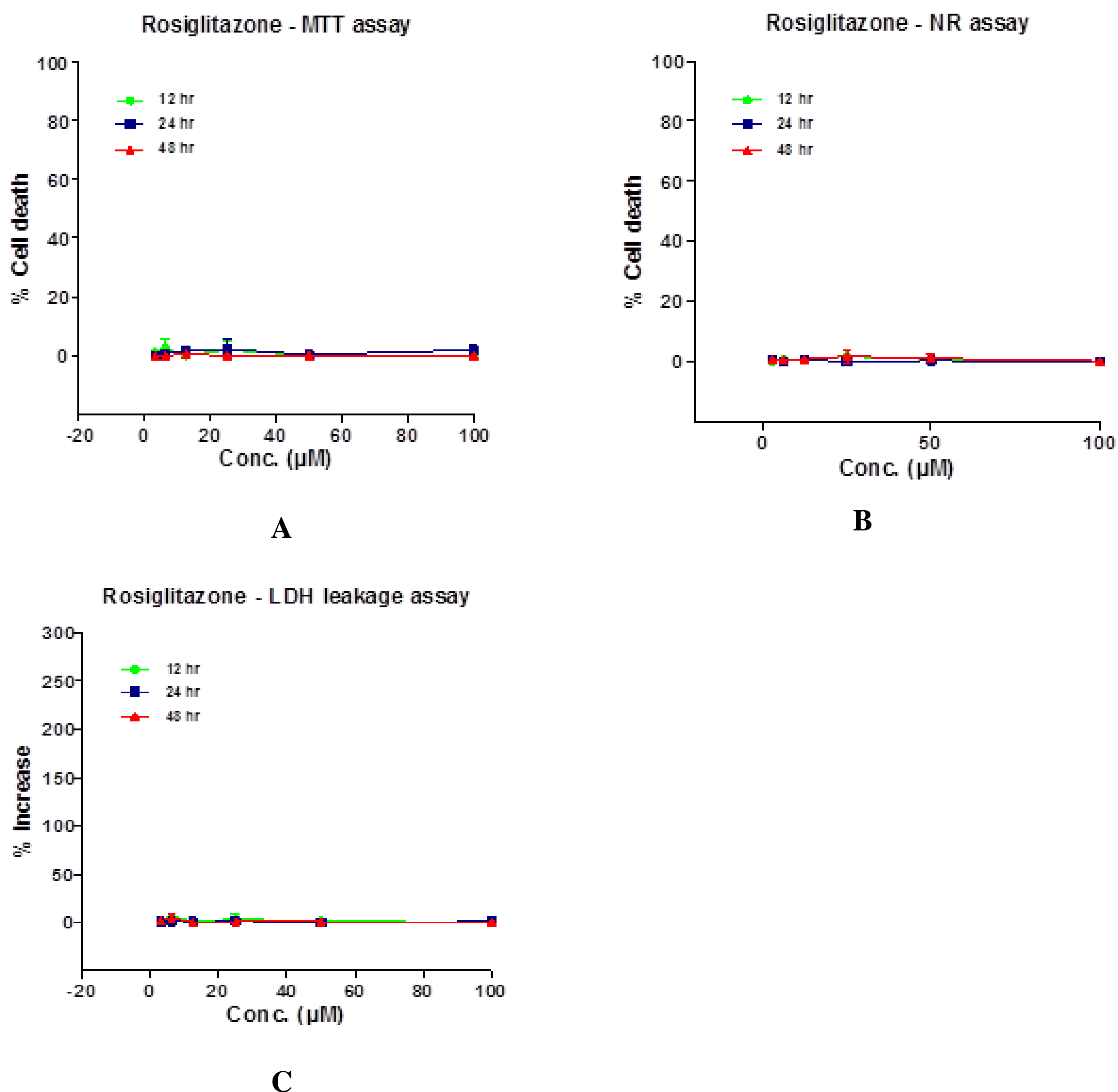

Figure 2: Concentration and time dependent effects of rosiglitazone on HepG2 cells viability using different cytotoxicity end pints A) MTT assay B) Neutral red assay C) LDH

leakage assay. Values represent Mean ± SD from 3 separate experiments.

Cytotoxicity of pioglitazone

The concentration and time dependent effects of pioglitazone on the viability of HepG2 cells determined by three different assays are summarized in the Figure 3. Pioglitazone did not affect the viability of HepG2 cells at any time points in all three cytotoxicity end points up to the concentration of 50 µM. Mild cytotoxicity (<20 %) was noted at 100 µM concentration at all time points and in all assays. The qualitative microscopic evaluation of the cells treated with different concentrations of troglitazone indicated mild morphologic changes in the pioglitazone treated cells at 100 µM concentration compared to the control cells (Figure 4D). The precipitation of test compound was also noted at 100 µM concentration after addition to the cells with culture medium.

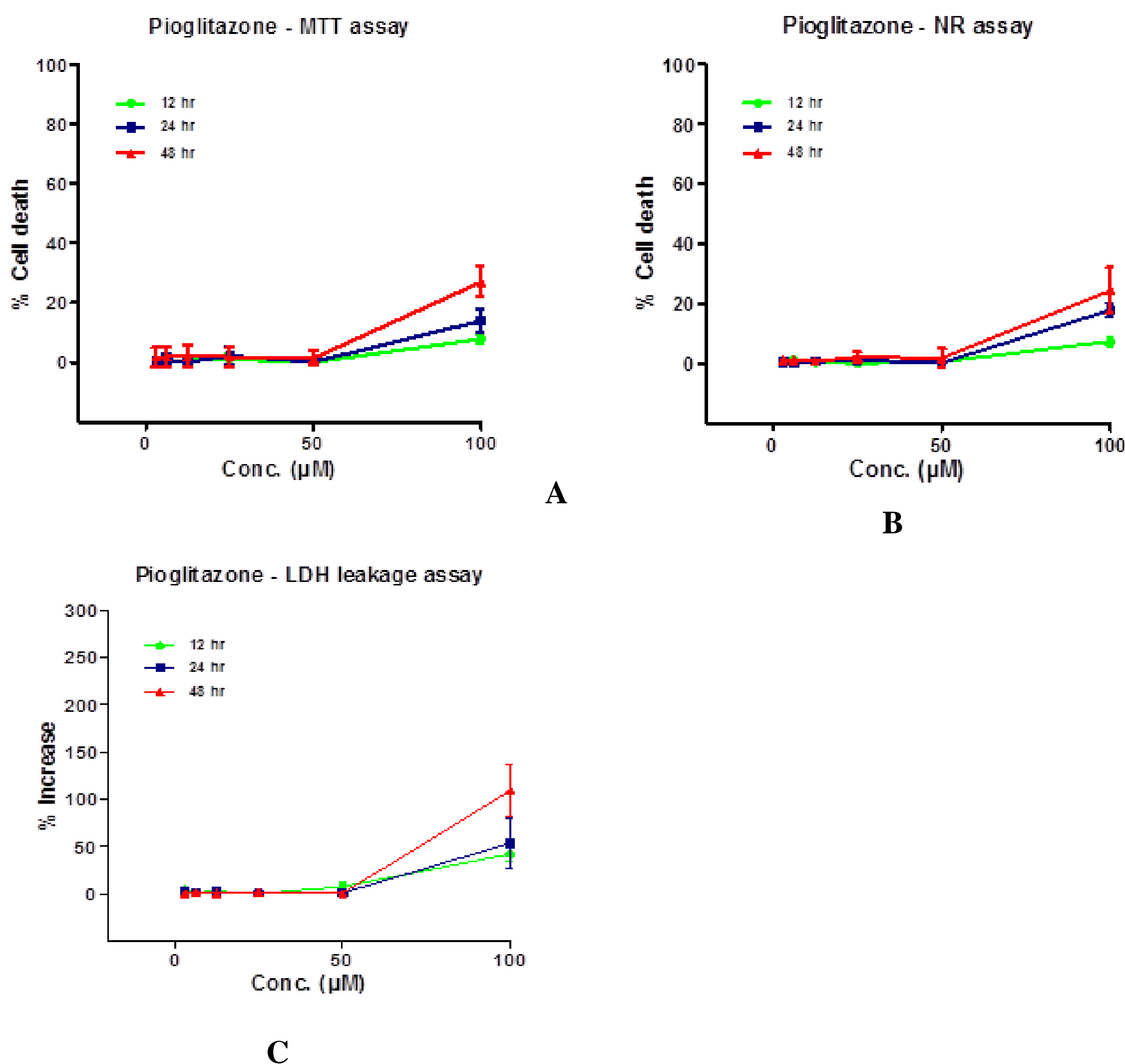

Figure 3: Concentration and time dependent effects of pioglitazone on HepG2 cells viability using different cytotoxicity end pints A) MTT assay B) Neutral red assay C) LDH leakage

assay. Values represent Mean ± SD from 3 separate experiments.

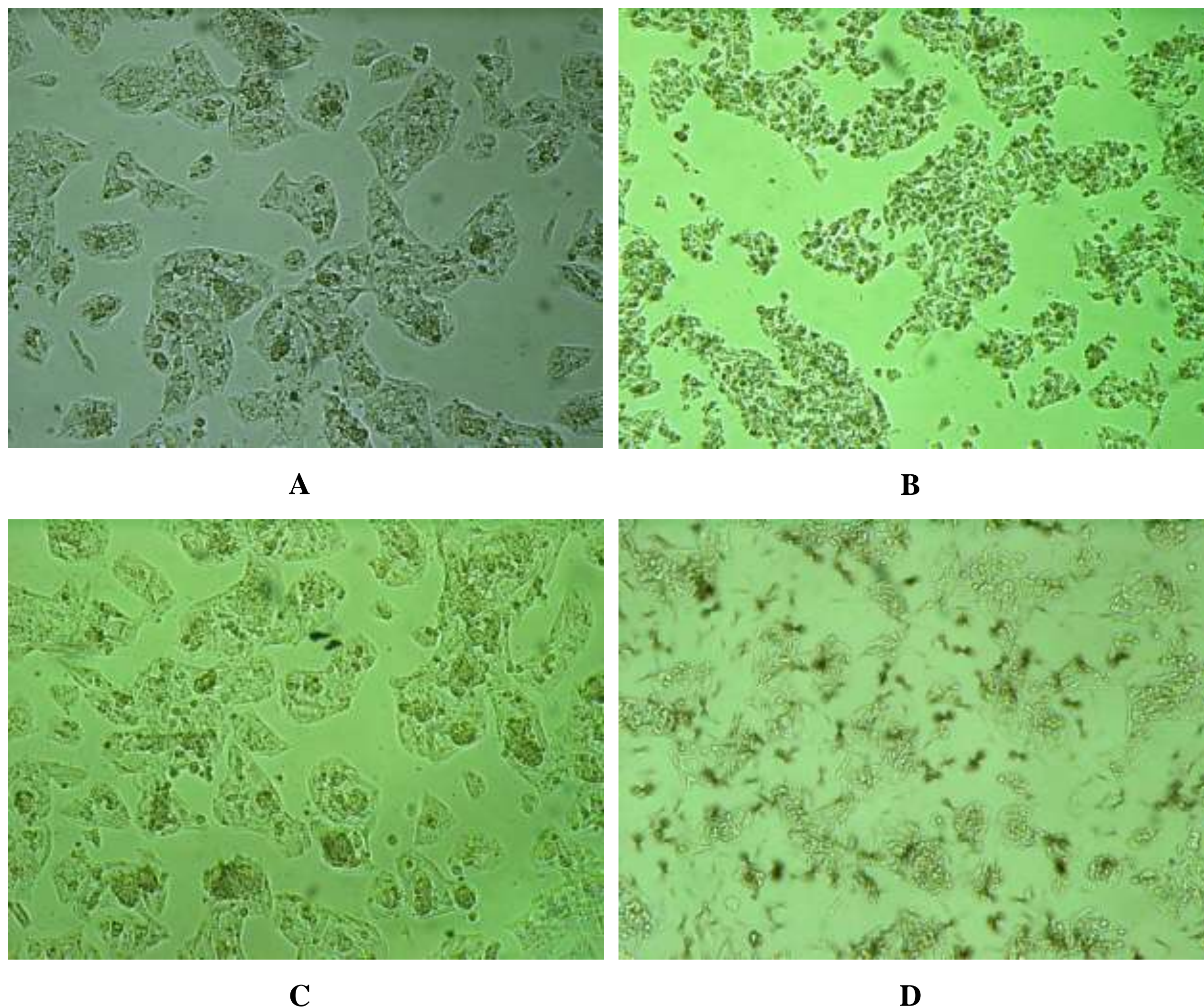

Figure 4: Representative pictures of HepG2 cells at 10 x magnification after treatment with A) Vehicle control (culture media with 0.5% DMSO) B) Troglitazone (100 µM) C) Rosiglitazone (100 µM) D) Pioglitazone (100 µM - Precipitation is visible).

The three thiazolidinones (troglitazone, rosiglitazone and pioglitazone) tested in this study exhibited very different cytotoxicity profile across the concentration range at multiple time points using multiple end points (Figure 1, 2 and 3). The results indicated that troglitazone showed time and concentration dependent cytotoxicity in all the end points whereas rosiglitazone and pioglitazone did not exhibit any cytotoxicity across the tested concentration range. The data for troglitazone indicated that the different end point assays employed to evaluate the cytotoxicity revealed slight differences in the profiles with regards to the onset and extent of the cytotoxicity. MTT assay appeared to be the more sensitive cytotoxicity assay showing higher cytotoxicity and early onset of cytotoxicity compared to neutral red assay. As the MTT assay is

mainly based on the enzymatic conversion process in the mitochondria, interference of mitochondrial function might be the first and major event contributing to the cytotoxicity exhibited by troglitazone. Comparison between MTT and NR assay at the concentrations of 50 and 100 µM indicate that cytotoxicity is evident at all the time points however the % cell death is higher MTT assay compared to NR assay. Similarly, at the concentration of 25 µM, MTT assay exhibited ~ 14% cell death at 24 h whereas NR assay and LDH leakage assay did not show any change indicative of cell death at that time point. The LDH leakage assay is based on the determination of leakage of enzyme due to permanent cell membrane damage which might happen after the initial intracellular effects in mitochondria due to exposure to troglitazone. Troglitazone has been reported to affect the mitochondrial homeostasis[13].

The maximum plasma concentrations in the patients taking troglitazone at therapeutic doses of 400 and 600 mg/day have been reported to be 3.6 to 6.3 µM14 which are similar to the lower concentrations (3.125 µM and 6.25 µM) tested in this study. Even though it is difficult to equate the in vitro concentrations with the patient plasma concentrations, it is important to note that there were no structural or functional effects in the HepG2 cells at these concentrations which are equivalent to the maximum therapeutic concentration achieved in humans. Some indication of cytotoxicity was evident at the concentrations of 25 µM which is ~4 fold above the maximum therapeutic concentration in humans but cytotoxicity (indicated by MTT assay, NR assay and LDH leakage assay) was evident at the higher concentrations of 50 and 100 µM which are approximately 8 to 16 fold above the maximum therapeutic concentration of 6.3 µM after 600 mg/day dose.

It has been reported that troglitazone accumulates in liver following oral administration in the animal studies[15]. Kawai et al[16] concluded that orally administered troglitazone is extracted by liver and subsequently undergoes enterohepatic circulation in rats. Further, the tissue distribution studies in rats have shown that the concentrations of troglitazone within liver tissues are much higher (10-12 fold) than those in the plasma[17]. If the similar effects occur for troglitazone in humans, the hepatocytes may be exposed to higher concentrations of troglitazone than those measured in plasma. Further, it is also possible that the patients with impaired mitochondrial function may be more susceptible to the mitochondrial effects of troglitazone leading to the hepatotoxicity at the lower therapeutic concentrations in few patients.

It has been reported that the affinity of pioglitazone to PPARγ is equal to that of troglitazone and that the affinity of rosiglitazone to PPARγ is 10 fold higher than that of troglitazone[18]. In addition, the rank order of affinity to PPARγ of thiazolidinedione chemicals has been shown to

closely match their glucose-lowering activity[19-20]. As pioglitazone and rosiglitazone did not induce cell death or cytotoxicity in the present study, it can be assumed that the affinity to PPAR$^{\gamma}$ may not affect the induction of cytotoxicity by troglitazone.

CONCLUSION

In conclusion, it should be noted that even though troglitazone, rosiglitazone and pioglitazone belong to the same class of thiazolidinones, they have significant difference in their potential to cause cytotoxicity in the HepG2 cells. Troglitazone induces concentration and time dependent cytotoxicity with MTT assay being the most sensitive assay for indicating the effect of troglitazone on mitochondrial function whereas rosiglitazone and pioglitazone do not induce any such cytotoxicity. This also correlates well with the clinical experience with these drugs indicating the utility of appropriately designed in vitro cytotoxicity assays.

ACKNOWLEDGEMENT

The authors would like to thank Glenmark Pharmaceuticals Ltd for the support provided for this research work.

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