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Benzylidene thiazolidinediones: Synthesis, *in vitro* investigations of antiproliferative mechanisms and *in vivo* efficacy determination in combination with Imatinib

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ABSTRACT

Thiazolidinedione (TZD) has been an interesting scaffold due to its proven antidiabetic activity and encouraging findings in anticancer drug discovery. We synthesised benzylidene thiazolidinedione derivatives which exhibited excellent antiproliferative effects in chronic myeloid leukemic cells K562 and the most active compounds 3t and 3x had GI_{50} value of 0.9 and 0.23 μM respectively. Both the compound was found to arrest the growth of K562 cells in G0/G1 phase in a time and dose dependent manner. Further, western blot analysis revealed that 3t and 3x could also inhibit the expression of cell proliferation markers, PCNA and Cyclin D1 and compound 3x up-regulated apoptosis markers, cleaved PARP1 and activated caspase 3, which could be a possible mechanism for the excellent antiproliferative effects exhibited by these compounds. *In vitro* combination studies of 3t and 3x with Imatinib found to potentiate the antitumor effects of Imatinib. Further *in vivo* efficacy in K562 xenografts, of 3t and 3x alone and in combination with Imatinib was found to be promising and far better than control group and combination treatment was found to be more effective as compared to only Imatinib treated or test compound treated animals. Thus, our findings suggest that these compounds are promising antitumor agents and could help to enhance the anticancer effects of Imatinib and other tyrosine kinase inhibitors, when used in combination.

Cancer is a dreadful multicellular disease which can arise from any cell types and organs with multi-factorial etiology. Cancer cells often obtain their proliferative advantage over their normal counterparts at least, in part, because of their inability to undergo terminal differentiation. They remain in the proliferative pool, providing themselves with a growth advantage.[1] Activation of nuclear receptors has been identified as an approach to induce differentiation and inhibit proliferation of cancer cells.[2]

Activation of PPARγ results in decreased concentration of serum glucose in diabetes, which led to the development of PPARγ agonists, thiazolidinediones (TZDs) containing glitazones such as Pioglitazone that are in clinical use as antidiabetic drugs.[3] Apart from established metabolic actions, PPARγ agonists induces apoptosis, cell cycle arrest, and terminal differentiation in several malignant cell lineages. During last two decades, studies have reported antitumor effects of various PPARγ ligands on almost every kind of tumor cell.[4]

Some glitazones have also been inducing apoptosis by the intrinsic pathway in a PPARγ- independent manner.[5–7] To help discern the PPARγ-dependent and PPARγ-independent properties of TZD containing glitazones, Shiau et al. developed Δ2-PG, Δ2-TG, Δ2-CG, benzylidene derivatives of Pioglitazone, Troglitazone and Ciglitazone , respectively, lacking PPAR-γ activity (Fig. 1). These derivatives have a double bond adjoining the terminal thiazolidine-2,4-dione ring (benzylidene double bond) which abolishes/decreases ligand binding to PPAR-γ.[8] Both Troglitazone and Δ2-TG induced cytochrome *C* release and DNA fragmentation in PC3 and LNCaP cells, attributing to the growth inhibition by apoptosis. These results and further several reports suggest that TZDs can induce apoptosis and cell cycle arrest independent of PPARγ activation.[4,5,9,10] Interestingly, these TZD derivatives have also been reported to possess anti-inflammatory effects,[11,12] which could be of benefit to relieve the pain associated with cancer. These findings initiated further research to assess the potential of Thiazolidinediones, as promising new therapeutics for cancer.

Since more than a decade, we are exploring the therapeutic potential of TZDs as anticancer agents targeting various pathways in cancer. In one of our reports, we had reported the derivatives of benzylidene

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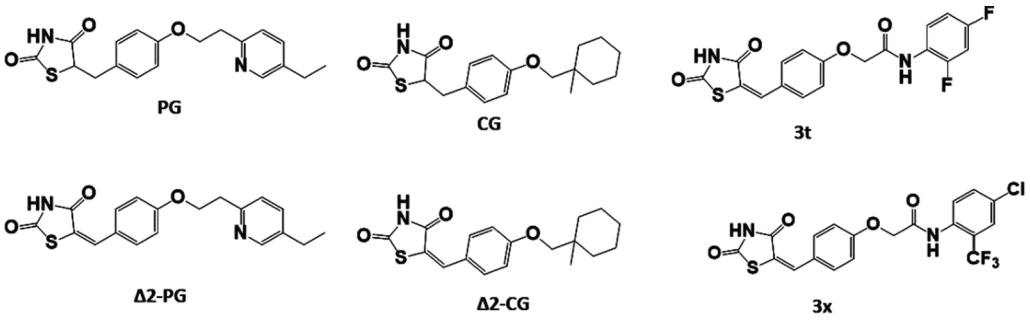
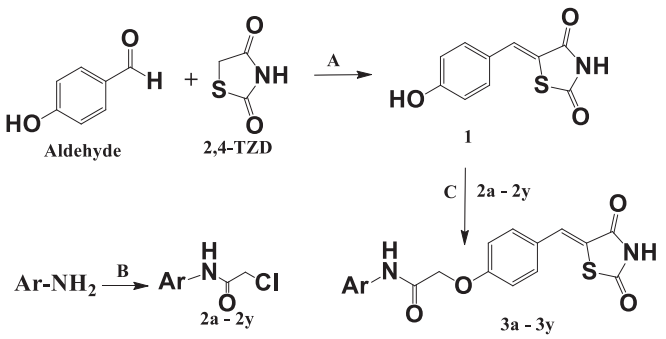


Fig. 1. Structures of most active compounds 3t and 3x, Pioglitazone (PG), Ciglitazone (CG) and their PPARγ inactive analogues Δ2-PG and Δ2-CG respectively.

TZD's with antiproliferative effects in sub-micromolar range in panel of cell lines[13] and the promising antiproliferative effects exhibited by these compounds gave us impetus to take this work further to find the exact molecular mechanism involved in the antiproliferative effects of these derivatives. These derivatives were designed as to circumvent the toxicity concerns associated with marketed glitazones.[14,15] We started designing of our compounds with Pioglitazone by considering two major modifications. Firstly, introduced of easily metabolizable amide linkage as linker between lipophilic tail and central aryl ring. This amide linkage may act as metabolic soft spot (diverting metabolism from TZD ring) and cleave in the presence of amidases in liver, thus rendering them inactive and improving their metabolic profile as compared to Pioglitazone. Also, toxicity of glitazones has lately been correlated with the full agonistic activity of the TZDs. Discovery of novel PPARγ sparing TZDs such as MCC-555 or Δ2 derivatives similar to that of reported by Shiau *et al.* might help in dodging the PPARγ related side effects.[6] Hence, secondly, we considered the addition of benzylidene double bond in between central aryl ring and TZD ring, as this may give rise to the compounds which may act by PPARγ independent manner or partial agonistic manner.

To further study the structure activity relationship, in addition to previously reported 3a-3j, we synthesized more derivatives **3k-3y** by retaining of the amide linkage and benzylidene double bond, and by varying the aromatic (Ar) moiety (Scheme 1). We tried substituting various electron donating and withdrawing groups on already active molecules from previous series. In newly selected Ar group, we also incorporated amines having mono substituted halogens like chlorine, fluorine and bromine. Further, the derivatives 3a-3y were evaluated *in vitro* to find out the molecular mechanism of antiproliferative effects and *in vivo* efficacy.

The final derivatives were synthesised in three steps as described earlier[13] and detailed synthetic procedures has been included in Supplementary Section 1.1. Molecular structures of 3k-3y were confirmed by using infrared (IR) spectroscopy, proton nuclear magnetic resonance (1H NMR) spectroscopy, carbon nuclear magnetic resonance



Scheme 1. Synthetic scheme for synthesizing 3a-3y. Reagents and conditions. (A)Toluene, piperidinium benzoate, reflux 5–6 hrs., (B) Chloroacetyl chloride, DCM, K_2CO_3, stir rt., (C) DMF, K_2CO_3- stir, rt. overnight.

(^{13}C NMR) and mass spectroscopy. The formation of Knoevenagel product was confirmed on the basis of proton NMR wherein the benzylidene proton exhibits a singlet signal in the range of at δ 7.8–7.9 ppm. Formation of chloroacetylated product was confirmed by the presence of singlet of $-CH_2$ proton resonating at δ 4.0–4.8 ppm. The IR spectra of the chloroacetylated aryl amines exhibited characteristic band in the range of 1680–1660 cm^{-1} corresponding to (C═O)−NH. The presence of resonance assigned to the $-CH_2$ protons provide evidence for formation of -CH_2-O-linkage in the final product, these protons resonated in 4.64–5.60 ppm region as singlet. ^{13}C NMR spectrum showed characteristic peaks of $-CH_2$-O in the range of 67 ppm and carbonyl peaks between 167 ppm for all the moieties. Spectral details have been included in Supplementary Section 3.

Toxicity to the untransformed hepatocytes was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Details of the experimental methods are presented in Supplementary Data. The TZDs, 3a-3y portrayed a differential inhibition; with MTT reduction ranging between 70 and 93% (Fig. 2, Supplementary Table S1). Since, all the compounds showed MTT reduction of above 65% against the vehicle control, we could significantly state that none of the compounds exerted any severe hepatotoxic effect. With this data, we moved ahead and screened the molecules for their antiproliferative potential.

The antiproliferative effects of 3a-3j have already been reported[13] and have been included for comparison purpose in Table 1 below, 3k-3y were evaluated in same panel of cell lines to find antiproliferative activity.

Interestingly, all the molecules displayed considerable activity in MCF7 (breast cancer), oral Cancer (GURAV) and chronic myeloid leukemia cell line (K562). GI_{50} values of molecules 3k-3y for MCF7 cell line were in the range of 28–74.5 μM and for GURAV GI_{50} were in the range of 35.6–75 μM. However, lower range of GI_{50} (0.23–24 μM) were found for K562 cell line, suggesting potential of these molecules in chronic myeloid leukemia (CML). Few molecules also showed activity in other cell lines. Molecule 3f from previous series was found to be active on PC3 and MCF7 cell lines with GI_{50} values 28.18 μM and 25.11 μM respectively. Addition of methyl group on para position of phenyl ring of compound 3f leads to 3k, which exhibited higher GI_{50} values on PC3 (> 100 μM) and MCF7 (61.2 μM) cell lines. However, 3k showed moderate GI_{50} on K562 (45.8 μM) and GURAV (58.7 μM) cell lines. Another interesting molecule is 3i from previous series. In the R group of 3i, we substituted methyl group which led to 3 l. Compound 3i had showed notable antiproliferative activity on HOP62 (GI_{50}-2.29 μM)), PC3 (GI_{50}-29.51 μM)) and MCF7 (GI_{50}-29.51 μM)) cell lines. However, methyl substituted derivative, 3 l exhibited higher GI_{50} values on HOP62 (GI_{50}-81.4 μM), PC3 (GI_{50}-58.3 μM)) and activity on MCF7 (GI_{50}-28.3 μM)) remained unchanged. It is also important to note that, 3l exhibited substantial effect on K562 (GI_{50}-10.7 μM)), GURAV (GI_{50}-60.9 μM)) and KB (GI_{50}-75 μM)) cell lines, whereas 3i had not shown activity on these three cell lines. Addition of methyl as R group of 3f and 3i increased the activity in some cell lines and decreased activity in others. Molecule 3t which has di-substituted fluorine showed

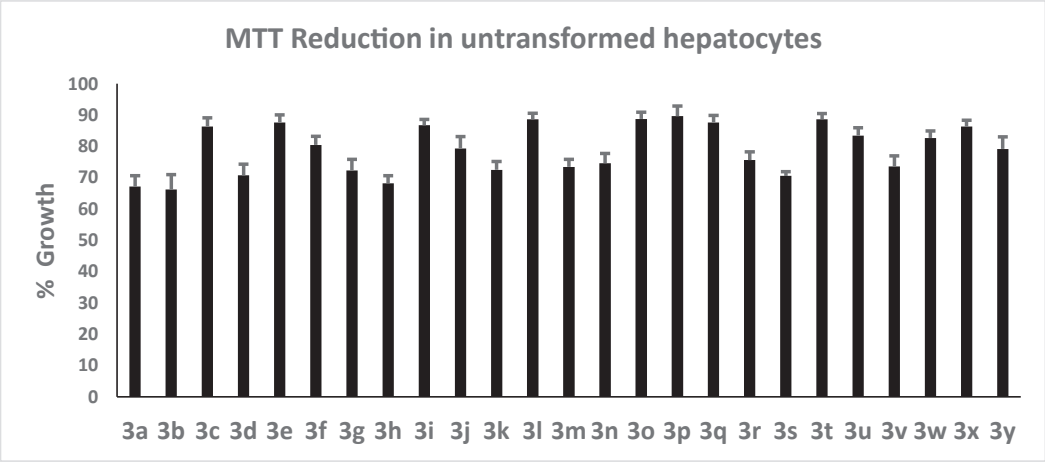


Fig. 2. Reduction of MTT by 3a-3y against control value of 100% in untransformed hepatocytes. Values are presented as Mean ± SD. The error bars represent the standard error in individual determinations.

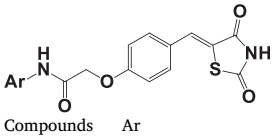
significant GI_{50} value on K562 cell line. Addition of chlorine at para position of R group of 3e leads to 3x, which was also found to be active on K562 cell line with significant GI_{50} value of 0.23 μM. When compared to Pioglitazone, almost 70% of the compounds exhibited better antiproliferative effects in K562 cells.

In second level of antiproliferative assays, the promising compounds from 3a to 3y were further treated with the other variants of lung cancer (A549, NCI-226), prostate (DU145), breast (MDA-MB-435 – triple negative breast cancer cell) and leukemic (Jurkat, Mot-4, HL60, U937) cell lines, GI_{50} values are presented in Table 2.

Although compounds 3a, 3c and 3f exhibited significant GI_{50} values in HOP62 cell line, they did not show greater or equal activities on other selected lung cancer cell lines (A549, NCI-H266). Rest of the compounds (3e, 3o, 3q, 3t, 3x, 3y) which were found to be active on K562 cell line, showed activity at higher concentrations on selected leukemic cell lines (Jurkat, MOLT-4, HL-60, U973). Molecule 3e was found to be active on prostate cancer (PC3) cell line with lower GI_{50} value and was further evaluated on DU145 cell line. 3e exhibited GI_{50} value at 59.2 μM on DU145 cell line. Since all the synthesized compounds found to exhibit discernible activity on K562, we continued our work with this cell line.

To find the effect on phase distribution of cell cycle in K562 cells,

Table 1
GI_{50} values of 3 k-3y on panel of 6 cancer cell lines.



Compounds	Ar	HOP62 GI_{50} μM	PC3 GI_{50} μM	MCF7 GI_{50} μM	K562 GI_{50} μM	GURAV GI_{50} μM	KB GI_{50} μM
3a	10*H*-Phenothiazin-10-yl	0.19	29.5	29.51	> 100	> 100	> 100
3b	1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl	0.186	> 100	> 100	> 100	> 100	> 100
3c	5-Methylthiazol-2-yl	0.169	2.2	> 100	2.23	0.194	> 100
3d	2-Butyl-4-oxo-1,3-diazaspiro[4,4]non-1-en-3-yl	29.51	> 100	28.18	> 100	> 100	> 100
3e	3-(Trifluromethyl) phenyl	> 100	2.51	0.194	0.186	0.186	2.2
3f	Benzo[*d*]thiazol-2-yl	0.99	28.18	25.11	> 100	> 100	> 100
3g	5-Nitrothiazol-2-yl	> 100	> 100	> 100	> 100	> 100	> 100
3h	1,2-Benzothiazol-3(2H)-one-1,1-dioxide	> 100	> 100	37.15	2.69	31.62	> 100
3i	Pyridine-2-yl	2.29	29.51	29.51	> 100	> 100	> 100
3j	5-Methylisoxazol-3-yl	> 100	> 100	39.81	2.75	38.9	> 100
3k	4-Methyl-benzothiazol-2-yl	> 100	> 100	61.2	45.8	58.7	> 100
3l	6-Methyl-pyridin-2-yl	81.4	58.3	28.3	10.7	60.9	75
3m	4-Nitro-phenyl	> 100	> 100	72.5	29.5	> 100	> 100
3n	3-Nitro-phenyl	> 100	> 100	58.5	20.5	71.5	> 100
3o	4-Fluoro-phenyl	68	> 100	54.6	9.6	56.8	> 100
3p	Phenyl	> 100	> 100	74.5	17.6	57	> 100
3q	4-Chlorophenyl	36.1	83.4	51.1	7.4	35.6	50.3
3r	4-Bromo-phenyl	45.4	86.6	55.1	23.4	54.4	32.1
3s	4-Bromo-2-methylphenyl	> 100	> 100	66.8	20.7	69.7	> 100
3t	2,4-Difluorophenyl	> 100	> 100	65.8	0.9	61.4	> 100
3u	3-Chloro-4-methylphenyl	> 100	> 100	61.8	19.6	71.4	99.6
3v	4-Bromo-2,6-diflurophenyl	75.2	> 100	57.3	23.1	64.1	> 100
3w	2-Chloro-4-trifluromethylphenyl	> 100	> 100	67.4	16.3	99.3	78.6
3x	4-Chloro-2-trifluromethylphenyl	> 100	> 100	49.7	0.23	86.8	> 100
3y	2,6-Dichlorophenyl	> 100	> 100	61.7	6.7	56.8	> 100
Doxorubicin	–	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Pioglitazone	–	NE	NE	NE	40.07	NE	NE

GI_{50} values are presented as mean of three independent experiments. HOP62- Lung cancer, PC3-Prostate cancer, MCF7- Breast cancer, K562- acute myeloid leukemia, Gurav- Oral cancer, KB-Oral cancer cell lines. Values are presented as mean of 3 independent experiments.*NE-not evaluated.

Table 2
GI_{50} values of selected compounds on variants of lung, prostate and leukemic cells.

Compounds	GI_{50} values in μM							
	A549	NCI-H226	Jurkat	Molt-4	HL60	U937	DU145	MDA-MB 435
3a	> 100	82.1	NE	NE	NE	NE	NE	NE
3c	88.7	57.8	NE	NE	NE	NE	NE	NE
3f	46.9	26.5	NE	NE	NE	NE	NE	NE
3e	NE	NE	26.5	47.3	73.3	49.1	59.2	> 100
3o	NE	NE	59.9	53.5	61.1	63.9	NE	NE
3q	NE	NE	> 100	42.7	52.4	46.3	NE	NE
3t	NE	NE	34.6	40.3	47.8	35.6	NE	NE
3x	NE	NE	28.9	32.5	56.7	67.5	NE	NE
3y	NE	NE	21.9	> 100	97.6	60	NE	NE

*NE- not evaluated. HOP62, A549, NCI-H266-non-small cell lung carcinoma cell line, Jurkat, Molt-4, HL60, U973- leukemic cell line, DU145- Prostrate cancer cell line, MDA-MB-435 – breast triple negative cell line. Values are presented as mean of 3 independent experiments.

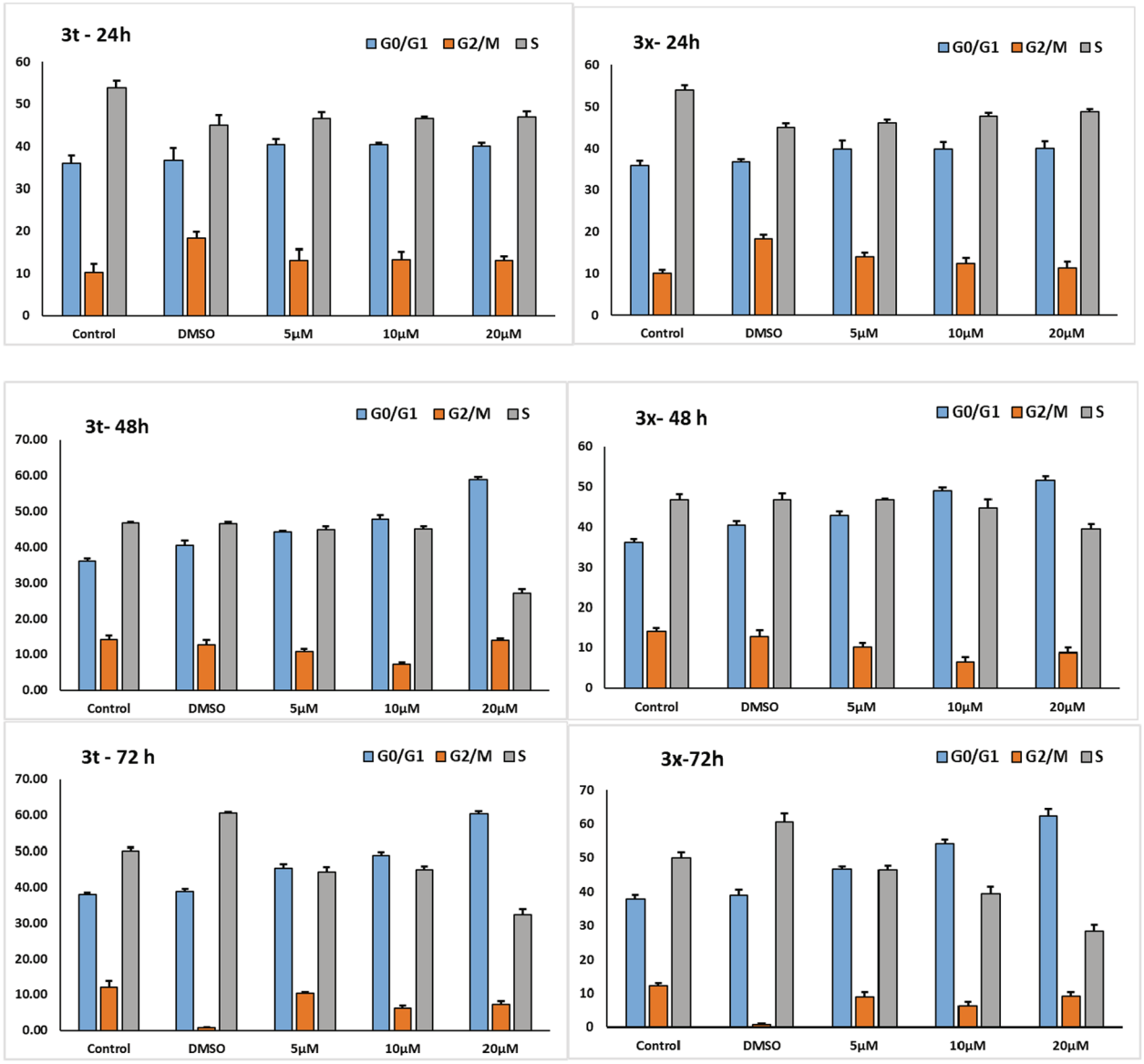


Fig. 3. Effect of treatment of 3t and 3x on cell cycle of K562 cells. The cells were treated with 3 concentrations 5 μM, 10 μM and 20 μM and the analysis was done a three time points of 24 h, 48 h and 72 h. The untreated cells serve as negative control and DMSO treated cells as vehicle control. The error bars represent standard errors of mean (SEM). The statistical analysis was performed with two tail students '*t*' test, n = 3, P < 0.05.

flowcytometric analysis of compounds 3x and 3t was carried out. Cells were incubated with three concentrations of test compounds Viz., 5 μM, 10 μM and 20 μM and samples were withdrawn at 24-, 48- and 72-hours time points. Details of experimental methodology and statistical analysis have been included in Supplementary section (Supplementary Tables S2–S7). At lower dose of 5 μM and 24 h of treatment with 3t and 3x, the K562 cells started to accumulate in G0/G1, S, and G2/M phases. As the treatment time increased to 48 and 72 h and treatment concentrations increased to 10 μM and 20 μM, the cell distribution was significantly altered in all the phases compared to the control cells. Thus, as shown in Fig. 3, after exposure of K562 cells 3t and 3x, there was dose dependent and time dependent effects on distribution of cells in various phases. The number of cells in G0/G1 phase was found to increase significantly in comparison with control cells. Also, a subsequent decrease in the number of cells in the S and G2/M phases relative to the control cells were observed. Thus, compounds 3t and 3x causes the growth arrest of K562 cells in G0/G1 phase in dose and time dependent manner. The G1 phase is considered a cell differentiation phase. The arrest of the G1 phase for the cell cycle can be used as indicator of induced differentiation.[16,17] Effect of thiazolidinedione treatment on distribution of cells in various phases of cell cycle have been studied and reported previously. The glitazones like Pioglitazone, Ciglitazone and Troglitazone were found to arrest the growth of cells in G1/G0 phase in cancer cell lines from colon,[18] ovarian,[19] melanoma[20] and adrenocortical[21] cancers. Thus, our newly synthesized TZD analogs, 3t and 3x leads to cell cycle arrest in G0/G1 phase, which is consistent with the reported results.

Based on cell cycle analysis and antiproliferative data we decided to analyze the protein levels of proliferative cell nuclear antigen (PCNA) and cyclin D1. The co-relation of these proteins and PPARγ in hematological cancer is well explained.[22–24] Moreover, many studies substantiate that PPARγ ligands inhibits the expression of these proteins by PPARγ dependent or independent mechanisms. PCNA an auxiliary factor of DNA polymerase, is not only essential for eukaryotic replication but also plays pivotal role in several DNA damage responsive pathways. PCNA is a well-known marker for cell proliferation. Glitazones in the promotion of G1 cell-cycle arrest has been correlated to the downregulation of cyclin D1 in several cell lines.[25] Cyclin D1 is involved in regulation of G1 to S phase transition in the cell cycle. Overexpression of cyclin D1 in normal non cancerous cells leads to malignant phenotype whereas downregulation of cyclin D1 in malignant cells results in loss of the malignant phenotype.[26] Treatment of 10 μM and 20 μM concentrations of compound 3t inhibited cyclin D1 at 48 and 72 h, however it did not inhibit PCNA at 48 hrs. Effect of 3t on PCNA was observed at 20 μM at 72 h. Whereas, compound 3x inhibited PCNA with 48 h and at 72 h treatments at both concentrations, 10 and 20 μM. 3x significantly inhibited cyclin D1 at 10 μM & 20 μM. Both the molecules did not show any effect at 24 h (Fig. 4).

Accumulation of subG1 phase cells at higher doses suggests that the compounds could be inducing apoptotic death of the cells. To determine this, we studied the expression of apoptosis markers PARP1[27] and caspase 3[28,29] in the K562 cells treated with 3x and results were compared with Vehicle (DMSO) control cells. Compound 3x was treated at three concentrations of 5 μM, 10 μM and 20 μM, keeping DMSO control for 72 hrs. Fig. 5 elucidates the action of 3x on apoptotic markers. There was an increase in the expression of cleaved PARP1 and activated caspase 3 with increasing concentration of 3x, confirming the contribution of apoptotic death of cells to the antiproliferative effects exerted.

Recently in phase II trial, the addition of thiazolidinedione (Pioglitazone) to patients taking Imatinib leading to complete molecular remission has been reported. Out of 24 assessable patients, the cumulative incidence rate in the treated group reached 57% versus 27% ($P = 0.02$) for a historical group of patients having received Imatinib alone, thus indicating that clinical evidence of efficacy can be detected even after very brief treatment and early analysis.[30] Also, the addition

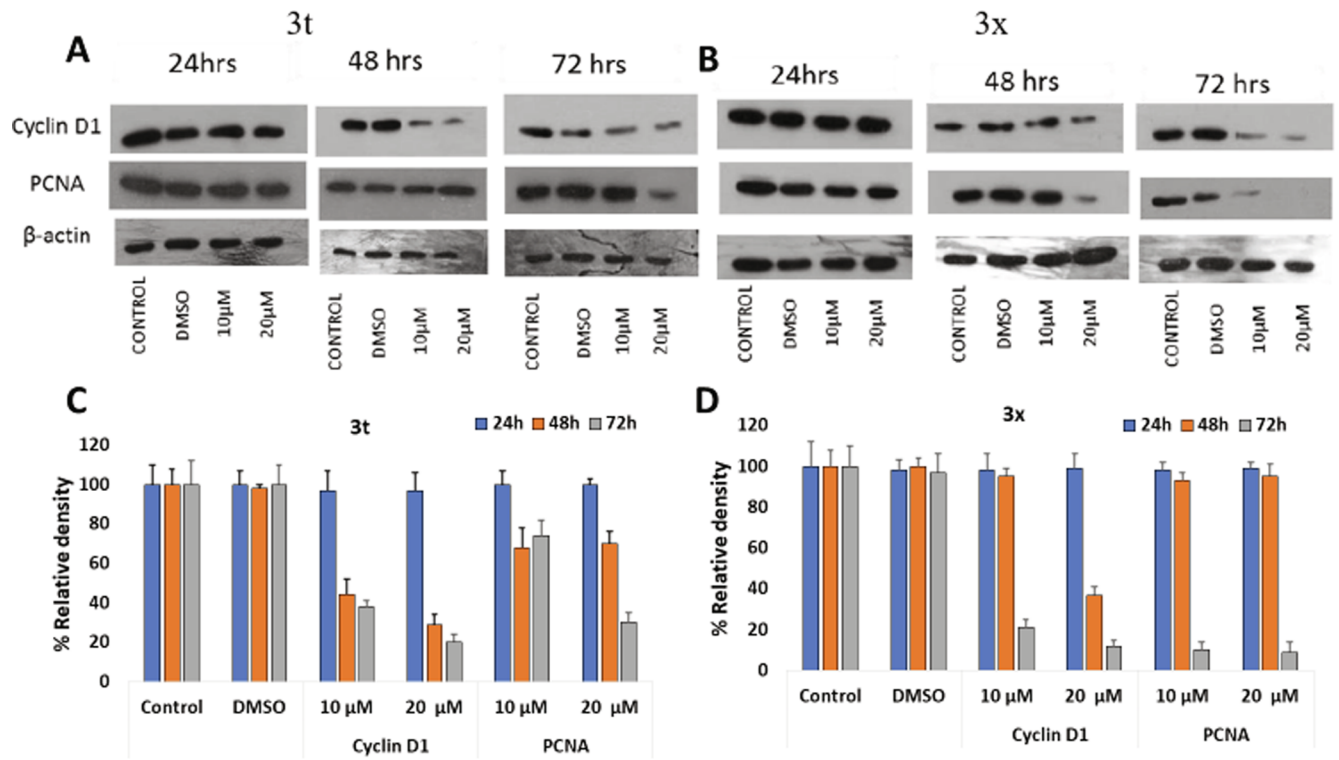


Fig. 4. Western blot analysis of markers associated with cell proliferation (PCNA and Cyclin D1), in 3t and 3x-treated cells and controls. (A) K562 cells were treated with 3t at concentrations of 10 μM and 20 μM for 24 h, 48 h and 72 h and subjected to Western blotting. (B) cells were treated with 3x at the concentrations of 10 μM and 20 μM for 24 h, 48 h and 72 h and subjected to Western blotting. (C) Cyclin D1 and PCNA bands were quantified and normalized to actin bands. Data are shown as means of three separate experiments, $P < 0.05$; bars, SD.

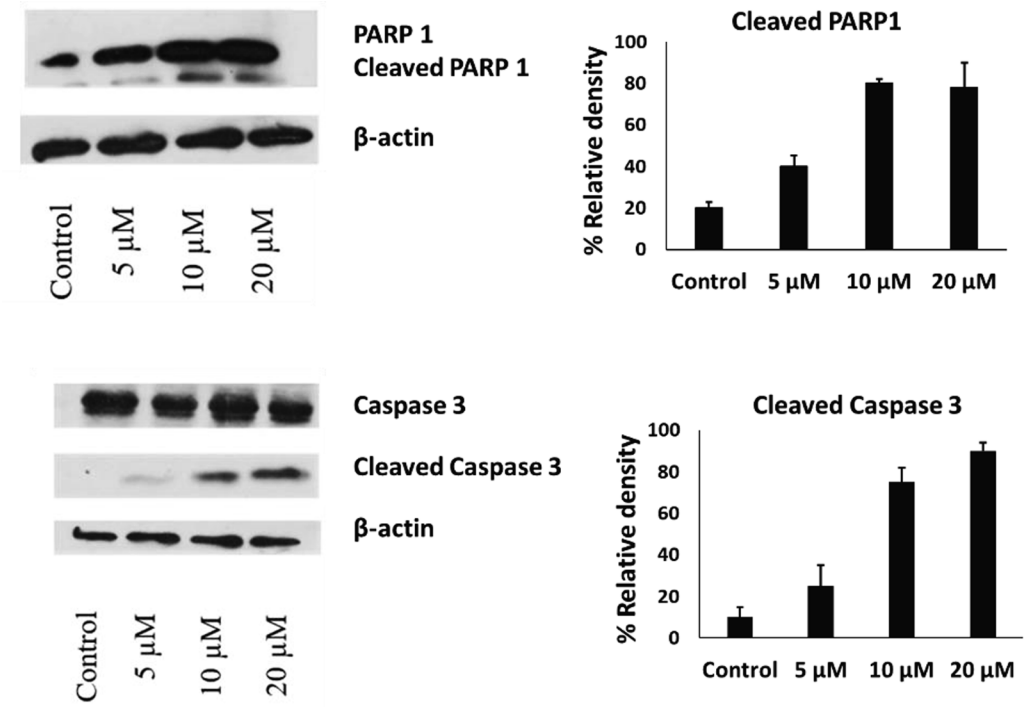


Fig. 5. Western blot analysis of 3x to determine the expression of apoptosis marker PARP1 and caspase 3. K562 cells were treated with 3x at concentrations of 5 μM, 10 μM and 20 μM at 72 h of cell treatment and subjected to western blotting to find the expression of apoptotic markers, PARP1 and caspase 3 which were quantified and normalized to actin bands. Data are shown as the means of at least two separate experiments $P < 0.05$; bars, SD.

of various TZDs to Imatinib, Nilotinib and Dasatinib has been found to increase the activity of these tyrosine kinase inhibitors as evident by latest reports.[31,32] Thus, it was of an interest to find if our compounds which are modified Pioglitazone derivatives, also potentiates the anti-tumor effects of Imatinib when used in combination with it, hence *in vitro* and *in vivo* efficacy was determined in K562 cell line and xenograft model respectively. The *in vitro* combination study was performed by treating K562 cells with Imatinib (1 μM), 3t (1 μM) and 3x (1 μM) alone and the combination of Imatinib (1 μM) + 3t (1 μM) and Imatinib (1 μM) + 3x (1 μM) by MTT assay as described above. The treatment of K562 cells with Imatinib, 3t and 3x led to 71%, 62% and 65% inhibition of proliferation, respectively. However, 3t and 3x in combination with Imatinib led to 79% and 82% inhibition respectively. Thus, it was observed that these compounds hold the capacity to potentiate the anti-tumor effects of Imatinib.

With hopeful *in vitro* effects of combination were observed, we proceeded further to determine *in vivo* efficacy of compounds 3t and 3x alone and in combination with Imatinib. First the acute toxicity and maximum tolerated dose was determined in healthy mice after oral administration of increasing doses of 3t and 3x. All the animals were observed for weight loss, behavioral changes and mortality. None of the mice in any group showed decrease in weight or mortality. Dose was gradually increased over the period if previous dose was found to be well tolerated. Both the molecules were found to be well tolerated upto 2000 mg/kg. MTD results are summarized in Supplementary section, Table S8. Further to assess the *in vivo* efficacy, tumors were induced by subcutaneously injecting 6×10^6 K562 cells/100 μl/mouse mixed with 50% matrigel into the right rear flank of the mouse. All animal work was approved by Institutional Animal Ethics Committee (IAEC/PR/2014-2015/03). Treatment was initiated when the tumors were around 300 mm^3 in size. Body weight and tumor size were measured every other day until day 30 after treatment. Tumor size or volume was calculated as $V = (L \times W2) \times 0.52$, where L is the length and W is the width of the xenograft. The Dunnet's test was used to determine the significant reduction in tumor volume as compared to control. Tumor growth data are expressed as mean tumor volumes ± SEM. For all data, differences were considered significant at $P \leq 0.05$. Encouraging results were obtained when 3t and 3x were combined with Imatinib. The combination of treatment results in significant ($p < 0.01$) decrease in the tumor volume at 30 days. Single drug (without Imatinib) oral dose administration of 3t and 3x also moderately inhibited the tumor volume ($p < 0.05$) (Fig. 6, Supplementary Table S9). Our results suggest that 3t and 3x could be used along with Imatinib to overcome resistance and enhance the activity in chronic myeloid leukemia. However, clinical studies are required to validate the results.

Pre-clinical pharmacokinetic characteristics can help predict drug behavior. The rapid HPLC method was developed and validated by using rat plasma to study the pharmacokinetic behavior of 3t and 3x and has been reported elsewhere.[33] Both the compounds found to exhibit comparable Pharmacokinetic behavior to the clinical TZD containing glitazones, Pioglitazone.

In conclusion, a series of benzylidene thiazolidine-2,4-diones were synthesized. All the derivatives were screened to determine their *in vitro* cytotoxic activity against panel of tumor cell lines consisting of HOP62, K562, GURAV, KB2, Hep G2, MCF7, PC3 and their variants. The compounds 3c, 3e, 3o, 3q, 3t, 3x and 3y exhibited promising

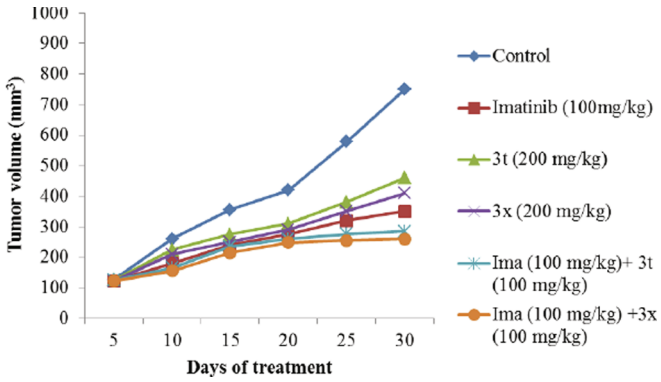


Fig. 6. Graph of tumor volume vs. time in K562 xenografts treated intra peritoneally with 3t and 3x, alone at 200 mg/kg i.p. (up triangle and cross respectively), 3t and 3x in combination with Imatinib, 100 mg/kg + 100 mg kg Imatinib (asterisk and circle respectively), Imatinib 100 mg/kg (square), saline (diamond), The Dunnet's test was used to determine the significant reduction in tumor volume as compared to control, $n = 6$. $p < 0.05$.

antiproliferative effects. Several molecules exhibited better antiproliferative profile in K62 cells as compared to non-benzylidene TZD derivative, Pioglitazone proving that the benzylidene derivatives are more effective anticancer agents. The most potent antiproliferative compounds were 3t and 3x against K562 cells with GI_{50} value of 0.9 μM and 0.23 μM respectively. Both the derivatives, further in flow cytometric analysis, were found to exhibit growth arrest of K562 cells in G0/G1 phase in time and dose dependent manner. The western blot analysis revealed that 3t and 3x reduces the expression of cell proliferation markers, PCNA and Cyclin D1. Additionally, antiapoptotic effects were proved with the modulation of apoptosis marker proteins PARP1 and caspase 3. Thus, excellent antiproliferative effects of 3t and 3x can be correlated with the dose dependent inhibition of expression of these cell proliferation and apoptosis markers. Further the *in vitro* cell line study and *in vivo* efficacy study in K562 xenografts in combination with Imatinib was found to be promising, in line with the reported effects of combination of TZD's and tyrosine kinase inhibitors. The compounds 3t and 3x also possesses the desirable pharmacokinetic profile making them drug like molecules. Thus, our findings indicate that these compounds are promising antitumor agents and could help to enhance the anticancer effects of Imatinib and other tyrosine kinase inhibitors when used in combination.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

1. Andreeff M, Goodrich DW, Pardee AB. Proliferation. Holland-Frei Cancer Medicine 6th edition. Published online 2003. Accessed July 9, 2020. https://www.ncbi.nlm.nih.gov/books/NBK13035/.
2. Sherman MH, Downes M, Evans RM. Nuclear receptors as modulators of the tumor microenvironment. *Cancer Preven Res.* 2012;5(1):3–10.
3. Karak M, Bal N, Bal C, Sharon A. Targeting peroxisome proliferator-activated receptor gamma for generation of antidiabetic drug. *CDR.* 2013;9(4):275–285. https://doi.org/10.2174/1573399811309990065.
4. Grommes C, Landreth GE, Heneka MT. Antineoplastic effects of peroxisome proliferatoractivated receptor γ agonists. *Lancet Oncol.* 2004;5(7):419–429.
5. Feinstein DL, Spagnolo A, Akar C, et al. Receptor-independent actions of PPAR thiazolidinedione agonists: Is mitochondrial function the key? *Biochem Pharmacol.* 2005;70(2):177–188.
6. Joshi H, Pal T, Ramaa CS. A new dawn for the use of thiazolidinediones in cancer therapy. *Expert Opin Invest Drugs.* 2014;23(4):501–510.
7. Weng J-R, Chen C-Y, Pinzone JJ, Ringel MD, Chen C-S. Beyond peroxisome proliferator-activated receptor γ signaling: the multi-facets of the antitumor effect of thiazolidinediones. Endocrine-Related Cancer. Published online June 2006:401-413. doi:10.1677/erc.1.01182.
8. Shiau C-W, Yang C-C, Kulp SK, et al. Thiazolidenediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARγ. *Cancer Res.* 2005;65(4):1561–1569.
9. Asati V, Mahapatra DK, Bharti SK. Thiazolidine-2,4-diones as multi-targeted scaffold in medicinal chemistry: potential anticancer agents. *Eur J Med Chem.* 2014;87:814–833.
10. Grillier Vuissoz I, Mazerbourg S. PPARγ-independent Activity of Thiazolidinediones: A Promising Mechanism of Action for New Anticancer Drugs? J Carcinog Mutagen. 2012;01(S8). doi:10.4172/2157-2518.S8-002.
11. Ma L, Pei H, Lei L, He L, Chen J, Liang X, et al. Structural exploration, synthesis and pharmacological evaluation of novel 5-benzylidenethiazolidine-2,4-dione derivatives as iNOS inhibitors against inflammatory diseases. *Eur J Med Chem.* 2015;92:178–190.
12. Ma L, Xie C, Ma Y, Liu J, Xiang M, Ye X, Zheng H, Chen Z, Xu Q, Chen T, Chen J, Yang J, Qiu N, Wang G, Liang X, et al. Synthesis and biological evaluation of novel 5-benzylidenethiazolidine-2,4-dione derivatives for the treatment of inflammatory diseases. *J Med Chem.* 2011;54(7):2060–2068.
13. Patil V, Tilekar K, Mehendale-Munj S, Mohan R, Ramaa CS. Synthesis and primary cytotoxicity evaluation of new 5-benzylidene-2,4-thiazolidinedione derivatives. *Eur J Med Chem.* 2010;45(10):4539–4544.
14. Crunkhorn S. Bypassing the side effects of thiazolidinediones? *Nat Rev Drug Discov.* 2007;6(7):517.
15. Isley WL. Hepatotoxicity of thiazolidinediones. *Exp Opin Drug Saf.* 2003;2(6):581–586.
16. Rizzardi LF, Cook JG. Flipping the switch from G1 to S phase with E3 ubiquitin ligases. *Genes & Cancer.* 2012;3(11-12):634–648.
17. Song E, Ma X, Li H, et al. Attenuation of Krüppel-Like Factor 4 Facilitates Carcinogenesis by Inducing G1/S Phase Arrest in Clear Cell Renal Cell Carcinoma. Müller R, ed. PLoS ONE. 2013;8(7):e67758. doi:10.1371/journal.pone.0067758.
18. Yoon J-K, Byeon HE, Ko SA, Park B-N, et al. Cell cycle synchronisation using thiazolidinediones affects cellular glucose metabolism and enhances the therapeutic effect of 2-deoxyglucose in colon cancer. *Sci Rep.* 2020;10(1) https://doi.org/10.1038/s41598-020-61661-4.
19. Al-Alem L, Southard RC, Kilgore MW, Curry TE. Specific Thiazolidinediones Inhibit Ovarian Cancer Cell Line Proliferation and Cause Cell Cycle Arrest in a PPARγ Independent Manner. Agoulnik I, ed. PLoS ONE. 2011;6(1):e16179. doi:10.1371/journal.pone.0016179.
20. Botton T, Puissant A, Bahadoran P, Annicotte J-S, Fajas L, Ortonne J-P, Gozzerino G, Zamoum T, et al. In Vitro and in vivo anti-melanoma effects of ciglitazone. *J Invest Dermatol.* 2009;129(5):1208–1218.
21. Ferruzzi P, Ceni E, Tarocchi M, Grappone C, Milani S, et al. Thiazolidinediones inhibit growth and invasiveness of the human adrenocortical cancer cell Line H295R. *J Clin Endocrinol Metabol.* 2005;90(3):1332–1339.
22. Body S, Esteve-Arenys A, Miloudi H, Recasens-Zorzo C, Tchakarska G, Moros A, Bustany S, Vidal-Crespo A, Rodriguez V, Lavigne R, Com E, Casanova I, Mangues R, Weigert O, Sanjuan-Pla A, Menéndez P, Marcq B, Picquenot J-M, et al. Cytoplasmic cyclin D1 controls the migration and invasiveness of mantle lymphoma cells. *Sci Rep.* 2017;7(1) https://doi.org/10.1038/s41598-017-14222-1.
23. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer.* 2007;6(1):24. https://doi.org/10.1186/1476-4598-6-24.
24. Smetana K, Gyorkey F, Chan P-K, Tan E, Busch H. Proliferating cell nuclear antigen (PCNA) and human malignant tumor nucleolar antigens (HMTNA) in nucleoli of human hematological malignancies. *Blut.* 1983;46(3):133–141.
25. C. Blanquicett J. Roman C.M. Hart Thiazolidinediones as anti-cancer agents Cancer Ther. 2008;6(A):25–34.
26. Prabhakar MM, Vasudevan K, Karthikeyan S, Baskaran N, Silvan S, Manoharan S. Anti-cell proliferative efficacy of ferulic acid against 7, 12-dimethylbenz(a) Anthracene induced hamster buccal pouch carcinogenesis. *Asian Pac J Cancer Prev.* 2012;13(10):5207–5211.
27. Qvarnström. γH2AX and cleaved PARP-1 as apoptotic markers in irradiated breast cancer BT474 cellular spheroids. *Int J Oncol.* 2009;35(01).
28. Kumar S. Mechanisms mediating caspase activation in cell death. *Cell Death Differ.* 1999;6(11):1060–1066.
29. Fischer U, Jänicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.* 2003;10(1):76.
30. Prost S, Relouzat F, Spentchian M, Ouzegdouh Y, Saliba J, Massonnet G, Beressi J-P, Verhoeyen E, Raggueneau V, Maneglier B, Castaigne S, et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature.* 2015;525(7569):380–383.
31. Prost S, Kirszenbaum M, Dantec ML, Rousselot P, LeBoulch P. Combination of an anti-cancer agent such as a tyrosinekinase inhibitor and a STAT5 antagonist, preferably a thiazolidinedione, for eliminating hematologic cancer stem cells in vivo and for preventing hematologic cancer relapse. Published online April 18, 2017. Accessed July 11, 2020. https://patents.google.com/patent/US9623015B2/en.
32. Glodkowska-Mrowka E, Manda-Handzlik A, Stelmaszczyk-Emmel A, et al. PPARγ ligands increase antileukemic activity of second- and third-generation tyrosine kinase inhibitors in chronic myeloid leukemia cells. *Blood Cancer J.* 2016;6(1):e377.
33. Joshi H. Simple and rapid HPLC method determination of CSR1 and CSR2, new Heterocyclic thiazolidinedione derivatives, in rat plasma. *Int J Pharm Pharm Sci.* 2016;8(8):273–276.