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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 2547–2550

Structure–activity requirements for the antiproliferative effect of troglitazone derivatives mediated by depletion of intracellular calcium

Yun-Hua Fan,[a] Han Chen,[a] Amarnath Natarajan,[a] Yuhong Guo,[a] Fred Harbinski,[a] Julia Iyasere,[a] William Christ,[a] Huseyin Aktas[a] and Jose A. Halperin[a,b,*]

[a]*Laboratory for Translational Research, Harvard Medical School, Cambridge, MA 02139, USA*
[b]*Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA*

Received 7 January 2004; revised 16 February 2004; accepted 26 February 2004

Abstract—Depletion of calcium from the endoplasmic reticulum has shown to affect protein synthesis and cell proliferation. The anticancer effect of troglitazone was reported to be mediated by depletion of intracellular calcium stores resulting in inhibition of translation initiation. The unsaturated form of troglitazone displays similar anticancer properties in vitro. In this letter, we report our findings on the minimum structural requirements for both compounds to retain their calcium release and antiproliferative activities.

1. Introduction

Calcium ions play a critical role in key biological processes.[1–3] The major intracellular calcium store is the endoplasmic reticulum (ER). Depletion of ER Ca^{2+} stores promote ER stress[4–6] and that leads to the phosphorylation of eukaryotic initiation factor 2 (eIF2) and thereby to inhibition of translation initiation.[7–11] Translation initiation plays an important role in regulation of cell proliferation and malignant transformation and is therefore an attractive target for the development of mechanism-specific anticancer drugs.

We have previously demonstrated that eicosapentaenoic acid (EPA),[12] thiazolidinediones (TZD),[13] and clotrimazole (CLT),[14] exhibit anticancer properties mediated by inhibition of translation initiation. These compounds cause partial and sustained depletion of intracellular Ca^{2+} stores because they simultaneously release Ca^{2+} from internal stores and block store-operated-calcium channels (SOC) that open to refill the internal Ca^{2+} stores. This partial depletion of intracellular Ca^{2+} stores causes activation of eIF2 kinases (PKR or/and PERK) leading to the phosphorylation of the α subunit of eIF2 (eIF2α) and inhibition of translation initiation. Both CLT and TZD inhibit growth of cancer cells in vitro at micromolar concentrations, and also tumor growth in experimental cancer models.

Troglitazone (TRO), an agonist ligand for the nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ), improves insulin sensitivity, induces terminal differentiation of normal pre-adipocytes and human liposarcoma cells in vitro[15] and also exhibits anticancer activity in other cancer cell types.[13,16] We have previously shown that the anticancer activity of TRO is independent of PPAR-γ and mediated by Ca^{2+} release mediated inhibition of translation initiation.[13,17] Recently, we have observed that the unsaturated form of TRO (**1b**), and ciglitazone release Ca^{2+} from intracellular stores and inhibit cell growth in the lung cancer cell line, A549, in a manner similar to TRO (**1a**), while rosiglitazone, a more potent member of the TZD family, does not release Ca^{2+} nor inhibit cell proliferation (Fig. 1). Ciglitazone and rosiglitazone differ from TRO only in the substitution on the 4′-hydroxy. We have, therefore, investigated the minimum requirement on the 4′-hydroxy substitution of the TZD type compounds needed to maintain their translation initiation inhibitory activity (Fig. 2).

Keywords: Troglitazone; Intracellular calcium; Thiazolidinediones.
* Corresponding author. Tel.: +1-6176216104; fax: +1-6176216148; e-mail: yunhuafan@yahoo.com

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doi:10.1016/j.bmcl.2004.02.087

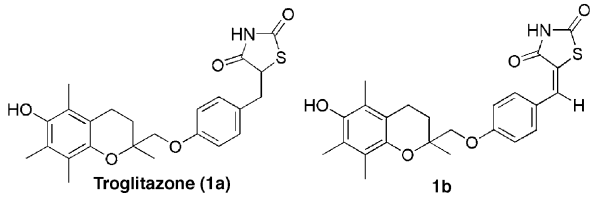


Figure 1.

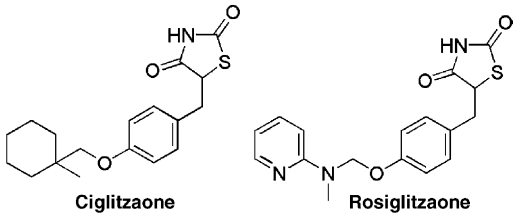
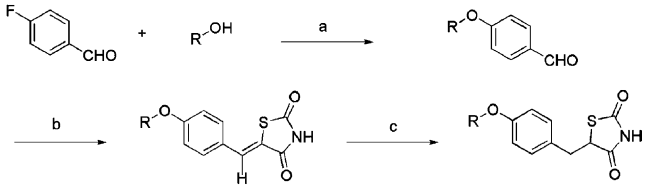


Figure 2.

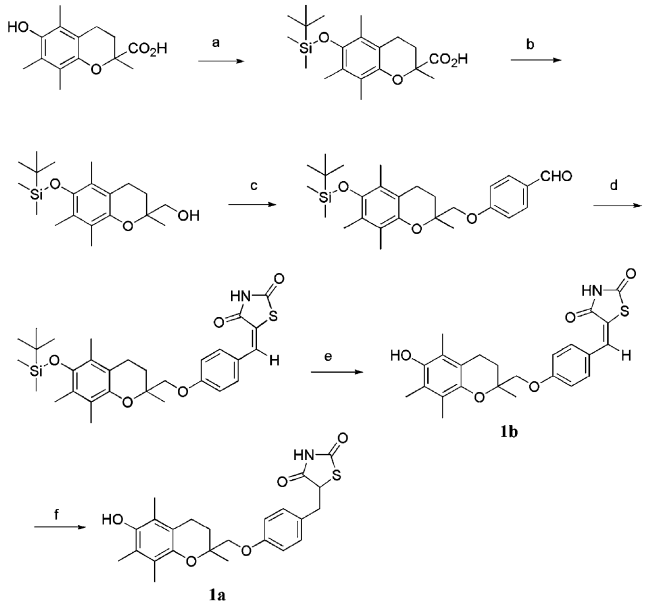
In the past we have observed a good correlation among intracellular Ca^{2+} release, phosphorylation of eIF2α, and inhibition of cell proliferation.[12–14] Therefore, in this communication we decided to use these three bioassays to measure the activities of our compounds and to determine their mechanisms of action.

Compounds **2**–**13**[18] were all synthesized in a similar fashion as outlined in Scheme 1. The reaction conditions were not optimized for the individual compounds. Different linear and cyclic alkanols were O-alkylated with 4-fluorobenzaldehyde using potassium *tert*-butoxide as base in DMSO at 80 °C for 8 h. The desired crude O-alkylated aldehydes underwent Knoevenagel condensation with 2,4-thiazolidinedione in refluxing toluene for 3–4 h. The 5-benzylidene thiazolidinedione derivatives (**b**) were purified by recrystallization from acetonitrile. The 5-benzylidene thiazolidinediones was then reduced with $CoCl_2$ and $NaBH_4$ to yield the 5-benzyl derivatives (**a**). The final products (**a**) were purified by silica gel flash chromatography. Compounds **1a** and **1b** were synthesized in six steps with moderate yield as shown in Scheme 2.[19,20] All the compounds were dried under vacuum overnight before use. All the compounds had greater than 95% purity as determined by ^1H NMR and LC–MS.

Mechanism related biological activities were measured by testing all compounds for intracellular Ca^{2+} release and phosphorylation of eIF2α at 80 and 15 μM, respectively. Different concentrations were used to compensate for the different durations of the assays. In the Ca^{2+} release assay cells were treated for 10 min. In the phosphorylation assay, cells were treated with drugs for 2 h. Inhibition of cell growth was determined at concentrations ranging from 0.5 to 100 μM. In the cell growth assay, the cells were treated with drug for 5 days. The intracellular Ca^{2+} release was assayed using fluo-4 loaded cells,[21] and phosphorylation of eIF2α was determined by western blotting extracts of treated cells with anti-phospho-serine 51 eIF2α-specific antibodies.[14] Inhibition of cell proliferation was measured using the sulforhodamine B (SRB) assay.[22] The results are summarized in Table 1.



Scheme 1. (a) K*t*OBu, DMF, 80 °C; (b) 2,4-thiazolidinedione, AcOH, piperidine, toluene, reflux; (c) $CoCl_2$, DMG.



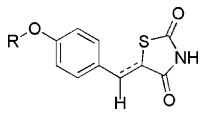
Scheme 2. (a) *t*-Butyldimethylsilyl chloride, imidazole, DMF; (b) LAH, rt, 3 h (75.9%, two steps); (c) 4-fluorobenzaldehyde, K*t*OBu, DMF, 80 °C, 8 h; (d) 2,4-thiazolidinedione, AcOH, piperidine, toluene, reflux, 4 h (37%, two steps); (e) HCl, MeOH, 15 min; (f) $CoCl_2$, DMG (84%).

2. Results and discussion

The parent compounds (**1a** and **1b**) are the most potent compounds in the series in terms of their Ca^{2+} release and antiproliferative activities. The unsaturated thiazolidinediones are generally more potent than their saturated counterparts in the cell proliferative assay. Interestingly, Reddy et al.[23] have observed a similar trend in the euglycemic and hypolipidemic activities for some of the unsaturated thiazolidinediones. The reason for the enhanced potency of the unsaturated thiazolidinediones is still not clear. However, this result suggests that the exocyclic double bond may improve the antiproliferative activities of these compounds, although it is not required.

Some of the structurally simpler analogs in this series still caused the release of Ca^{2+}, phosphorylation of eIF2α, and inhibition of cell growth. Compounds **12a**,**b** and **13a**,**b** have completely lost the ability to induce the release of Ca^{2+} from intracellular stores suggesting that the substitution on the 4′-hydroxyl group is essential for

Table 1. Lipophilicity and structure–activity relationships of the troglitazone-derived series (TRO)



Compounds[a]	R	*c* Log *P*[b]	Ca^{2+}[c]	eIF2-P[d]	IC_{50} (μM)[e]	Yield (%)[f]	Mp (°C)[g]
1a (Tro)		4.46	+	+	15 ± 2.1	84	179.6–182.1
1b		5.31	+	+	5 ± 1.2	24	205.5–208.2
2a	Tetrahydropyranylmethyl	2.29	–	–	>100	50	125.4–127.3
2b	Tetrahydropyranylmethyl	2.02	+	+	22 ± 2.1	49	184.6–187.4
3a	Cyclohexylmethyl	3.94	+	±	26 ± 1.9	52	122.2–124.1
3b	Cyclohexylmethyl	3.66	+	+	14 ± 1.7	41	190.4–192.8
4a	Cyclopentylmethyl	3.38	+	+	25 ± 3.6	45	101.7–102.9
4b	Cyclopentylmethyl	3.21	+	+	10 ± 1.0	33	155.3–158.1
5a	Cyclobutylmethyl	2.81	+	–	>100	46	98.6–101.2
5b	Cyclobutylmethyl	2.54	+	+	13 ± 1.6	39	177.7–179.4
6a	Cyclopropylmethyl	2.26	–	–	>100	48	86.9–89.2
6b	Cyclopropylmethyl	1.99	–	±	23 ± 4.5	32	189.7–191.3
7a	*tert*-Amyl	3.76	+	+	23 ± 1.6	54	160.6–161.9
7b	*tert*-Amyl	3.49	+	+	15 ± 0.9	30	182.6–184.1
8a	*iso*-Butyl	3.36	+	–	25 ± 3.3	46	87.7–89.5
8b	*iso*-Butyl	3.09	–	±	16 ± 2.8	38	142.7–145.2
9a	*n*-Propyl	2.96	–	±	45 ± 3.4	44	81.4–83.4
9b	*n*-Propyl	2.69	±	±	7 ± 0.9	32	186.3–188.4
10a	*n*-Ethyl	2.43	–	±	>100	50	109.5–110.9
10b	*n*-Ethyl	2.16	–	±	27 ± 2.6	51	145.4–148.0
11a	Methyl	1.91	–	–	>100	35	111.5–113.6
11b	Methyl	1.63	–	–	23 ± 1.2	62	216.5–217.9
12a	H	1.32	–	±	>100	43	140.7–144.0
12b	H	1.05	–	–	20 ± 1.0	66	301.9–303.3
13a	(Without OH)	1.99	–	–	>100	39	78.5–80.1
13b	(Without OH)	1.71	–	–	>100	58	122.9–124.3
14a	Benzyl	3.67	–	–	>100	46	123.4–125.6
14b	Benzyl	3.40	–	–	15 ± 0.9	63	223.7–225.2
Ciglitazone[h]		5.07	+	+	20 ± 2.4	—	—

[a] In compounds **1a**–**13a** the thiazolidine ring is substituted by a benzyl moiety, and in compounds **1b**–**13b** the thiazolidine ring is substituted by a benzylidene moiety.

[b] *c* Log *P*'s are calculated log *P* values generated by CHEMDRAW program.

[c] Release of Ca^{2+} from intracellular stores was observed using fluo-4 loaded cells. The +, –, and ± notations indicate release, no release, and insignificant release, respectively. The experiment was done with clotrimazole, a known Ca^{2+} releaser, as a positive control.

[d] Phosphorylation of eIF2α was determined by western blotting. The +, –, and ± notations indicate phosphorylation, no phosphorylation, and insignificant phosphorylation, respectively. The experiment was done with Tro and DMSO as the positive and negative controls, respectively.

[e] IC_{50} was measured using sulforhodamine B assay. The values indicate the concentration needed to inhibit 50% of cell proliferation. The experiment was done in triplicate.

[f] The yields for the **b** series are the overall yield. The yields for the **a** series are the yield of the reduction step from the correspond compounds from **b** series.

[g] The melting points were determined on a Mel-Temp® apparatus from Electrothermal with a thermocouple thermometer from Barnant.

[h] Ciglitazone was purchased from Sigma, St. Louis, MO.

the monitored activities. The size of the substituents at the 4′-hydroxyl group also seems to be important for their activities. In most cases the potencies improve as the *O*-alkyl substituents become longer and bulkier. The cyclopropyl analogs (**6a** and **6b**) in the cycloalkyl series and compounds **9a**,**b**–**13a**,**b** with fewer than four carbons in the linear alkyl chain series completely lost their ability to deplete Ca^{2+} from the intracellular stores.

Contrary to our prediction, compound **2a** substituted by the tetrahydropyranyl moiety was devoid of any intracellular Ca^{2+} releasing activity, possibly explained by the lower lipophilicity of the compound. Fischer et al.[24] have observed a similar phenomenon in the rat basophilic leukemia cell (RBL-2H3) in which compounds with high lipophilicity released Ca^{2+} from intracellular stores and inhibited SOC channels. In the saturated series **a**, only compounds with *c* log *P* greater than 2.7 induce the release of Ca^{2+}; in the unsaturated series **b**, with the exception of **2b**, only compounds with *c* log *P* greater than 2.5 induce the release of Ca^{2+}. However, this observation holds true only in the aliphatic substituted compounds. When the substituent was a benzyl group, both compounds (**14a** and **14b**) lost their ability to release Ca^{2+}.

Drug-induced phosphorylation of eIF2α suggests inhibition of translation initiation. In most cases, compounds that release Ca^{2+} also phosphorylate eIF2α and inhibit cell proliferation, and compounds that do not release Ca^{2+} also fail to cause phosphorylation of eIF2α and inhibition of cell proliferation. A few compounds

(**5a** and **8a**), which cause some release of intracellular Ca^{2+} without phosphorylating eIF2α, as well as those compounds that inhibit cell growth without releasing Ca^{2+} from intracellular stores are likely to affect cell proliferation by a mechanism(s) other than inhibition of translation initiation.

These observations suggest that the combination of Ca^{2+} release and eIF2α phosphorylation assays allow us to distinguish between antiproliferative compounds that act via Ca^{2+} induced inhibition of translation initiation and those that employ other mechanisms. In summary, we were able to identify the minimal structural requirement for TRO as Ca^{2+} depleting translation initiation inhibitors.

Acknowledgements

The authors wish to thank Drs. Daniel C. Tosteson and Magdalena Tosteson for their continuous support. In addition, we thank Dr. Michael Chorev for his comments and discussion. These studies were supported in part by NIH National Cooperative Drug Discovery Group (NCDDG) grant U19 CA87427 and NIH CA78411.

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18. All the compounds were prepared following the outlined procedure preparation of **2b**: Potassium *tert*-butoxide (1 g, 8.9 mmol) in 7.5 mL of DMF was added to a solution of tetrahydropyran-2-methanol (1 mL, 8.4 mmol) at ambient temperature followed by the addition of 4-fluorobenzaldehyde (0.95 mL, 8.9 mmol). The reaction mixture turned dark as the benzaldehyde was added. The final mixture was heated to 80 °C under N_2 for 8 h. The mixture was concentrated in vacuo then dissolved in toluene and washed with water and brine. The crude product was then concentrated to dryness, and was taken to the next step without further purification. The entire crude product was dissolved in fresh toluene (8 mL) then 2,4-thiazolidiendione (1 g, 8.5 mmol) was added followed by catalytic amount of piperidine (0.08 mL) and glacial acetic acid (0.08 mL). The final mixture was refluxed for 4 h. The product (precipitated out upon cooling) was filtered and washed with acetonitrile and recrystallized with hot acetonitrile. The product was obtained as yellow needles (mp 184.6–187.4 °C, 1.35 g 49% yield): ^{1}H NMR (DMSO-d_6, 500 mHz) δ 12.51 (s, 1H), 7.74 (s, 1H), 7.53 (d, $J = 8.5$ Hz, 2H), 7.09 (d, $J = 8.5$ Hz, 2H), 3.97 (t, $J = 3$ Hz, 2H), 3.87 (m, 1H), 3.63 (m, 1H), 3.36 (m, 1H), 1.81 (m, 1H), 1.63 (m, 1H), 1.43–1.52 (m, 1H), 1.31 (m, 1H); MS (APCI$^+$): m/z 319.80 (M+H).
Preparation of **2a**: Compound **2b** (0.13 g, 0.4 mmol), $CoCl_2$ (0.067 mg, 0.28 μmol) and trace amount of DMG were dissolved in a mixture of solution composed of H_2O/THF/1 N NaOH (0.44:0.26:0.28 mL) at ambient temperature followed by the addition of $NaBH_4$ (23.6 mg, 0.62 mmol) in 0.2 N NaOH (0.36 mL). Reaction progress was accompanied by change of color from deep-purple to yellow. A few drops of acetic acid were added to the mixture when the reaction turned yellow (the reaction should turn purple if not completed). The reaction was monitored by TLC and terminated 3 h after completion. The reaction was quenched by acetone (1 mL) then allowed to stir for 15 min. The product was extracted into CH_2Cl_2 (3×15 mL) and then concentrated in vacuo. The crude product was purified by chromatography on a silica gel column with a hexane/TBME (3:1) solution. A white powder was obtained (62 mg, 50%, mp 125.4–127.3 °C): ^{1}H NMR (DMSO-d_6, 500 mHz) δ 11.99 (s, 1H), 7.12 (d, $J = 8$ Hz, 2H), 6.85 (d, $J = 8$ Hz, 2H), 4.85 (dd, $J = 9.5$, 4 Hz, 1H), 3.82–3.88 (m, 3H), 3.59 (m, 1H), 3.36 (m, 1H), 3.28 (dd, $J = 14$, 4 Hz, 1H), 3.04 (dd, $J = 14$, 9.5 Hz, 1H), 1.80 (m, 1H), 1.62 (m, 1H), 1.42–1.51 (m, 1H), 1.29 (m, 1H); MS (APCI$^+$): m/z 321.94 (M+H).
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