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Control of the intracellular levels of prostaglandin E_2 through inhibition of the 15-hydroxyprostaglandin dehydrogenase for wound healing ☆

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ABSTRACT

Excessive scar formation is an aberrant form of wound healing and is an indication of an exaggerated function of fibroblasts and excess accumulation of extracellular matrix during wound healing. Much experimental data suggests that prostaglandin E_2 (PGE_2) plays a role in the prevention of excessive scarring. However, it has a very short half-live in blood, its oxidization to 15-ketoprostaglandins is catalyzed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Previously, we reported that 15-PGDH inhibitors significantly increased PGE_2 levels in A549 cells. In our continuing attempts to develop highly potent 15-PGDH inhibitors, we newly synthesized various thiazolidine-2,4-dione derivatives. Compound **27**, **28**, **29**, and **30** demonstrated IC_{50} values of 0.048, 0.020, 0.038 and 0.048 μM, respectively. They also increased levels of PGE_2 in A549 cells. Especially, compound **28** significantly increased level of PGE_2 at 260 pg/mL, which was approximately fivefold higher than that of control. Scratch wounds were analyzed in confluent monolayers of HaCaT cells. Cells exposed to compound **28** showed significantly improved wound healing with respect to control.

1. Introduction

The healing of skin wounds is complex and involves the formation of a clot, an inflammatory response, accumulation of granulation tissue, and the deposition and remodeling of an extracellular matrix.[1] It also requires the interaction of cells in the epidermis and the dermis and mediators released from inflammatory cells, fibroblasts, and keratinocytes.[2] Re-epithelialization by keratinocytes, which is achieved by migration and mitosis of cells in the epidermis proximal to the wound margin, is a central event in wound healing.[3] Many factors influence wound healing, including growth factors, cytokines, metalloproteinases, and extracellular matrix proteins.[2] The exogenous application of some of these factors has been shown to aid healing.[4]

Two cytokines, transforming growth factor-β (TGF-β) and prostaglandin E_2 (PGE_2), are lipid mediators of inflammation involved in wound healing. Clinical data suggests that the overproduction of TGF-β and suppression of PGE_2 are found in cases of excessive wound scarring compared with normal wound healing. Hsu et al.[5] reported that PGE_2 basal levels in hypertrophic burn scars were significantly lower than those present in normotrophic burn scars. Reno et al.[6] showed that the prevention and control of hypertrophy, especially in burn scars, is achieved using elastocompression. This compression induced a significant increase in the release of PGE_2, in both the remission and active stages, suggesting a role for PGE_2 in the process of hypertrophy remission induced by pressure therapy. Kolodsick et al.[7] reported that prostaglandins, mainly PGE_2, were important mediators of dermal wound healing with specific effects on fibroblast behavior and that PGE_2 can inhibit the differentiation of fibroblast into myofibroblasts via the EP2 receptor pathway through the up-regulation of cyclic adenosine monophosphate (cAMP). Therefore, PGE_2 might play an important role in the prevention of excessive scarring.

Abbreviations: cAMP, cyclic adenosine monophosphate; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothretitol; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; GST, glutathione S-transferase; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); PG, prostaglandin; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PPARγ, peroxisome proliferators-activated receptor; SDS, sodium dodecylsulfate; TGF-β, transforming growth factor-β; THF, tetrahydrofuran; TLC, thin layer chromatography.

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Prostaglandins are a family of biologically potent fatty acids derived from arachidonic acid through the cyclooxygenase pathway. The wide variety of effects of prostaglandins in many different cell types is explained with binding to a variety of mainly G protein-coupled receptors.[8] Prostaglandins have a short lifetime in vivo because they are metabolized rapidly by oxidation to 15-ketoprostaglandins catalyzed by the cytosolic enzyme NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH).[9] Therefore, inhibitors of 15-PGDH will be valuable for the therapeutic management of diseases requiring elevated prostaglandin levels. Previously, we reported that the antidiabetic drug ciglitazone and its analogue 5-(4-(2-cyclohexylethoxy) benzylidene)thiazolidine-2,4-dione were potent inhibitors of 15-PGDH and the inhibition of 15-PGDH may lead to increase accumulation of PGE_2.[10,11] Recently, we synthesized different thiazolidinedione analogues of 15-PGDH inhibitors and evaluated: the levels of PGE_2, the influence of 15-PGDH inhibitor on cochlear blood flow, and the effects on wounds healing.[12] However, the relationship between thiazolidine-2,4-dione derivatives and wound healing activity is still unclear.

Several previous studies have evaluated wound healing activity using various derivatives and natural products for the development of an efficient wound healing agent. Jeong[13] synthesized various derivatives of asiatic acid and evaluated the structure–activity relationship of asiatic acid derivatives for new wound healing agent. Among them, ethoxymethyl 2-oxo-2,23-isopropylideneasiatate showed the strongest and the fastest wounding healing activity. Furthermore, it left the smallest scar after healing. Halehatty et al.[14] characterized a new class of 2-mercapto/2-selenobenzo[*h*]quinoline-3-carbaldehyde and show significant wound healing activity and protect oxidative DNA damage from harmful free radical reactions. Antonov et al.[15] studied the natural polysaccharide chitosan for the wound healing agent in thermal and chemical burns and possibility of its use. They found that derivatives of chitosan regulate proliferation of fibroblasts and stimulate normal regeneration of skin. Cristiano et al.[16] evaluated the effects of topical tetracycline on the wound healing process in the skin of normal and diabetic animals and compared the wound healing areas in diabetics and their controls after local tetracycline use. Even though several therapeutic effects from various derivatives have been documented, there has been no information about the wound healing effect of thiazolidine-2,4-dione derivatives as 15-PGDH inhibitors.

In this study, for the development of a potential new drug for treatment of skin wounds, various thiazolidine-2,4-dione derivatives were newly synthesized and their efficacies and selectivity were examined by analyzing their structure–activity relationships. We also evaluated the expression level of PGE_2 in A549 cells using some synthetic derivatives. One of the more potent compounds was further investigated to elucidate the wound healing effect in HaCaT confluent monolayers and was compared to the effects of TGF-β1, the positive control.

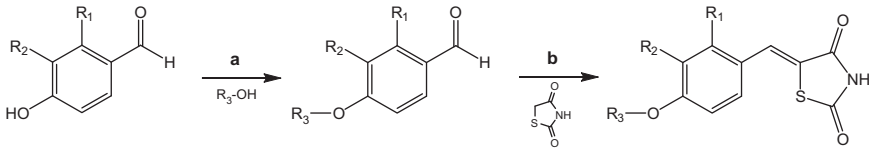
2. Results and discussion

Currently, heterocyclic compounds have been extensively studied due to their unique properties and applications in the pharmaceutical industry. Among these compounds, thiazolidine derivatives have become especially noteworthy in recent years. The thiazolidine-2,4-dione derivatives, especially, have acquired much importance because of their diverse pharmaceutical applications. They are used in various treatments such as: in hypoglycemic and hypolipidemic,[17] and as antibactericidal,[18] pesticidal,[19] antifungal,[20] insecticidal,[21] anticonvulsant,[22] antitubercular,[23] antiproteinuric and anti-inflammatory,[24] antithyroidal and antihistaminic agents.[25] Previously, we reported that several chemopreventive agents such as cyclooxygenase inhibitors, flavanoids, phytophenolic compounds, and peroxisome proliferator activated receptor agonists were able to inhibit 15-PGDH at low micromolar range.[26] The results showed that ciglitazone was the most potent inhibitor of 15-PGDH among those cyclooxygenase inhibitors, PPARγ agonists, and chemopreventive agents such as flavanoids and phytophenolic compounds, which were also shown to be 15-PGDH inhibitors. It was also interesting to discover that the inhibitory potency of ciglitazone was higher than those of other thiazolidinediones such as rosiglitazone and troglitazone. It appears that the nature of the moiety linking through the ether linkage to benzyl-2,4-thiazolidinedione plays an important role in inhibitory potency.[10] Ding et al.[27] suggested that 15-PGDH decreased the level of proliferative PGE_2, induced apoptosis and functioned like a tumor suppressor. We also examined the structure–activity relationship of various thiazolidinedione derivatives and designed a powerful thiazolidinedione that inhibited 15-PGDH. Based on the structures of the thiazolidinedione derivatives, we developed a protocol for synthesization of the various benzylidene thiazolidinedione derivatives using a series of reactions. The thiazolidinedione derivatives had different substituents on the phenyl ring and we investigated the inhibition of 15-PGDH on accumulation of PGE_2.[10]

Scheme 1 summarizes the general synthetic routes of thiazolidine-2,4-dione derivatives. *p*-Hydroxybenzaldehyde, as a starting material, was reacted with various substituents to afford the substituted benzaldehyde intermediate in good yield. The intermediate obtained was then used for a coupling reaction with thiazolidine-2,4-dione to afford the appropriate thiazolidine-2,4-dione derivatives. Previously, CT-8, a thiazolidinedione derivative, was found to be a potent inhibitor of 15-PGDH.[10] Structure–activity analysis indicated that the N-methylation of thiazolidine-2,4-dione, CT-8, abolished the inhibitory activity, whereas the introduction of an ethyl hydroxyl group at amine in CT-8 still had a good inhibitory effect. Based on the structures of the thiazolidinedione derivatives and inhibitory activity, a range of benzylidene thiazolidinedione derivatives were synthesized with different substituents on the phenyl ring and their inhibitory activity was evaluated. Replacement of the cyclohexylethyl group of CT-8 with the hetero five-member ring increased the inhibitory potency. However, replacement of the cyclohexylethyl group with a hetero six-member ring decreased the inhibitory potency significantly. We suggested that the hydrogen bond donating groups of thiazolidine-2,4-dione are essential in order to orient the molecule more favorably toward the binding site in the enzyme.[11]

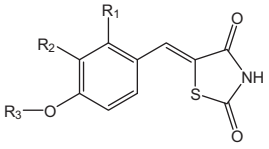
Based on our previous results, we synthesized different derivative of thiazolidine-2,4-dione and investigated the inhibitory activities of these various synthetic thiazolidine-2,4-diones. All the synthesized compounds were assayed in vitro against 15-PGDH. The inhibitory results of synthesized thiazolidine-2,4-dione derivatives are shown Table 1.

The inhibitory activity was strongly affected by the C2 position of the benzyl ring. Previous studies have shown that the introduction of a chlorine atom at the C3 of CT-8 showed a great increase in inhibitory potency. We also introduced a chlorine atom at the C2 of CT-8 resulting in significantly increased inhibitory potency with an IC_{50} values of 0.013 μM, as indicated by compound **1**. On the other hand, replacement of the cyclohexylethyl group with a 2-(propan-2-yloxy) ethyl (**2**), 2-(pyridin-2-yl)ethyl (**3**), 4-nitrobenzyl (**4**), 2-(propan-2-yloxy)ethyl (**9**), 2-(pyridin-2-yl)ethyl (**11**), 2-(4-methyl-1,3-thiazolidin-5-yl)ethyl (**12**), or 2-(5-ethylpyridin-2-yl)ethyl group (**20**) significantly decreased inhibitory potency. However, replacement of the cyclohexylethyl with a cyclobutylmethyl (**16**) or cyclopentylmethyl group (**18**) increased inhibitory potency with IC_{50} values of 0.009 and 0.011 μM, respectively.



Scheme 1. Synthesis of compounds **1**–**34**. Reagents and conditions: (a) PPh_3, DEAD, THF, 25 °C, 18 h; (b) piperidine, AcOH, reflux, 12 h.

Table 1
Inhibitory potency of various synthetic thiazolidine-2,4-diones

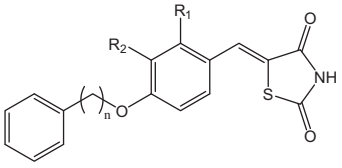


Compound	R_1	R_2	R_3	IC_{50} (μM)
CT-8	H	H	Cyclohexylethyl	0.051
1	Cl	H	Cyclohexylethyl	0.013
2	Cl	H	2-(Propan-2-yloxy)ethyl	1.050
3	Cl	H	2-(Pyridin-2-yl)ethyl	8.915
4	Cl	H	4-Nitrobenzyl	4.540
5	Cl	H	2-Cyclopentylethyl	0.042
6	Cl	H	2-(Cyclohexyloxy)ethyl	0.211
7	H	Cl	2-Thiomorpholine 1,1-dioxideethyl	0.223
8	H	Cl	2-(Thiophen-2-yl)ethyl	0.084
9	H	Cl	2-(Propan-2-yloxy)ethyl	2.738
10	H	Cl	4-Nitrobenzyl	0.930
11	H	Cl	2-(Pyridin-2-yl)ethyl	4.440
12	H	Cl	2-(4-Methyl-1,3-thiazolidin-5-yl)ethyl	5.165
13	H	Cl	2-Cyclopentylethyl	0.099
14	H	Cl	Thiophene-2-carbonyl	0.264
15	H	Cl	2-(Cyclohexyloxy)ethyl	0.020
16	H	Cl	Cyclobutylmethyl	0.009
17	H	Cl	4-(Prop-1-en-2-yl)cyclohexyl]methyl	0.075
18	H	Cl	Cyclopentylmethyl	0.011
19	H	Cl	1-Cyclopropylethyl	0.132
20	H	Cl	2-(5-Ethylpyridin-2-yl)ethyl	2.077
21	H	Cl	3-Nitrophenyl	0.100
22	H	Cl	5-Oxo-5,6,7,8-tetrahydronaphthalen-1-yl	0.071
23	H	Cl	2-(5-Nitrofuran-2-yl)ethyl	0.123
24	H	Cl	3-(-2-Nitroethenyl)phenyl	0.059
25	H	Cl	1,3-Benzodioxol-5-ylmethyl	0.193
26	H	Cl	Bicyclo[2.2.1]hept-2-ylmethyl	0.165

The enzyme was assayed fluorometrically as described in the text. IC_{50} values were determined using NAD^+ (250 μM) as coenzyme and PGE_2 (21 μM) as substrate. 15-PGDH was expressed as a GST fusion enzyme using pGEX-2T vector as described in the text.

For further structure–activity analysis, phenyl was introduced with different chain lengths to the hydroxyl group of 5-(2 or 3-chloro-4-hydroxybenzylidene)thiazolidine-2,4-dione and inhibitory activities of these derivatives were tested. The inhibitory results of the synthesized thiazolidine-2,4-dione derivatives are shown in Table 2. The inhibitory activity was strongly affected by the methylene group between phenyl ring and oxygen of 5-(2 or 3-chloro-4-benzyloxybenzylidene) thiazolidine-2,4-dione. Specifically, addition of one more methylene group between phenyl ring and the oxygen of 5-(3-chloro-4-(2-cyclohexylethoxy)benzylidene)thiazolidine-2,4-dione increased the inhibitory potency. Further addition of methylene groups gradually decreased the inhibitory potency, as indicated by compounds **29** and **30**. It appears that two methylene groups (**28**) between the phenyl ring and oxygen of 5-(3-chloro-4-benzyloxybenzylidene) thiazolidine-2,4-dione are optimal for inhibitory activity. However, a series of 2-chlorothiazolidine analogues showed that three methylene groups (**33**) between the phenyl ring and oxygen are optimal for inhibitory activity.

Table 2
Inhibitory potency of various synthetic thiazolidine-2,4-diones



Compound	*n*	R_1	R_2	IC_{50} (μM)
27	1	H	Cl	0.048
28	2	H	Cl	0.020
29	3	H	Cl	0.038
30	4	H	Cl	0.048
31	1	Cl	H	0.487
32	2	Cl	H	0.178
33	3	Cl	H	0.052
34	4	Cl	H	0.081

The enzyme was assayed fluorometrically as described in the text. IC_{50} value were determined using NAD^+ (250 μM) as coenzyme and PGE_2 (21 μM) as substrate. 15-PGDH was expressed as a GST fusion enzyme using pGEX-2T vector as described in the text.

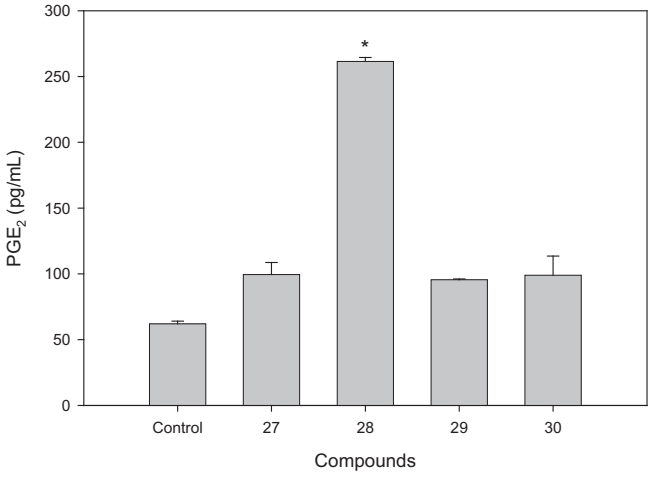


Figure 1. Inhibition of 15-PGDH in A549 cells. A549 cells were seeded in 6-well culture plates in RPMI medium containing fetal bovine serum and antibiotic and incubated overnight in incubator containing 5% CO_2 at 37 °C. The A549 cells were treated with the 15-PGDH inhibitors at 5 μM and the PGE_2 levels were assayed by PGE_2 enzyme immunoassay as described in the text. Data were analyzed from results of 3 independent experiments. $^*p$ <0.05, Statistically significant versus the control.

We evaluated the inhibition of 15-PGDH using various synthetic thiazolidine-2,4-diones in IL-1β-stimulated A549 cells. The synthetic thiazolidine-2,4-diones were added at various concentrations and incubated for 5 h. Medium was then collected and the level of PGE_2 was analyzed by enzyme immunoassay. The results show that the level of PGE_2 was dose dependently increased (data not shown). In addition, A549 cells were seeded in 6-well culture plates in RPMI medium containing fetal bovine serum and antibiotic for overnight in incubator containing 5% CO_2 at 37 °C. These

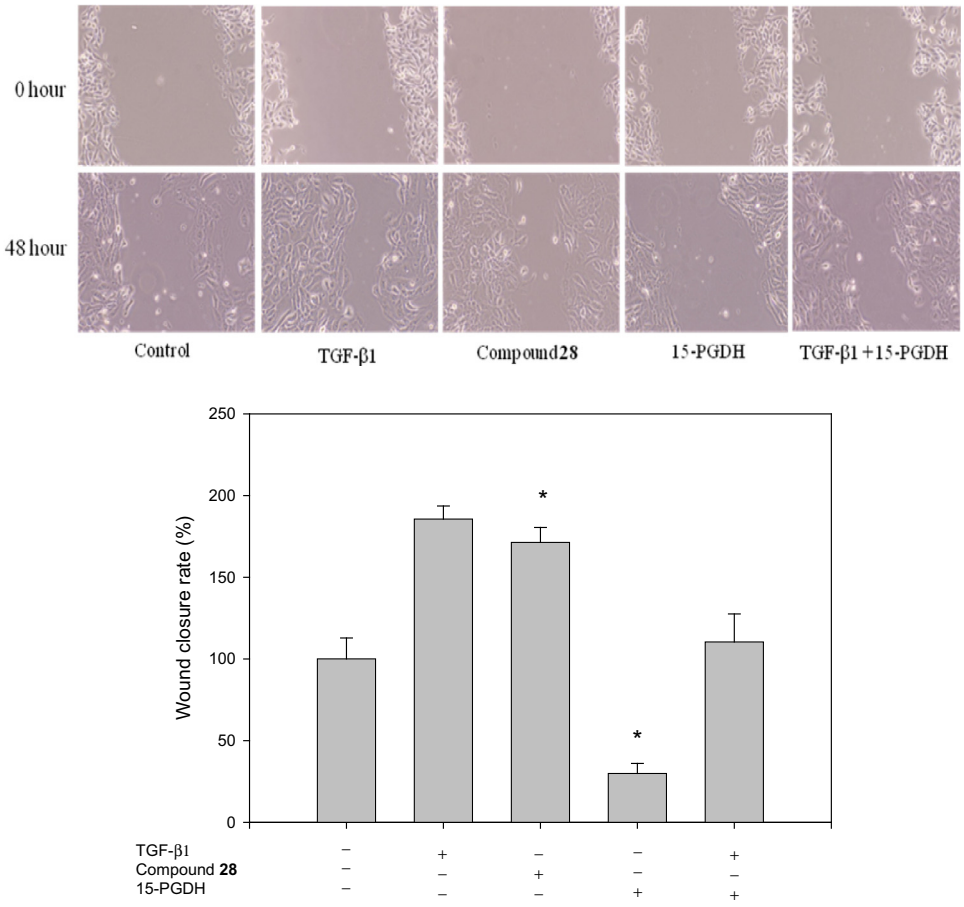


Figure 2. Scratch wound healing of HaCaT confluent monolayers. HaCaT cells were plated in a 6-well plate at ca. 5×10^5 cells per well and maintained in DMEM medium supplemented with 10% fetal bovine serum and 10 μg/mL mitomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were incubated until ca. 80% confluence was reached. The cells were mechanically scratched with a sterile 200 μL pipette tip and incubated under control conditions, in the presence of 1 ng TGF-β1 as positive control, and in the presence of 5 μM compound **28**, 15-PGDH, and TGF-β1 + 15-PGDH. The cells were observed 48 h after wounding. *p <0.05, Statistically significant versus the control.

A549 cells were then treated with compound **27**, **28**, **29**, and **30**, each at 5 μM and the PGE_2 levels were assayed by PGE_2 enzyme immunoassay. The results show that levels of PGE_2 were greatly increased as shown in Figure 1. Especially, compound **28** significantly increased the level of PGE_2 (260 pg/mL), which was about fivefold higher than that of control. These results suggest that thiazolidinedione derivatives as 15-PGDH inhibitors may have utility for the therapeutic management of diseases requiring elevated prostaglandin levels.

To study wound healing, a scratch was made using a sterile 200 μL pipette tip in a HaCaT cell line. Confluent monolayers of HaCaT were scratch wounded as described in the Methods and then allowed to re-epithelialize for 48 h at 37 °C in the presence or absence of compound **28** at 5 μM. Cells exposed to compound **28** showed significantly improved wound healing after 48 h with respect to a control (Fig. 2). However, in the presence of 15-PGDH, wound closure rate were significantly decreased, as shown in Figure 2. These findings indicate that PGE_2 is an important mediator of wound healing.

Adult wounds heal with imperfect repair and scar formation, whereas tissue repair in fetal wounds is regenerative. This result is thought to be the product of both intrinsic fetal fibroblast properties and extrinsic soluble mediators such as growth factors, cytokines, and chemokines. Many studies have reported that PGE_2 is an important mediator of dermal wound healing with specific effects on fibroblast behavior. Therefore 15-PGDH inhibitors can be used to help heal wounds, which require elevated prostaglandin levels.

3. Conclusion

Much experimental data suggests that PGE_2 might play a role in the prevention of excessive scarring. However, it has a very short half-live in blood, oxidizing to 15-ketoprostaglandins catalyzed by 15-PGDH. Based on the structures of previously synthesized thiazolidinedione analogues and their 15-PGDH inhibitory activities, a series of thiazolidinedione analogues were prepared using a series of reactions and tested for 15-PGDH inhibitory activity. Among the various synthetic thiazolidinedione compounds, compound **28** was found to be the most active candidate that significantly increased level of PGE_2 with 260 pg/mL, which was about fivefold higher than that of control. These results suggest that thiazolidinedione derivatives as 15-PGDH inhibitors may have utility for the therapeutic management of diseases requiring elevated prostaglandin levels. 15-PGDH inhibitors can be used to treat diseases such as Raynaud's and Burerger's diseases, and diabetic neuropathy, which require elevated prostaglandin levels. In addition, cell exposed to compound **28** showed significantly improved wound healing and the wound closure rate was about twofold higher than that of control. Hence, it could also be useful in wound healing therapy as it is a directly acting wound healing agent.

4. Experimental procedures

4.1. Chemistry

The thiazolidine-2,4-dione derivatives listed in Tables 1 and 2 were prepared by the method shown in Scheme 1. The connection of cyclohexylalcohol to the central aryl aldehyde was accomplished via Mitsunobu coupling to produce an intermediate aldehyde with a yield between 70% and 90%. Knoevenagel condensation between intermediate aldehyde and thiazolidine-2,4-dione in refluxing toluene, containing a catalytic amount of piperidinium acetate, produced the benzylidene-2,4-thiazolidinedione, which crystallized from the reaction mixture in high purity. *p*-Hydroxybenzaldehyde, as a starting material, was reacted with various substituents to produce an intermediate substituted benzaldehyde with good yield. The intermediate was then used in the coupling reaction with thiazolidine-2,4-dione to provide the appropriated thiazolidinedione derivatives. All the compounds were characterized by ^{1}H NMR.

4.2. Materials

PGE_2, NAD^+, NADH, Glutathione-Sepharose 4B, Dithiothretitol (DTT), Sodium dodecylsulfate (SDS), EDTA and reduced glutathione were obtained from Sigma. PGE_2 ELISA Kit was purchased from Thermo Scientific (Rockford, IL, United States). GST gene fusion pGEX-2T expression vector was obtained from Pharmacia Corp. The cDNA of human 15-PGDH was cloned from a human placenta cDNA library as described previously.[28] All chemical reagents were commercially available. UV spectra were obtained using a UV–vis spectrophotometer (SHIMADZU). TLC plates were prepared using Kieselgel 60 PF254. Column chromatography was performed using silica gel (230–400 mesh, Whatman Inc). NMR spectra were recorded on a JEOL JNM-LA 300 spectrometer (JOEL, Tokyo, Japan). Chemical shifts were reported in parts per million (δ) and signals were quoted as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Various thiazolidinediones were synthesized according to published procedures.[29]

4.3. Expression and purification of 15-PGDH

The sequence of 15-PGDH cDNA plasmid containing BamHI and EcoRI sites of the pGEX-2T expression vector was used to transform *Escherichia coli* BL-21 LysS. Cells were grown in 500 mL LB medium containing 50 μg/mL ampicillin at 37 °C and 220 rpm until OD_{600} reached 0.6. Isopropyl β-D-thiogalactoside (1 mM) was added and cells were allowed to grow for 12 h at 25 °C. Cells were then harvested by centrifugin at 4000×*g* for 30 min at 4 °C. The cell pellets were resuspended in 20 mL of cold cell lysis buffer [1×PBS buffer (pH 7.4) containing 1 mM EDTA and 0.1 mM DTT] and sonicated (14 × 10 s at 4 °C). Disrupted cells were centrifuged at 4000×*g* for 20 min at 4 °C. The supernatant was slowly applied to the Glutathione-Sepharose 4B column (about 3 mL) which was equilibrated at 4 °C with lysis buffer [1×PBS buffer (pH 7.4) containing 1 mM EDTA and 0.1 mM DTT]. After washing with lysis buffer until the OD_{280} reached less than 0.005. The 15-PGDH was eluted from the Glutathione-Sepharose 4B column by incubation at room temperature for 5 min with the elution buffer [50 mM Tris–HCL (pH 8.0) containing 10 mM reduced glutathione 1 mM EDTA and 0.1 mM DTT]. The concentration of the purified enzyme was determined and the purity of the 15-PGDH was assessed by SDS–PAGE and Coomassie Blue staining method.

4.4. 15-PGDH assay

Assays for the activity of the 15-PGDH inhibitors were performed using a fluorescence spectrophotometer by measuring the formation of NADH at 468 nm following excitation at 340 nm. 50 mM Tris–HCl (pH 7.5), 0.1 mM DTT, 0.25 mM NAD^+, 10 μg of the purified enzyme, 21 μM PGE_2, and various concentrations of inhibitors (total 2 mL) were added to the cells. Each concentration of inhibitor was assayed in triplicate. The fluorescence of the reaction mixture was read at 340 nm and the activity of the 15-PGDH inhibitors was determined from a standard curve prepared from the fluorescence of various concentrations of NADH at 340 nm.

4.5. Cell culture

A549 cells, derived from a human alveolar type II like adenocarcinoma, were cultured in RPMI media supplemented with 10% heat inactivated fetal bovine serum (Sigma) and 100 μg/mL penicillin. Cells were maintained and subcultured in 5% CO_2 at 37 °C.

4.6. PGE_2 enzyme immunoassay

A549 cells (5×10^5 cells/well) were seeded in 6-well culture plates in RPMI medium containing fetal bovine serum and antibiotic overnight in CO_2 incubator at 5% CO_2 and 37 °C. The following day, 15-PGDH inhibitors (5 μM) were added and media was collected after 12 h of drug treatment. The PGE_2 levels were determined by using PGE_2 enzyme immunoassay kits (Thermo Scientific, Rockford, IL, United States) according to manufacturer's protocol.

4.7. Scratch wound test

Scratch wound analysis was performed in confluent monolayers of HaCaT cells. HaCaT cells were plated in a 6-well plate at ca. 5×10^5 cells per well and maintained in DMEM medium supplemented with 10% fetal bovine serum and 10 μg/mL mitomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were incubated until ca. 80% confluence was reached. The cells cultured in the 6-well plate were mechanically scratched with a sterile 200 μL pipette tip and incubated under control conditions in the presence of 1 ng TGF-β1 as positive control or in the presence of 5 μM compound **28**. The width of the wound was measured upon wounding and after treatment using an inverted microscope equipped with a digital camera.

4.8. General procedures for the synthesis of compounds 1–34

Diethyl azodicarboxylate (40% in toluene, 3.74 g, 8.58 mmol) was slowly added to a stirring solution of cyclohexane ethanol (1.0 g, 7.80 mmol), 2-chloro-4-hydroxybenzaldehyde (1.22 g, 7.80 mmol) and triphenylphosphine (2.25 g, 8.58 mmol) in THF (20 mL) over 10 min. at 0 °C. The mixture was then stirred at room temperature until all of the starting materials dissolved (TLC analysis). The resulting solution was concentrated under reduced pressure and purified by column chromatography over silica gel (elution with hexane/ethyl acetate) to produce 1.80 g of intermediate, 2-chloro-4-(2-cyclohexylethoxy)benzaldehyde (1.64 g, 87%) as yellow oil. A mixture of 2-chloro-4-(2-cyclohexylethoxy)benzaldehyde (1.0 g, 4.06 mmol), 2,4-thiazolidinedione (475 mg, 4.06 mmol), piperidine (0.20 mL, 2.03 mmol) and acetic acid (0.13 mL, 2.03 mmol) in toluene (20 mL) was then added to a round bottom flask fitted with a Dean–Stark water trap and stirred under reflux for overnight. After cooling to room temperature, the

precipitate was washed with hexane and dried to produce compound **1**.

4.8.1. 5-(2-Chloro-4-(2-cyclohexylethoxy)benzylidene)-thiazolidine-2,4-dione (1)

Compound **1** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.24 g, 85%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.654 (s, 1H), 7.877 (s, 1H), 7.517 (d, J = 9.0 Hz, 1H), 7.110 (s, 1H), 7.012 (d, J = 9.0 Hz, 1H)), 4.111 (t, J = 13.2 Hz, 2H), 1.434–1.642 (m, 6H), 1.263–1.434 (m, 1H), 1.094–1.221 (m, 4H), 0.847–0.989 (m, 2H).

4.8.2. 5-(2-Chloro-4-(2-(propan-2-yloxy)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (2)

Compound **2** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.00 g, 71.4%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.49 (s, 1H), 7.963 (s, 1H), 7.908 (d, J = 8.4 Hz, 1H), 6.951 (m, 1H), 6.908 (d, J = 8.4 Hz, 1H), 4.195 (t, J = 9.6 Hz, 2H), 2.844 (t, J = 9.6 Hz, 4H), 2.359 (m, 1H) 1.024 (d, J = 5.7 Hz, 6H).

4.8.3. 5-(2-Chloro-3-(2-(pyridin-2-yl)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (3)

Compound **3** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.1 g, 79.7%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.461 (s, 1H), 7.966 (s, 1H), 7.722 (t, J = 13.8 Hz, 1H), 6.836–7.291 (m, 6H), 4.008 (d, J = 2.7 Hz, 2H), 2.982–3.033 (m, 2H), 1.185 (t, J = 13.8 Hz, 2H).

4.8.4. 5-(2-Chloro-4-(4-nitrobenzyloxy)benzylidene)thiazolidine-2,4-dione (4)

Compound **4** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (0.88 g, 65.7%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.640 (s, 1H), 7.887 (s, 1H), 7.704 (d, J = 13.2 Hz, 2H), 7.554 (d, J = 13.2 Hz, 2H), 7.510 (s, 1H) , 7.387 (d, J = 11.4 Hz, 1H), 7.223 (d, J = 11.4 Hz, 1H), 5.419 (s, 2H).

4.8.5. 5-(2-Chloro-4-(2-cyclopentylethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (5)

Compound **5** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 83.2%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.192 (s, 1H), 7.456 (d, J = 8.7 Hz, 1H), 7.089 (d, J = 3 Hz, 1H), 7.034 (d, J = 2.7 Hz, 1H), 4.047 (t, J = 13.2 Hz, 2H), 3.664–3.701 (m, 2), 1.811–1.917 (m, 2H), 1.562–1.649 (m, 2H), 1.285 (s, 1H), 1.172–1.261 (m, 3H).

4.8.6. 5-(2-Chloro-4-(2-(cyclohexyloxy)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione) (6)

Compound **6** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.176 (s, 1H), 7.211 (d, J = 9, 1H), 7.026 (d, J = 2.4, 1H), 6.920 (d, J = 10.5, 1H), 4.122 (t, J = 6.9, 2H), 3.843 (t, J = 9, 2H), 1.930 (s, 3H), 1.764 (d, J = 5.7, 3H), 1.253–1.311 (m, 4H).

4.8.7. 5-(3-Chloro-4-(2-thiomorpholine 1,1-dioxideethoxy)benzylidene)-2,4-thiazolidinedione (7)

Compound **7** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.26 g, 86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.564 (s, 1H), 7.724 (s, 1H), 7.706 (s, 1H), 7.547 (d, J = 9.0 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 4.263 (t, J = 10.5 Hz, 2H), 3.031–3.119 (m, 8H), 2.990 (t, J = 10.5 Hz, 2H).

4.8.8. 5-(3-Chloro-4-(2-(thiophen-2-yl)ethoxy)benzylidene)thiazolidine-2,4-dione (8)

Compound **8** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.21 g, 88.3%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.585 (s, 1H), 7.725 (s, 1H), 7.708 (d, J = 2.4 Hz, 1H), 7.536 (d, J = 11.1 Hz, 1H), 7.315–7.363 (m, 2H), 7.017 (d, J = 3.3 Hz, 1H), 6.978 (d, J = 8.4 Hz, 1H), 4.354 (t, J = 12.6 Hz, 2H), 3.322 (t, J = 12.6 Hz, 2H).

4.8.9. 5-(3-Chloro-4-(2-(propan-2-yloxy)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (9)

Compound **9** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.09 g, 77.3%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.607 (s, 1H), 7.507 (s, 1H), 7.373 (d, J = 8.4 Hz, 1H), 7.052 (d, J = 8.4 Hz, 1H), 4.255 (t, J = 10.2 Hz, 2H), 3.881 (t, J = 10.2 Hz, 2H), 1.216 (d, J = 3.9 Hz, 6H).

4.8.10. 5-(3-Chloro-4-(4-nitrobenzyloxy)benzylidene)thiazolidine-2,4-dione (10)

Compound **10** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.09 g, 81.3%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.680 (s, 1H), 8.407 (s, 1H), 8.302 (d, , J = 8.7 Hz, 2H), 7.760 (d, J = 8.7 Hz, 2H), 7.730 (s, 1H), 7.561 (d, J = 8.4 Hz, 1H), 7.451(d, J = 8.4 Hz, 1H), 5.473 (s, 2H).

4.8.11. 5-(3-Chloro-4-(2-(pyridin-2-yl)ethoxy)benzylidene}-1,3-thiazolidine-2,4-dione (11)

Compound **11** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.03 g, 73.9%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.971(s, 1H), 8.451 (d, J = 4.8 Hz, 1H), 7.776 (s, 1H), 7.720 (t, J = 17.4 Hz, 1H), 7.649 (s, 1H), 7.416 (d, J = 8.4 Hz, 1H), 7.068 (d, J = 8.4 Hz, 1H), 7.191–7.254 (m, 2H), 4.008 (d, J = 14.4 Hz, 2H), 3.048 (t, J = 14.4 Hz, 2H).

4.8.12. 5-(3-Chloro-4-(2-(4-methyl-1,3-thiazolidin-5-yl)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (12)

Compound **12** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.14 g, 84.4%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.586 (s, 1H), 8.832 (s, 1H), 7.724 (s, 1H), 7.697 (s, 1H), 7.532 (d, J = 9.0 Hz, 1H), 7.320 (d, J = 9.0 Hz, 1H), 4.318 (t, J = 11.7 Hz, 2H), 3.274 (t, J = 11.7 Hz, 2H), 2.359 (s, 3H).

4.8.13. 5-(3-Chloro-4-(2-cyclopentylethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (13)

Compound **13** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.18 g, 84.9%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.992 (s, 1H), 7.720 (s, 1H), 7.689 (s, 1H), 7.545 (d, J = 11.1 Hz, 1H), 7.317 (d, J = 8.7 Hz, 1H), 4.161 (t, J = 13.2 Hz, 2H), 1.189–1.921 (m, 9H), 1.053–1.212 (m, 2H).

4.8.14. 4-((2,5-Dioxopyrrolidin-3-ylidene)methyl)phenyl thiophene-2-carboxylate (14)

Compound **14** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.695 (s, 1H), 7.730 (s, 1H), 7.636 (d, J = 3.0 Hz, 1H), 7.355 (d, J = 3.6 Hz, 1H), 7.121 (d, J = 8.4 Hz, 2H), 6.968 (d, J = 7.8 Hz, 1H).

4.8.15. 5-(3-Chloro-4-(2-(cyclohexyloxy)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (15)

Compound **15** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.886 (s, 1H), 7.727 (s, 1H), 7.349 (d, J = 8.7 Hz, 1H), 4.259 (t, J = 9.3 Hz, 2H), 3.780 (t, J = 9 Hz, 2H), 1.897–1.975 (m, 2H), 1.628–1.643 (m, 2H), 1.461 (s, 1H), 1.185–1.212 (m, 4H).

4.8.16. 5-(3-Chloro-4-(cyclobutylmethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (16)

Compound **16** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.585 (s, 1H), 7.726 (d, J = 8.7, 1H), 7.523 (d, J = 6.6,

1H), 7.215 (d, J = 7.2, 1H), 4.114 (d, J = 6.3, 2H), 1.952–2.029 (m, 2H), 1.828–1.8947 (m, 4H).

4.8.17. 5-(3-Chloro-4-((4-(prop-1-en-2-yl)cyclohexyl)methoxy)benzylidene)-1,3-thiazolidine-2,4-dione (17)

Compound **17** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.599 (s, 1H), 7.785 (s, 1H), 7.515 (d, J = 6.6, 1H), 7.299 (d, J = 4.8, 1H), 5.858 (s, 1H), 4.695 (s, 2H), 4.509 (s, 2H), 1.902–2.099 (m, 4H), 1.639 (s, 3H), 1.338–1.505 (m, 2H).

4.8.18. 5-(3-Chloro-4-(cyclopentylmethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (18)

Compound **18** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.601 (s, 1H), 7.726 (d, J = 8.4, 1H), 7.518 (d, J = 6.3, 1H), 7.313 (d, J = 8.7, 1H), 4.022 (d, J = 6.9, 2H), 2.073–2.380 (m, 1H), 1.577–1.754 (m, 2H), 1.381–1.516 (m, 4H), 1.315–1.340 (m, 2H).

4.8.19. 5-(3-Chloro-4-(2-cyclopropylpropyl)benzylidene)-1,3-thiazolidine-2,4-dione (19)

Compound **19** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.598 (s, 1H), 7.732 (d, J = 6.6, 1H), 7.504 (d, J = 6.6, 1H), 7.303 (s, 1H), 4.200 (s, 1H), 1.319 (d, J = 4.2, 3H), 1.101–1.154 (m, 2H).

4.8.20. 5-(3-Chloro-4-(2-(5-ethylpyridin-2-yl)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (20)

Compound **20** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 9.022 (s, 1H), 8.312 (s, 1H), 7.731 (s, 1H), 7.556 (d, J = 2.4, 1H), 7.492 (s, 1H), 7.296 (s, 1H), 7.005 (d, J = 3.3, 1H), 4.055 (d, J = 7.2, 2H), 3.952 (d, J = 7.5, 2H), 2.496 (s, 3H), 1.183 (s, 2H).

4.8.21. 5-(3-Chloro-4-(3-nitrophenoxy)benzylidene)-1,3-thiazolidine-2,4-dione (21)

Compound **21** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 11.447 (s, 1H), 7.913 (s, 1H), 7.658 (d, J = 6.9, 2H), 7.399 (d, J = 8.4, 1H), 7.110 (d, J = 8.1, 1H), 6.177 (s, 1H).

4.8.22. 5-(3-Chloro-4-((5-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)oxy)benzylidene)-1,3-thiazolidine-2,4-dione (22)

Compound **22** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 11.071 (s, 1H), 7.628 (d, J = 2.1, 1H), 7.381 (d, J = 2.4, 1H), 7.113 (d, J = 8.7, 1H), 3.30 (m, 2H), 2.490 (d, J = 5.4, 2H), 1.351 (d, J = 6.9, 1H).

4.8.23. 5-(3-Chloro-4-(2-(5-nitrofuran-2-yl)ethoxy)benzylidene)-1,3-thiazolidine-2,4-dione (23)

Compound **23** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 11.099 (s, 1H), 8.978 (s, 1H), 7.634 (d, J = 2.1, 1H), 7.406 (s, 1H), 7.116 (d, J = 8.4, 1H), 4.007 (d, J = 6.9, 4H), 1.096–1.185 (m, 4H).

4.8.24. 5-(3-Chloro-4-(3-((2-nitroethenyl)phenoxy)benzylidene)-1,3-thiazolidine-2,4-dione (24)

Compound **24** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 11.099 (s, 1H), 8.085 (s, 1H), 7.871 (s, 1H), 7.721 (d, J = 6.3, 1H), 7.394 (s, 1H), 7.229 (d, J = 7.5, 2H), 6.933 (s, 1H).

4.8.25. 5-(4-(1,3-Benzodioxol-5-ylmethoxy)-3-chlorobenzylidene)-1,3-thiazolidine-2,4-dione (25)

Compound **25** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO) δ 11.597 (s, 1H), 7.716 (s, 1H), 7.372 (s, 1H), 7.114 (d, J = 8.4, 1H), 6.979 (s, 1H), 6.935 (d, J = 8.1, 2H), 6.021 (s, 2H), 5.168 (s, 2H).

4.8.26. 5-(4-(Bicyclo[2.2.1]hept-2-ylmethoxy)-3-chlorobenzylidene)-1,3-thiazolidine-2,4-dione (26)

Compound **26** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.15 g, 79.2%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.596 (s, 1H), 7.732 (s, 1H), 7.533 (d, J = 2.1, 1H), 7.156 (t, J = 6.3, 1H), 4.084 (d, J = 6.3, 2H), 2.490 (s, 2H), 2.31 (s, 1H), 1.68 (s, 1H), 1.445–1.510 (m, 2H), 1.318–1.388 (m, 3H).

4.8.27. 5-(4-(Benzyloxy)-3-chlorobenzylidene)thiazolidine-2,4-dione (27)

Compound **27** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.21 g, 86.4% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.024 (s, 1H), 7.936 (s, 1H), 7.747 (d, J = 10.2 Hz, 1H), 7.324–7.479 (m, 5H), 7.093 (d, J = 10.2 Hz, 1H), 5.259 (s, 2H).

4.8.28. 5-(3-Chloro-4-phenylethoxybenzylidene)thiazolidine-2,4-dione (28)

Compound **28** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.19 g, 86.2% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.547 (s, 1H), 7.894 (s, 1H), 7.694 (s, 1H), 7.533 (d, J = 11.1 Hz, 1H), 7.311–7.348 (m, 5H), 7.248 (d, J = 11.1 Hz, 1H), 4.367 (t, J = 13.5 Hz, 2H), 3.109 (t, J = 13.5 Hz, 2H).

4.8.29. 5-(3-Chloro-4-(3-phenylpropoxy)benzylidene)thiazolidine-2,4-dione (29)

Compound **29** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.13 g, 83.1% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.538 (s, 1H), 7.714 (s, 1H), 7.706 (s, 1H), 7.533 (d, J = 10.8 Hz, 1H), 7.158–7.311 (m, 6H), 4.143 (t, J = 12.3 Hz, 2H), 2.802 (t, J = 12.8 Hz, 2H), 2.022–2.093 (m, 2H).

4.8.30. 5-(3-Chloro-4-(4-phenylbutoxy)benzylidene)thiazolidine-2,4-dione (30)

Compound **30** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.04 g, 77.6% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.577 (s, 1H), 7.720 (s, 1H), 7.693 (s, 1H), 7.529 (d, J = 10.5 Hz, 1H), 7.135–7.304 (m, 6H), 4.155 (t, J = 5.7 Hz, 2H), 2.671 (t, J = 7.2 Hz, 2H), 1.741–1.761 (m, 4H).

4.8.31. 5-(4-(Benzyloxy)-2-chlorobenzylidene)thiazolidine-2,4-dione (31)

Compound **31** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (0.98 g, 70.1%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.850 (s, 1H), 7.561 (d, J = 9.9 Hz, 1H), 7.332–7.499 (m, 6H), 7.221 (d, J = 9.9 Hz, 1H), 5.224 (s, 2H).

4.8.32. 5-(2-Chloro-4-phenethoxybenzylidene)thiazolidine-2,4-dione (32)

Compound **32** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (0.96 g, 69.6%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.645 (s, 1H), 7.863 (s, 1H), 7.500 (d, J = 7.8 Hz, 1H), 7.188–7.274 (m, 6H), 7.127 (d, J = 7.8 Hz, 1H), 4.323 (t, J = 13.5 Hz, 2H), 3.064 (t, J = 13.8 Hz, 2H).

4.8.33. 5-(2-Chloro-4-phenpropoxybenzylidene)thiazolidine-2,4-dione (33)

Compound **33** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (1.0 g, 72.7%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.548 (s, 1H), 7.924 (s, 1H), 7.482 (d, J = 7.8 Hz, 1H), 7.200–7.368 (m, 6H), 7.212 (d, J = 7.8 Hz, 1H), 4.139 (t, J = 13.5 Hz, 2H), 3.156 (t, J = 13.8 Hz, 2H), 2.591–2.622 (m, 2H).

4.8.34. 5-(2-Chloro-4-phenbutoxybenzylidene)thiazolidine-2,4-dione (34)

Compound **34** was obtained by recrystallization from hexane/ethyl acetate as a yellow solid (0.98 g, 73.1%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.64 (s, 1H), 7.81 (s, 1H), 7.511 (d, J = 9.3 Hz, 1H), 7.161–7.296 (m, 6H), 7.105 (d, J = 9.3 Hz, 1H), 4.084 (t, J = 12.9 Hz, 2H), 2.654 (t, J = 13.8 Hz, 2H), 1.653–1.724 (m, 4H).

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