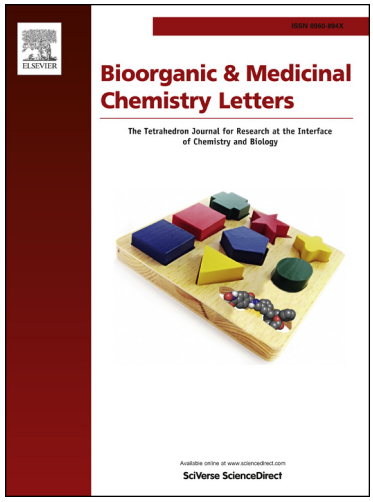

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Hit-to-lead optimization of phenylsulfonyl hydrazides for a potent suppressor of PGE_2 production: Synthesis, biological activity, and molecular docking study

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Keywords: inflammation; prostaglandin E_2; phenylsulfonyl hydrazide; mPGES-1; molecular docking study

Abstract: Preliminary hit-to-lead optimization of a novel series of phenylsulfonyl hydrazide derivatives, which were derived from the high throughput screening hit compound **1** (IC_{50} = 5,700 nM against PGE_2 production), for a potent suppressor of PGE_2 production is described. Subsequent optimization led to the identification of the potent lead compound **8n** with IC_{50} values of 4.5 and 6.9 nM, respectively, against LPS-induced PGE_2 production and NO production in RAW 264.7 macrophage cells. In addition, **8n** was about 30- and >150-fold more potent against mPGES-1 enzyme in a cell-free assay (IC_{50} = 70 nM) than MK-886 and hit compound **1**, respectively. Molecular docking suggests that compound **8n** could inhibit PGE_2 production by blocking the PGH_2 binding site of human mPGES-1 enzyme.

PGE_2 (prostaglandin E_2) has long been considered the principal prostaglandin of acute inflammation and of chronic diseases such as rheumatoid arthritis[1] and inflammatory bowel disease.[2] Macrophages play particularly important roles in inflammation because they produce many pro-inflammatory molecules such as PGE_2. Therefore, the pharmacological interference of PGE_2 production has been postulated as a means of alleviating a number of disease states mediated by excessive and/or protracted macrophage activation. As PGE_2 is produced in relative abundance by COX-2 (cyclooxygenase-2) action, selective COX-2 inhibitors such as celecoxib and rofecoxib suppress PGE_2 levels relative to other prostaglandins. However, coxib drugs such as Bextra® and Vioxx® were withdrawn from the market in 2004 and 2005, respectively, because they have been linked with an increased risk of adverse cardiovascular events with high doses and chronic usage.[3] Therefore, the exploration of alternative pharmacological approaches leading to safer anti-inflammatory drugs that do not alter COX-2 activity is of urgent need. One promising approach to circumvent COX-related side effects while maintaining anti-inflammatory efficacy is the interference with microsomal prostaglandin E_2 synthase (mPGES)-1. This mPGES-1 enzyme, a PG synthase downstream of COX-2, specifically catalyzes the biosynthesis of COX-2-derived PGE_2 from PGH_2 (prostaglandin H_2)[4] and thus, its inhibition would ideally not affect the formation of house-keeping PGs. Therefore, small molecule inhibitors of mPGES-1 are considered valuable for anti-inflammatory therapy with reduced side effects.[5-15] Several compounds like MF-63, MK-866, and Triclosan are mPGES-1 inhibitors, which were assayed in *in vitro* studies, but some of them have shown poor bioavailability and hepatotoxicity.[16-17]

In our previous research, we had initiated both virtual screening and real high throughput screening (HTS) using the master library from Korean Chemical Bank and identified hit compound **1** featuring a phenylsulfonyl hydrazide core (Figure 1).[18] Since compound **1** displayed strong suppression of LPS-induced PGE_2 production (IC_{50} = 5,700 nM against PGE_2) in RAW 264.7 macrophage cells, our efforts were focused on the hit-to-lead optimization via structure–activity relationship (SAR) study on the phenylsulfonyl hydrazide template to identify novel compounds with

improved *in vitro* potency. After analysis in a cell-free mPGES-1 enzyme assay, a molecular docking study on the putative active site of mPGES-1 was carried to shed light into the mPGES-1/inhibitor binding interactions.

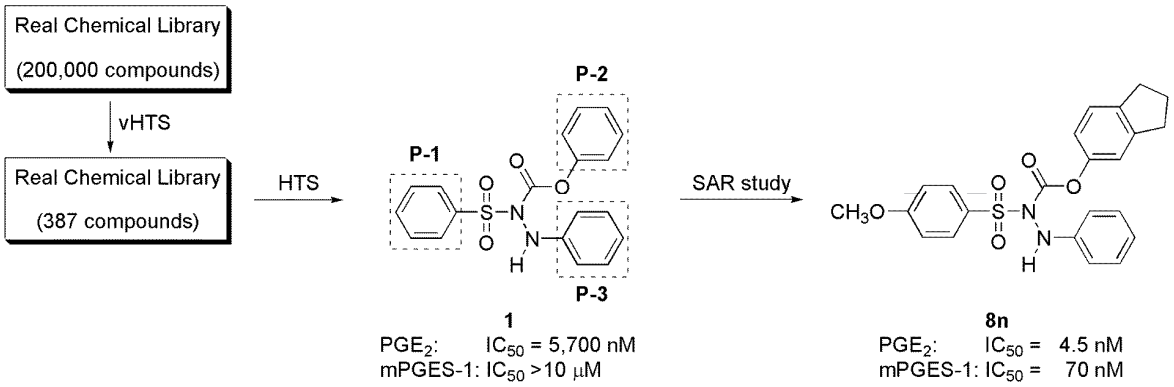
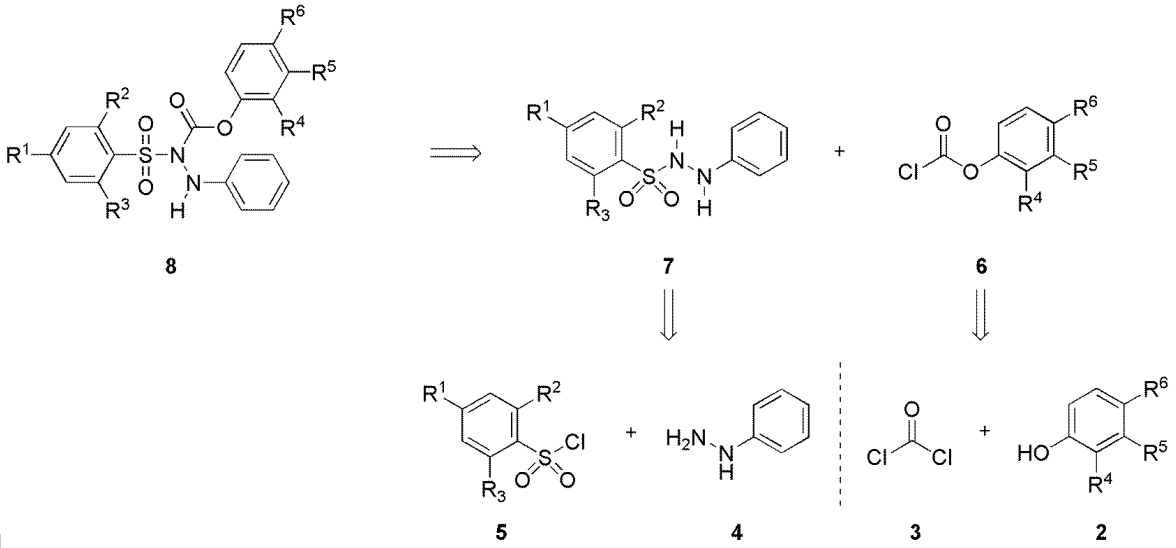


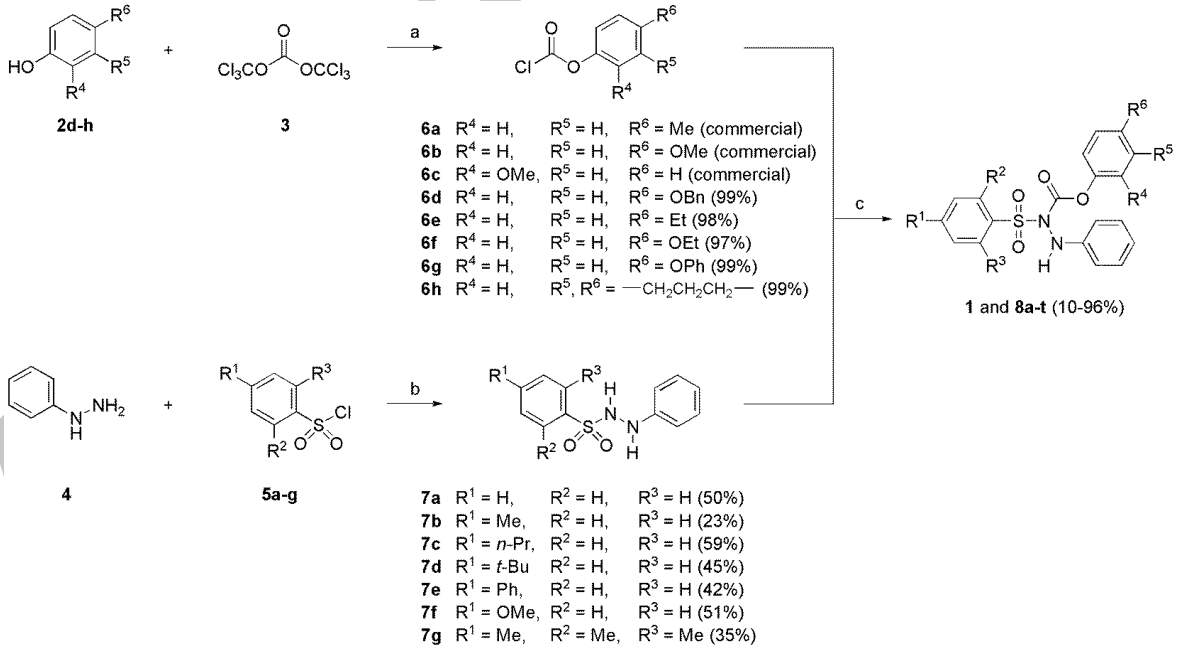
Figure 1. Discovery of HTS hit compound **1** and lead compound **8n** via SAR study



Scheme 1. Retrosynthetic analysis of phenylsulfonyl hydrazide derivatives **8**

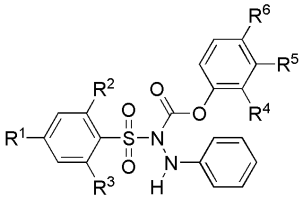
In order to improve the potency of hit compound **1**, only two areas were addressed with a fixed phenyl ring at P-3 region as a preliminary research: the effect of both substituents of phenyl ring and phenoxy ring at P-1 and P-2 region (Figure 1), respectively, on LPS-induced PGE_2

production. We designed synthetic route to obtain phenylsulfonyl hydrazide derivatives **8** via a retrosynthetic analysis (Scheme 1). The target compound **8** could be obtained from the nucleophilic substitution reaction of phenyl chloroformate **6** with phenylsulfonyl hydrazide **7**, which could be obtained from the reaction of phenylsulfonyl chloride **5** and phenylhydrazine **4**. The phenyl chloroformate **6** could also be obtained from the reaction of phosgene **3** and phenol **2**. As illustrated in Scheme 2, the reaction of appropriately substituted phenols **2d**-**h** with 0.6 equivalent of triphosgene **3** (instead of toxic phosgene gas) in the presence of *N*, *N*-diisopropylethylamine (Hünig's base) gave commercially unavailable phenyl chloroformates **6d**-**h** in excellent yields. Next, *N*-(phenyl)-phenylsulfonyl hydrazide **7a**-**g** were synthesized in low to moderate yields via the sulfonation of phenylhydrazine **4** using appropriately substituted phenylsulfonyl chlorides **5a-g** in the presence of triethylamine (TEA). Finally, *N*-(phenoxycarbonyl)-*N*'-(phenyl)phenylsulfonyl hydrazide **1** and **8a**-**t** were obtained in various yields by coupling of *N*-(phenyl)phenylsulfonyl hydrazide **7a**-**g** and phenyl chloroformates **6a**-**h** (commercially available **6a**-**c** and synthesized **6d**-**h**) in the presence of TEA under THF reflux condition.



Scheme 2. Reagents and conditions: (a) DIPEA, THF, 0 °C, 12 h for **6d**-**h**; (b) TEA, CH_2Cl_2, 0 °C to rt, 3-6 h; (c) TEA, THF, 60 °C, 4-8 h.

Table 1. Biological activity of *N*-(phenoxycarbonyl)-*N'*-(phenyl)phenylsulfonyl hydrazide derivatives



Entry	Structure						Yield (%) [a]	Viability (μM) [b]	PGE_2 IC_{50} (nM) [c]
	R^1	R^2	R^3	R^4	R^5	R^6			
1	H	H	H	H	H	H	82	> 10	5,700
8a	H	H	H	H	H	Me	96	> 10	1,020
8b	H	H	H	H	H	MeO	90	> 10	584
8c	Me	H	H	H	H	H	81	> 10	5,720
8d	Me	H	H	H	H	Me	63	> 10	1,010
8e	Me	H	H	H	H	BnO	36	≥ 10	32.6
8f	Me	H	H	MeO	H	H	48	> 10	34.1
8g	MeO	H	H	H	H	H	89	> 10	4,430
8h	MeO	H	H	H	H	MeO	49	> 10	490
8i	MeO	H	H	MeO	H	H	15	> 10	165
8j	MeO	H	H	H	H	Et	44	> 10	28.5
8k	MeO	H	H	H	H	EtO	52	> 10	20.5
8l	MeO	H	H	H	H	PhO	81	> 10	17.1
8m	MeO	H	H	H	H	BnO	17	≥ 10	50.0
8n	MeO	H	H	H	$CH_2CH_2CH_2$		10	> 10	4.5
8o	*n*-Pr	H	H	H	H	PhO	82	> 1	134
8p	*t*-Bu	H	H	H	H	BnO	78	> 1	56.9
8q	Ph	H	H	H	H	PhO	28	> 1	161
8r	Ph	H	H	H	H	BnO	17	> 1	93.0
8s	Me	Me	Me	H	H	PhO	14	> 1	177
8t	Me	Me	Me	H	H	BnO	50	> 1	81.5
NS398 [d]									7.0

[a] Isolated yield of final step for each compound; [b] Cytotoxicity of each compound using MTT assay; [c] IC_{50} value is the compound concentration required to produce 50% inhibition of LPS-induced PGE_2 production in RAW 264.7 macrophages; [d] Positive control as a selective COX-2 inhibitor for assay of PGE_2 production inhibition.

In order to check that the suppressive effects of all synthesized compounds on PGE_2 production could be attributable to non-specific cytotoxicity, initially, we examined the cytotoxicity of synthetic compounds in RAW 264.7 cells in the presence of LPS using MTT assays.[19] None of the

compounds affected the viabilities of RAW 264.7 cells at 1 or 10 μM concentration over 24 h (Table 1). Therefore, all compounds were screened for their ability to suppress PGE_2 production in LPS-induced RAW 264.7 cells at a concentration of 1 or 10 μM over 24 h using NS398 (@ 3 μM) as a positive control. PGE_2 concentration in the medium was measured using an EIA kit for PGE_2 according to the manufacturer's recommendation. Then, active compounds exhibiting >50% suppression of PGE_2 production at the given concentration were pushed forward for IC_{50} determinations.[20] All experiments were carried out at least twice and they gave similar results. The biological activities of all compounds are summarized as both cell viabilities and IC_{50} values of PGE_2 production in Table 1 with NS398 utilized as a positive control in PGE_2 assays. Our initial efforts focused on modifications of the P-1 region via the introduction of substituent on R^1 position of hit compound **1** (Table 1). Introduction of a simple methyl group led to a similar activity (**8c**) compared to hit compound **1,** whereas a slightly improved activity was found when a methoxy group was introduced (**8g**), indicating that small substituents were well tolerated at this position. Interestingly, compounds (**8f** and **8i**) bearing an additional methoxy group at the R^4 position in P-2 region exhibited a dramatically enhanced activity (in particular, IC_{50} = 34.1 nM for **8f**) compared to their corresponding mono-substituted analogs (**8c** and **8g**). With respect to the R^6 position in P-2 region, the introduction of methyl (**8a**) or methoxy groups (**8b**) resulted in an approximately 5- or 10-fold increase in activity compared to parental compound **1**. In addition, compounds (**8d** and **8h**) having the same methyl or methoxy groups at both R^1 and R^6 positions were also 5- and 10-fold more active than those of mono-substituted analogs (**8c** and **8g**), respectively. The introduction of methyl and benzyloxy groups on R^1 and R^6 positions, respectively, exhibited potent inhibitory activity (IC_{50} = 32.6 nM for **8e**) comparable to that of compound **8f** (IC_{50} = 34.1 nM). With these overall results in hand, we further investigated the role of R^6 substituents by incorporating ethyl (**8j**), ethoxy (**8k**), phenoxy (**8l**) or benzyloxy (**8m**) groups into this R^6 position, which led to a dramatically increase of activity (IC_{50} = 17.1 to 50 nM), indicating the importance of bulky substituents at this position that can be accommodated at the binding site of certain enzyme. In particular, the importance of a

benzyloxy substituent at this position was confirmed by comparing **8c** and **8d** (IC_{50} = 5,720 and 1,010 nM) with **8e** (IC_{50} = 32.6 nM). Furthermore, benzyloxy derivatives (**8p**, **8r** and **8t**) were generally more active than their corresponding phenoxy analogs (**8o**, **8q** and **8s**) with the exception of compounds **8l** (IC_{50} = 17.1 nM). When a bulky group (phenoxy or benzyloxy) was located as R^6 substituent, a small group (methyl or methoxy: **8e**, **8l** or **8m**) displayed better *in vitro* potency than bulky substituents (*n*-Pr, *t*-Bu or Ph: **8o-8r**) at R^1 position and a 2,4,6-trimethylphenyl ring (**8s-8t**) at P-1 region, indicating that only small groups as R^1 substituent were tolerated for stronger activity again. As observed in this preliminary hit-to-lead study, more potent activity was reached by the compound **8n** bearing a methoxy group at R^1 position and trimethylene unit at R^5-R^6 position. Compound **8n** (IC_{50} = 4.5 nM) was 1.5-fold more active than NS398 (IC_{50} = 7.0 nM) as positive control and also displayed about 1,100-fold better activity than the original hit compound **1**. Additionally, compound **8n** was tested for inhibition of NO production in LPS-stimulated RAW 264.7 cells and found to exhibit an IC_{50} value of 6.9 nM. Figure 2 shows the concentration–response inhibition of **8n** towards the secretion of PGE_2 and NO.

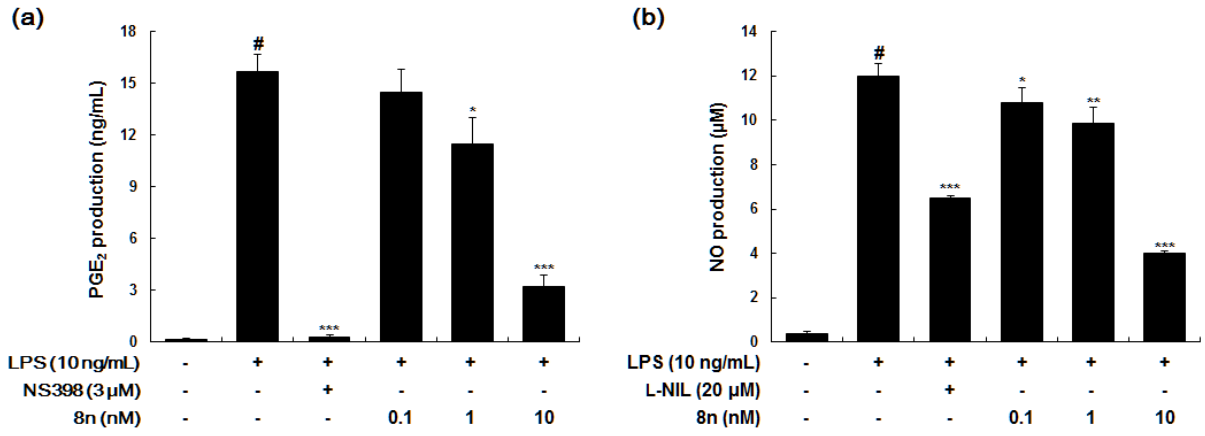


Figure 2. Effects of compound **8n** on (a) PGE_2 and (b) NO production in LPS-stimulated RAW 264.7 cells. The cells were co-incubated with LPS (10 ng/mL) and different concentrations of **8n** ranging from 0.1 to 10 nM. The supernatants were then collected for the measurement of PGE_2 and NO production using EIA kit and a Griess reagent, respectively. **8n** significantly inhibited PGE_2 and NO levels in LPS stimulated macrophages. The values are expressed as the means ± SD of three individual samples. $^{\#}p$ <0.05 versus the control cells; $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 versus LPS-stimulated cells; statistical significances were compared using ANOVA and Dunnett's post hoc test.

Among all the synthetic compounds, 8 selected compounds that potently suppressed PGE_2 production (IC_{50} < 60 μM) were investigated for interference with mPGES-1 activity in a cell-free assay, using the microsomal fraction of interleukin-1β-stimulated human A549 cells as enzyme source.[21] Compounds with low potency (**1** and **8d**) were tested as well for comparison, and MK-886 (10 μM) was used as a reference compound. The results summarized in Table 2 show that all compounds except **8e** and **8p** significantly inhibited mPGES-1 activity at a concentration of 10 μM. As compounds **8d**, **8l** and **8n** repressed mPGES-1 activity at 10 μM by more than 50%, we determined the IC_{50} values by more detailed concentration response studies. Compound **8n** turned out to be most potent with an IC_{50} = 70 nM, while **8d** and **8l** were less efficient (IC_{50} = 6,100 and 6,600 nM, respectively). The mPGES-1 reference inhibitor MK-886 reduced PGE_2 production by 84% at 10 μM with an IC_{50} = 2 μM (in line with literature data)[21-22] under our assay condition. Therefore, the most active compound **8n** showed about 30- and >150-fold higher potency than MK-886 and hit compound **1,** respectively, against mPGES-1 enzyme in a cell-free assay.

Table 2. Inhibition of human mPGES-1 in a cell-free assay by selected compounds

Entry	Remaining activity of mPGES-1 at 10 μM (%) [a]	mPGES-1 IC_{50} (μM) [b]	MolDock Score [c]
1	63.6 ± 2.5	> 10	-135.2
8d	41.3 ± 9.2	6.1 ± 5.7	-150.4
8e	81.2 ± 5.7	> 10	-160.6
8f	55.7 ± 12.4	> 10	-148.9
8j	63.6 ± 5.8	> 10	-151.3
8k	62.5 ± 2.6	> 10	-159.1
8l	37.1 ± 11.8	6.6 ± 4.8	-165.0
8m	62.6 ± 2.7	> 10	-167.6
8n	18.5 ± 6.8	0.07 ± 0.03	-168.8
8p	79.9 ± 9.0	> 10	-163.7
MK-886 [d]	16.3 ± 2.4	2.0 [e]	-182.4

[a] The remaining mPGES-1 activity is given as percentage of uninhibited control (= 100%) and presented as means ± S.E.; [b] IC_{50} values are presented as means ± S.E. of n = 3 independent experiments; [c] MolDock score during docking in the mPGES-1 (PDB code: 4AL0) active site by using MVD2013.6.0.1; [d] MK886 (10 μM) was used as a reference compound; [e] reported data for MK886[21-22]; [f] NS398 was used as a selective COX-2 inhibitor for the reliability of docking data.

As can be seen from Table 1 and 2, inhibition of mPGES-1 by the test compounds was in line with the suppression of PGE_2 formation except for compound **8d**. Thus, for **8l** and **8n** that were the most efficient mPGES-1 inhibitors, these compounds also efficiently inhibited PGE_2 production with IC_{50} values of 17.1 and 4.5 nM, respectively, confirming our preliminary results regarding the phenylsulfonyl hydrazide derivatives as putative mPGES-1 inhibitors.[18]

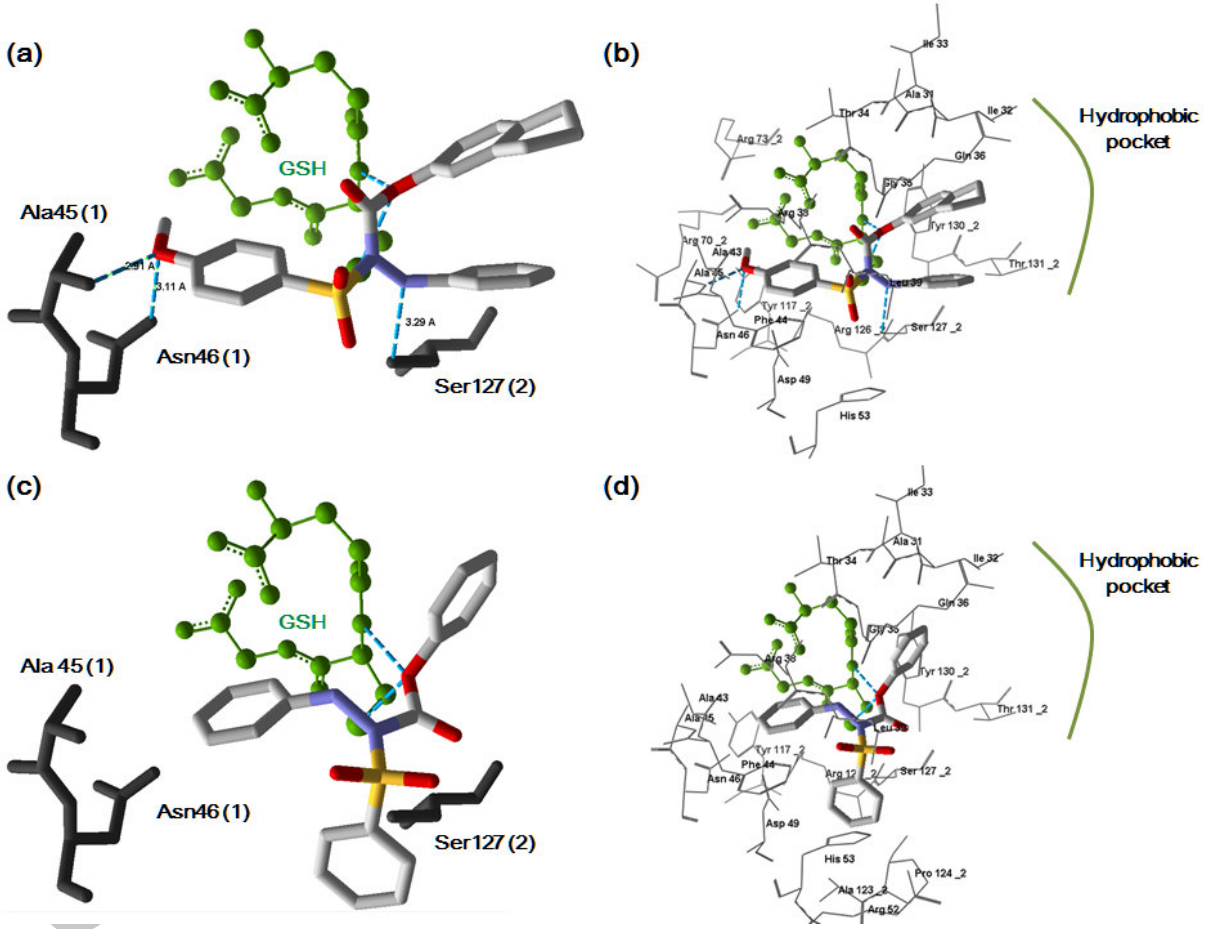


Figure 3. Predicted binding poses retrieved from flexible docking of compounds **8n** [(a) and (b)] and **1** [(c) and (d)] in human mPGES-1 (PDB: 4AL0) putative binding sites. The atom coloring for the compounds is the following: carbons in grey, oxygens in red, nitrogens in light purple, and sulfur in yellow. GSH (glutathione) as a cofactor is depicted as green ball and stick model. The key amino acids of chain 1 and 2 participating in hydrogen bonding are depicted as dark grey stick model. Hydrogen bonds are shown in blue dotted line. The hydrogens are omitted for the simplicity. In (b) and (d), the amino acids of chain 1 and 2 participating in hydrogen bonds and hydrophobic interactions are depicted as grey wireframe models.

Next, molecular docking studies were performed in order to rationalize the different biological activities of compounds tested against mPGES-1 enzyme by using the recently crystallized structure of mPGES-1 (PDB code: 4AL0)[23] because hit compound **1** was found to suppress PGE_2 production via the inhibition of mPGES-1 enzyme.[18] Recently, it has been reported that PGH_2 covalently binds together with GSH (glutathione) into the active site of mPGES-1 and isomerizes to PGE_2 with the help of several amino acids including key amino acid Ser127 close to GSH.[23] In addition, a hypothetical mPGES-1 inhibitor can act either as a false substrate (PGH_2) or as a cofactor analog (GSH), or it can behave in both ways.[24] Recently, we found that the appropriate correlation between real IC_{50} values and MolDock scores for known mPGES-1 inhibitors was found only in the presence of GSH.[25] In this study, therefore, molecular docking calculations were then performed in the presence of this cofactor. The docking studies were performed using Molegro Virtual Docker (MVD) 2013.6.0.1 for Windows.[26] First of all, we identified one cavity with a volume 188 $Å^3$ located in close proximity to GSH (glutathione) as a cofactor by the same software, which can automatically detect cavities from protein surfaces. The putative active site of the enzyme was defined to include residues within a 10 Å radius to this cavity. The docking wizard of 2013.6.0.1 was used to dock all compounds together with MK-886 and NS398 as mPGES-1 inhibitor and COX-2 inhibitor, respectively, for comparison. The best pose with the highest MolDock score by the MVD scoring function was selected for each compound and inserted in Table 2. As shown in Table 2, the appropriate correlation between IC_{50} value and MolDock Score was found for compound **1**, **8l**, **8n**, and MK-886. This docking result suggest that this series compounds rather inhibit PGE_2 production by blocking the active site (i.e., the binding site for PGH_2) of mPGES-1 enzyme than of COX-2 enzyme. This possibility was also supported by the relatively lowest MolDock score (-117.9) for **NS398**, a selective COX-2 inhibitor with an IC_{50} value of 7.0 nM against PGE_2 production under our assay condition. In particular, the most active compound **8n** with IC_{50} value of 70 nM against mPGES-1 was found to dock into the active site of mPGES-1 with higher MolDock Score of −168.8 compared to hit compound **1** (IC_{50} value of >10 μM and MolDock Score = -135.2). Based on these

results, we decided to further analyze the detailed binding modes of compounds **8n** and **1** with mPGES-1, respectively, because the understanding of their binding modes will be of great help in further lead optimization studies in the future. According to the docking results, the three ring systems of most active compounds **8n** occupied the similar positions as those of hit compound **1** in the active site of mPGES-1 enzyme as shown in Figure 3. Both hit compound **1** and active compound **8n** interacted with GSH through two hydrogen bond interactions with the oxygen atom of carbamate group in Figure 3 (a) and (c). In the case of compound **8n**, however, the oxygen atom of the methoxy group in P-1 region and the second nitrogen atom of the sulfonyl hydrazide group participated in additional hydrogen bond interactions with Ala45 (2.91 Å) and Asn46 (3.11 Å) of chain 1, and Ser127 (3.29 Å) of chain 2, respectively, which has been identified as a key residue in the catalytic activity of human mPGES-1 enzyme.[23] In addition, the indanyl ring in P-2 region of compound **8n** was well-positioned in the hydrophobic pocket, constituting of hydrophobic residues Ile32 (chain 1), Gly35 (chain 1), Leu29 (chain 1), Gln36 (chain 1), Tyr130 (chain 2), and Thr131 (chain 2) in the mPGES-1 active site when compared to the simple phenyl ring of hit compound **1** as shown in Figure 3 (b) and (d). The combination of additional hydrogen bonds and hydrophobic interactions could possibly increase the ligand binding affinity and thus result in higher mPGES-1 inhibitory activity of compound **8n** than hit compound **1**. In fact, compound **8n** was more than 100-fold more potent than hit compound **1** against mPGES-1 enzyme in a cell-free assay.[27] The presence of an indanyl ring could also explain why compound **8n** was more active than compound **8j** bearing simple 4-ethylphenyl ring at P-2 region.

In conclusion, a series of novel phenylsulfonyl hydrazide derivatives have been synthesized and evaluated for their inhibitory effects on LPS-induced PGE_2 production in RAW 264.7 cells through a preliminary hit-to-lead optimization strategy. Among them, compound **8n** bearing an anisole and indanyl ring at P-1 and P-2 regions, respectively, exhibited the most potent inhibition on PGE_2 production with an IC_{50} value of 4.5 nM and also was non-cytotoxic at up to 10 μM

concentration. Compound **8n** also showed an IC_{50} value of 70 nM against mPGES-1 enzyme in a cell-free assay. Molecular docking study revealed that **8n** could inhibit PGE_2 production by blocking the PGH_2 binding site of mPGES-1 enzyme and will serve as an important template for future chemical modification. Further optimization including P-3 region in Figure 1 and application of this series of compounds will be reported in due course.

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21. Koeberle, A.; Siemoneit, U.; Buehring, U.; Northoff, H.; Laufer, S.; Albrecht, W.; Werz, O. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 975: Preparations of interleukin(IL)-1β-stimulated A549 cells and determination of mPGES-1 activity. Briefly, cells were treated with 2 ng/mL Il-1β for 72 h, harvested and homogenized. The homogenate was subjected to differential centrifugation at 10,000 × g for 10 min and 174,000 × g for 1 h at 4 °C. The pellet (microsomal fraction) was re-suspended in 1 mL homogenization buffer. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4 °C reaction (100 µL total volume) was initiated by addition of 20 µM PGH_2. After 1 min at 4 °C, the reaction was terminated using stop solution (100 µL; 40 mM $FeCl_2$, 80 mM citric acid, and 10 µM 11β-PGE_2 as internal standard). PGE_2 was separated by solid-phase extraction and analyzed by RP-HPLC.

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26. MVD 2013.6.0.1 for Windows in Molegro ApS: With this purpose, crystal structures of human mPGES-1 (PDB code: 4AL0) was obtained from the Protein Data Bank in order to prepare the protein for docking studies. Docking procedure was followed using the standard protocol implemented in MVD 2013.6.0.1 and the geometry of resulting complexes was studied using the MVD's Pose Viewer utility.

27. Spectral data of *N*-(5-indanoxycarbonyl)-*N*'-(phenyl)-4-methoxyphenylsulfonyl hydrazide **(8n)**: mp 134.7-139.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.16 (1H, s), 7.94-7.91 (1H, m), 7.27-7.23 (2H, m), 7.22-7.18 (3H, m), 6.85 (1H, t, *J* = 7.2 Hz), 6.81-6.77 (3H, m), 6.72 (1H, dd, *J* = 8.0 and 2.4 Hz,), 3.89 (3H, s), 2.81 (2H, t, *J* = 7.2 Hz), 2.80 (2H, t, *J* = 7.2 Hz), 2.00 (2H, qu, *J* = 7.6 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.82, 151.40, 148.29, 146.87, 145.51, 141.85, 130.97, 129.09, 129.04, 124.84, 119.88, 118.72, 117.11, 114.46, 111.95, 55.94, 32.24, 31.62, 25.31; Low MS (ES): m/z 439.23 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{23}H_{23}N_2O_5S$ $[M+H]^+$ 439.1328, found 439.1322.

Graphical Abstract

Hit-to-lead optimization of phenylsulfonyl hydrazides for a potent suppressor of PGE_2 production: Synthesis, biological activity, and molecular docking study

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