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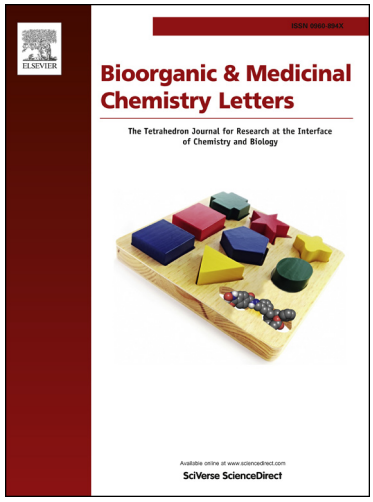

Structure–Activity Relationship Study of a Series of Novel Oxazolidinone Derivatives as IL-6 Signaling Blockers

Sarbjit Singh, Veeraswamy Gajulapati, Kondaji Gajulapati, Ja-Il Goo, Yeon-Hwa Park, Hwa Young Jung, Sung Yoon Lee, Young Kook Kim, Kyeong Lee, Tae-Hwe Heo, Yongseok Choi

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Structure–Activity Relationship Study of a Series of Novel Oxazolidinone Derivatives as IL-6 Signaling Blockers

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ABSTRACT

A series of oxazolidinone and indole derivatives were synthesized and evaluated as IL-6 signaling blockers by measuring the effects of these compounds on IL-6–induced luciferase expression in human hepatocarcinoma HepG2 cells transfected with p-STAT3-Luc. Among different compounds screened, compound **4d** was emerged as the most potent IL-6 signaling blockers with IC_{50} value of 5.9 μM which was much better than (+)-Madindoline A (IC_{50} = 21μM), a known inhibitor of IL-6.

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Interleukin 6 (IL-6), a phosphorylated glycoprotein, is a T cell-derived B cell stimulating factor which plays an important role in normal cell inflammatory processes, host immune responses, and modulation of cellular growth.[1-2] However, overexpression of IL-6 results in pathogenesis of autoimmunity, inflammation, and cancer.[3-4] Also, it has been seen in patients suffering from rheumatoid arthritis that activation of synoviocytes increases the production of proinflammatory cytokines including IL-6, resulting in destruction of cartilage and bones in the joints of patients.[5-7] IL-6 is also overexpressed during pancreatitis.[8-9] The therapeutic strategy of inhibition of IL-6 using anti-bodies has been emerged as an effective and alternative technique to cure inflammatory diseases that are refractory to conventional drugs.[10] Clinical studies targeting IL-6 using anti–IL-6 Abs such as sirukumab (CNTO136), olokizumab (CP6038), PF-423691, siltuximab (CNTO328), elsilimomab (BE-8), clazakizumab (BMS945429), and MEDI5117, and by anti–IL-6R Abs including sarilumab (REGN88) and tocilizumab (Actemra) are either ongoing or complete.[3] The use of antibodies targeting IL-6 has achieved a tremendous commercial successes,[11] however, suffer from major drawbacks such as high cost, invasive route of administration, and high rate of immunogenicity. Therefore, the search of small molecules as IL-6 inhibitors is warranted by their superiority in oral absorption and low antigenicity.

(+)-Madindoline A, a nontoxic small organic molecule, originally isolated by fermentation of *Streptomyces nitrosporeus* K93-0711 has displayed potent IL-6/IL-6R blocking properties (Figure 1).[12-13] Unfortunately, (+)-Madindoline A is produced in a low yield by fermentation and is difficult to synthesize chemically.

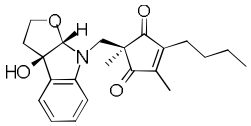
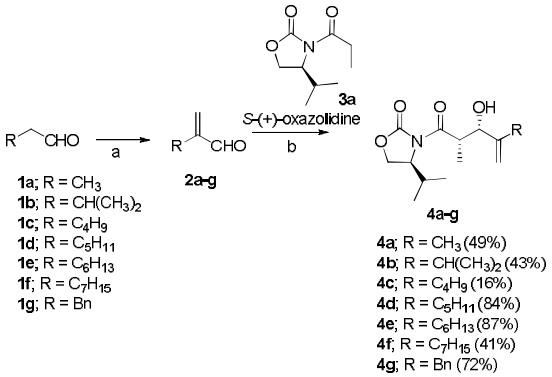


Figure 1. Structure of (+)-Madindoline A.

Recently, we reported the mode of action of compound **4d**, which suppressed the activation of STAT3 induced by IL-6, but not by leukemia inhibitory factor.[14] Furthermore, the direct interaction of **4d** with gp130 and specific reduction of IL-6/IL-6Rα complex binding to gp130 in the presence of **4d** was demonstarated by surface plasmon resonance analysis.[14] Herein, we report the structure-activity relationship and molecular modeling study of the IL-6 inhibitors.

Synthesis of IL-6 inhibitors is described in Schemes 1, 2, and 3. The building block acrolins (**2a-g**) were synthesized in 45-90% yields from commercially available aldehydes (**1a-g**) employing Mannich reaction of formaldehyde solution and dimethylamine hydrochloride with different aldehydes, followed by their subjection to Evans asymmetric aldol reaction as aldol acceptors employing (+)-oxazolidinone (**3a**) as aldol donor using Bu_2BOTf and DIPEA in CH_2Cl_2.[15,16] Compound **4h** was synthesized by Evans aldol coupling of commercially available aldehyde **2h** with (+)-oxazolidinone (**3a**) (Scheme 2).



Scheme 1. Reagents and conditions: (a) $(Me)_2NH{\cdot}HCl$, 37% HCHO; (b) *n*-Bu_2BOTf, DIPEA, CH_2Cl_2, -78 °C.[16]

Compound **4i** was synthesized by coupling of 2-pentylacrolein **2d** with *R*-(-)-oxazolidinone (**3b**) using the same reaction conditions (Scheme 1). Compound **4j** was synthesized by Evans aldol coupling of commercially available aldehyde **2i** with (+)-oxazolidinone (**3a**) (Scheme 2). Compounds **4k** and **4l** were synthesized in 68% and 14% yield, respectively by aldol reaction of aldehyde **2d** with compound **3c** using *n*-Bu_2BOTf and DIPEA in CH_2Cl_2 at -78°C (Scheme 2).

Compounds **10b** and **12b** were synthesized by using the synthetic route illustrated in scheme 3. First, tryptophol (**5**) was reduced into compound **6** in 90% yield using $NaBH_3CN$ in acetic acid, followed by protection of its free hydroxyl group with TBSCl and imidazole in dry DCM at 0°C to afford compound **7** in 93% yield (Scheme 3). Compound **7** was then coupled with chiral α-methyl β-hydroxy acid (**8**) using HATU and DIPEA in DMF to afford compound **9a** in 60% yield, followed by its oxidation with DDQ to afford compound **10a** in 74% yield. Silyl group deprotection of compound **10a** gave compound **10b** in 94% yield. Compound **12b** was synthesized from compound **9a** by reduction of its carbonyl group into CH_2 group using BH_3.DMS as reducing agent to afford **11a** in 73% yield, followed by its oxidation with DDQ to give compound **12a** in 37% yield. Silyl group deprotection of **12a** afforded compound **12b** in 36% yield (Scheme 3).

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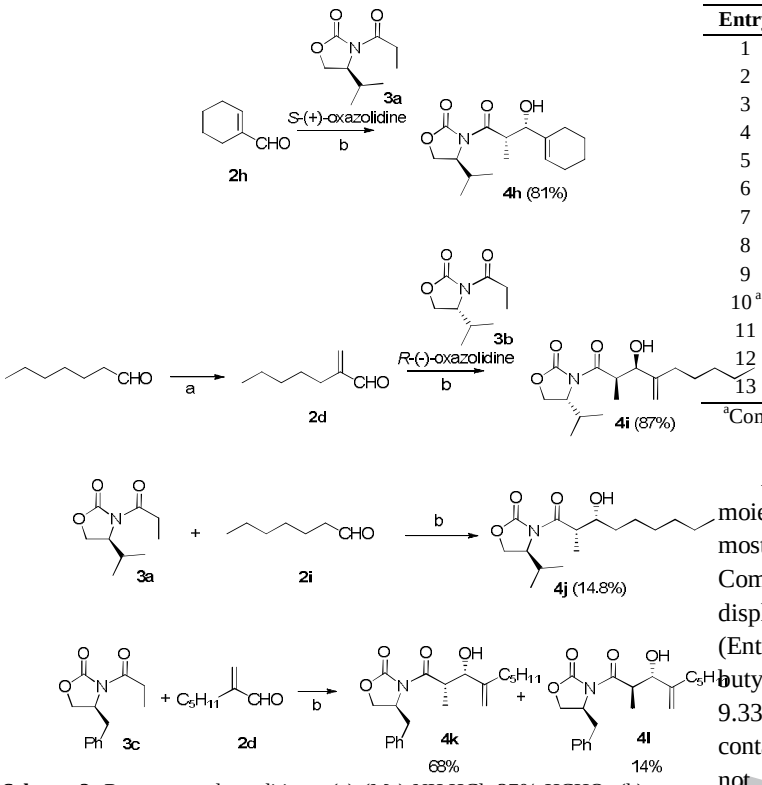
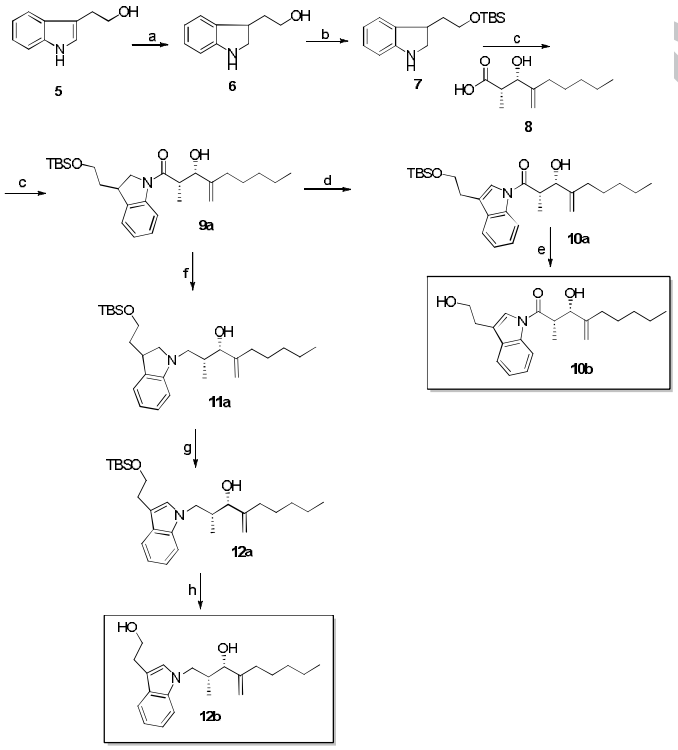


Scheme 2. Reagents and conditions: (a) $(Me)_2NH{\cdot}HCl$, 37% HCHO; (b) *n*-Bu_2BOTf, DIPEA, CH_2Cl_2, -78 °C.



Scheme 3. Reagents and conditions: (a) $NaBH_3CN$, AcOH, 37%; (b) Imidazole, TBSCl, CH_2Cl_2, 93%; (c) HATU, DIPEA, DMF, 60%; (d) DDQ, Benzene, 74%; (e) PTSA/MeOH, 94%; (f) BH_3.DMS, THF, 73%; (g) DDQ, Benzene, 37%; (h) TBAF, THF, 36%.

The synthesized compounds were screened by measuring the effects of each compound on IL-6–induced luciferase expression in human hepatocarcinoma HepG2 cells transfected with p-STAT3-Luc (Table 1).[14]

Table 1. IL-6 induced reporter gene assay of aldol products.

Entry	Compds	IC_{50} (μM)	CC_{50} (μM)
1	(+)-Madindoline A	21	>100
2	**4a**	>100	>100
3	**4b**	20.1	>100
4	**4c**	9.0	>100
5	**4d**	5.9	>100
6	**4e**	9.33	55.3
7	**4f**	>100	46
8	**4g**	6.55	>100
9	**4h**	>20	>100
10 [a]	**4i**	>20	>100
11	**4j**	>20	>100
12	**4k**	>20	>100
13	**4l**	>20	>100

[a]Compound **4i** is an enantiomer of compound **4d**.

Among compounds **4a-h** containing the *S*-(+)-oxazolidinone moiety, compound **4d** containing *n*-pentyl side chain displayed most potent activity with IC_{50} value of 5.9 μM (Entry 5, table 1). Compound **4g** (IC_{50}, 6.55 μM) containing benzyl moiety also displayed potent activity but slightly lesser than compound **4d** (Entry 8, table 1). Compounds **4b, 4c**, **4e** containing *i*-propyl, *n*-butyl, *n*-hexyl side chains displayed IC_{50} values of 20.1, 9.0, and 9.33 μM, respectively. However, compounds **4a** and **4f** containing the methyl and *n*-heptyl side chain, respectively did not show any inhibitory activity suggesting the importance of appropriate chain length for potent activity (Entries 2 and 7, Table 1). Compounds **4e** and **4f** exhibited some cytotoxicity with CC_{50} values of 55.3 and 46 μM, respectively (Entries 6-7, table 1). Compound **4h** containing 1-cyclohexene did not show inhibitory activity up to 20 μM (Entries 9, Table 1). From these results, it may be concluded that the length of side chain plays an important role in determining the activity of these compounds.

Interestingly, compound **4i** (enantiomer of compound **4d**) did not display any inhibitory activity up to 20 μM suggesting that fixed stereochemistry is required for appropriate interactions with target site (Entry 10, table 1). Compound **4j** lacking any double bond did not exhibit any inhibitory activity suggesting the importance of double bond for high activity (Entry 11, table 1). The two diastereomeric compounds **4k** and **4l** where the isopropyl moiety on oxazolidinone was replaced by benzyl moiety did not display any inhibitory activity up to 20 μM (Entries 12-13, table 1).

Compound **10b** where the oxazolidinone ring of **4d** was replaced by with indole ring showed potent inhibitory activity, but along with cytotoxicity of 17 μM (Entry 2, table 2). Compound **12b** without oxo group was more potent than **10b** but displayed CC_{50} of 10.8 μM (Entry 3, table 2).

Table 2. IL-6 induced reporter gene assay of compounds **10b** and **12b**.

Entry	Compds	IC_{50} (μM)	CC_{50} (μM)
1	(+)-Madindoline A	21	>100
2	**10b**	6.38	17
3	**12b**	1.48	10.8

In order to understand how compound **4d** inhibits IL-6/GP130/STAT3 signal transductions mechanism, we chose hexameric complex composed of the two trimers (PDB ID: 1P9M) as an initial computational modeling structure.[17-19] IL-6

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first binds to IL-6Rα to form the IL-6/IL-6Rα complex followed by binding to D2 and D3 domains of GP130 to form trimeric complex IL-6/IL-6Rα/GP130. Two trimeric complex IL-6/IL-6Rα/GP130D1 by homodimerization form hexameric complex *via* D1 domain (Figure 2A and 2B). MDL-A has been reported to show direct binding at the extracellular domain of GP130 by Saleh and co-workers.[20] MDL-A binds to the GP130 D1 domain and inhibits hexameric complex formation by disrupting interactions between IL-6 and the GP130 D1 domain.

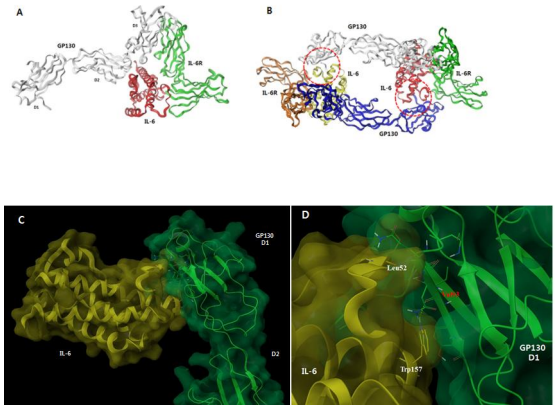

Figure 2. A) Crystal structure of the trimeric human IL-6/IL-6 alpha receptor/GP130 complex (PDB ID: 1P9M); B) Hexameric complex formed by homodimerization of IL-6/IL-6R/GP130 trimers *via* D1 domain. (Red dot circles are the region of IL-6/Gp130 interaction); C) PPIs docking between IL-6 and GP130 D1 Domain; D) Close up view of interaction sites of IL-6/GP130 complex.

Li and co-workers found that the "hot spot" was directly located in GP130 D1 domain and was represented by Leu57 and Trp157 included in the IL-6 (Figure 2C and 2D).[21]

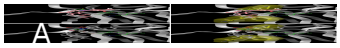

Figure 3. A) MDL-A bound to GP130 D1 domain; B) Overlapping of MDL-A with Leu52 and Trp157 of IL-6; C) Compound **4d** bound to GP130 D1 domain; D) Overlapping of **4d** with Leu52 and Trp157 of IL-6.

In our study, the binding sites of compound **4d** and MDL-A bound GP130 D1 domain were verified using Glide protein-protein docking application in Schrödinger suites. Ligand **4d** and MDL-A were drawn on the 2D sketcher and their 3D conformation was predicted using LigPrep (Figure 3).

MDL-A bound to the GP130 D1 domain showed H-bonding interactions with Asn92 and Cys6 residues. MDL-A was also located on the "hot spot" (Leu57, Trp157 in IL-6). When compound **4d** was bound to the GP130 D1 domain, it showed H-bonding interactions with Val93 and Cys6 residues. Compound **4d** was also located on the "hot spot" similar to MDL-A. Docking results revealed that **4d** is able to interrupt H-bonding interactions between IL-6 and GP130 complex similar to MDL-A.

To confirm the results of docking study, we constructed the mutant forms of gp130, V93A, and C6A, and performed the SPR assay.[22] The C6A mutation of gp130 did not change its binding properties to compound **4d** compared to wild type, whereas V93A mutation of gp130 significantly reduced the interaction with compound **4d** (Figure 4). Hydrogen bond between compound **4d** and Val93 on gp130 is critical for their interaction and seems to explain the interruption of the binding of IL-6 to gp130 by compound **4d**.

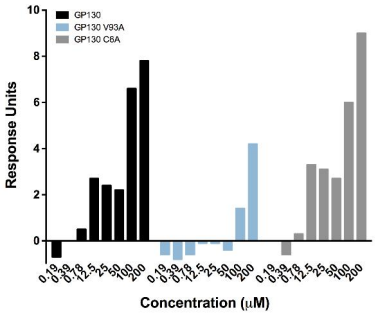

Figure 4. SPR analysis between **4d** and immobilized gp130 mutants. V93A or C6A mutation was introduced into gp130 wild type and immobilized onto the CM5 chip. **4d** was injected in the flow cells and evaluated its binding properties to gp130 mutants and wild type.

In summary, a series of compounds containing oxazolidinone and indole rings were synthesized and evaluated as IL-6 signaling blockers. The compound **4d** showed the most potent activities with IC_{50} of 5.9 μM which was much better than (+)-Madindoline A (IC_{50} = 21μM), a known inhibitor of IL-6. Also, no any cytotoxic effects with compound **4d** were observed. The indole analogues **10b** and **12b** also displayed potent IL-6 inhibitory activities but along with cytotoxic effects. It was also observed that the length of alkyl side chain and the stereochemistry is playing an important role in determining the activity and cytotoxicity of compounds. Also, binding sites of compound **4d** with gp130 were assessed by SPR analysis and docking studies.

Acknowledgments

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16. **Procedure for the synthesis of (*S*)-3-((2*S*,3*S*)-3-hydroxy-2-methyl-4-methylenenonanoyl)-4-isopropyloxazolidin-2-one (4d):** Aqueous formaldehyde solution (0.434 ml, 5.8 mmol, 37%) was mixed with **1d** (662 mg, 5.8 mmol). The mixture was stirred at room temperature for 5 minutes, followed by the addition of dimethylamine hydrochloride (440 mg, 5.0 mmol). The solution was refluxed for 48 h at 70°C. Then water (10 mL) was added and the mixture steam distilled. The distillate was extracted with diethyl ether (3x30 mL) and the whole organic phases were washed with saturated NaCl solution and dried over $MgSO_4$. The solvent was evaporated to get crude product **2d** which was used for next step without any purification (crude yield 85%): ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.52 (s, 1H), 6.23 (s, 1H), 5.97 (s, 1H), 2.21 (t, 2H, *J* = 7.8 Hz), 1.31-1.25 (m, 6H), 0.87 (t, 3H, *J* = 6.8 Hz). To the stirred solution of 4-oxazolidinone (**3a**) (2.5g, 13.5 mmol) in CH_2Cl_2 (60 ml) was added to Bu_2BOTf (16.37 ml, 16.37 mmol) and diisopropylethylamine (3.31 ml, 19.0 mmol) at 0°C. After stirring for 50 min, **2d** (3.53 g, 28.0 mmol) was added to the enolate reaction mixture at -78°C. The reaction mixture was stirred at -78°C for 20 min and then was allowed to warm at 0°C. After 1 hour of stirring at 0°C, pH 7 buffer solution (1 mL) and MeOH (2 mL) were added to the reaction mixture, followed by continuous addition of H_2O_2 (2 mL) and MeOH (2 mL) at the same temperature. After stirring the reaction mixture for 1 hour at 0°C, the solvent was removed in vacuo, followed by addition of water to the remaining mixture. The mixture was then extracted with CH_2Cl_2 (3 × 50 ml). The combined organic layer were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The organic residue was purified by column chromatography on silica gel (using 14 % ethyl acetate in hexane as eluting solvents) to afford (3.58 g, 84 %) of **4d**; Colorless oil; IR-FT (neat): ν 3527.5, 2960.0, 2929.8, 2873.4, 1773.8, 1696.8, 1685.4, 1457.6 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 5.18 (1H, s), 4.99 (1H, s), 4.50-4.47 (1H, m), 4.42 (1H, brs), 4.28 (1H, dd, *J* = 17.4, 9.0 Hz), 4.22 (1H, dd, *J* = 9.3, 2.9 Hz), 3.95 (1H, dq, *J* = 7.3, 2.9 Hz), 3.15 (1H, brs), 2.39 - 2.33 (1H, m), 2.04 - 1.90 (2H, m), 1.49 - 1.43 (2H, m), 1.34 - 1.28 (4H, m), 1.18 (3H, d, *J* = 7.3 Hz), 0.93 (3H, d, *J* = 7.1 Hz, 0.88 (3H, d, *J* = 7.1 Hz), 0.87 (3H, t, *J* = 5.4 Hz);
    ^{13}C NMR ($CDCl_3$, 125 MHz): δ 177.7, 153.4, 147.9, 110.5, 72.7, 63.3, 58.3, 40.1, 32.7, 31.6, 28.3, 27.6, 22.5, 17.9, 14.6, 14.0, 10.5; HRMS (ES+) $C_{17}H_{29}NO_4$, m/z 312.2261 $[M + H]^{1+}$
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18. **Binding Site Prediction:** Although the hexameric complex interaction of IL-6/GP130 (PDB ID : 1P9M) is known, but in order to study more detailed binding sites, we used a binding site prediction tool called “SiteMap” (SchrÖdinger Inc., Portland, OR, U.S.A.). SiteMap searches the surface of a protein for possible binding sites and scores them according to size, degrees of enclosure and exposure, tightness, hydrophobic and hydrophilic character, and physical description of hydrogen-bond donors and acceptors.
19. **Ligand docking:** We used a docking method with Glide 5.5 (SchrÖdinger Inc.). Glide is based on grids for energy scoring and ligand matching. One starts with receptor grid generation in which a grid is generated that conforms to the shape and properties of the receptor. Conformational search in Glide is done in a hierarchical way. Rough matching of ligand atom positions and grid points generates a set of possible ligand poses, which are refined through a successive optimization procedure.
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22. **Surface plasmon resonance analysis:** To examine the direct binding between compound **4d** and the extracellular domain of gp130, gp130 V93A, and gp130 C6A (ANRT, Daejeon, Korea), we performed surface plasmon resonance (SPR) analysis using BIAcore T200 model (GE Healthcare) at 25℃ with buffer HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20) containing 5% DMSO (Sigma-Aldrich). The pH scouting for immobilization was performed in 10 mM acetate buffer at pH 4.0, 4.5, 5.0, 5.5. gp130, V93A, or C6A was immobilized on a CM5 sensor chip to the 3970 response unit (RU), 4113 RU, or 4271 RU respectively with standard amine coupling at pH 4.5. Compound 4d was injected into the gp130 or mutants-immobilized flow cell at concentrations of 200, 100, 50, 25, 12.5, 0.78, 0.39, and 0.19 mM with a flow rate of 30 ml/min for 240 s and allowed to dissociate for 900 s. T-200 BIAevaluation software was used to obtain RU and subtract references.

Supplementary Material

Experimental details for the synthesis and characterization of all compounds

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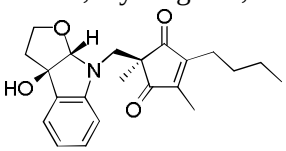
Graphical Abstract

Structure–Activity Relationship Study of a Series of Novel Oxazolidinone Derivatives as IL-6 Signaling Blockers

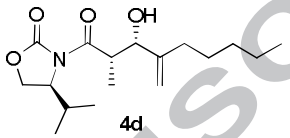
Sarbjit Singh, Veeraswamy Gajulapati, Kondaji Gajulapati, Ja-Il Goo, Yeon-Hwa Park, Hwa Young Jung, Sung Yoon Lee, Jung Ho Choi, Young Kook Kim, Kyeong Lee, Tae-Hwe Heo, Yongseok Choi



(+)-Madindoline A

IL-6–induced luciferase activity

IC_{50} = 21 μM



4d

IL-6–induced luciferase activity

IC_{50} = 5.9 μM

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