A New Application of Parallel Synthesis Strategy for Discovery of Amide-Linked Small Molecules as Potent Chondroprotective Agents in TNF-α-Stimulated Chondrocytes

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Abstract

As part of an effort to profile potential therapeutics for the treatment of inflammation-related diseases, a diversity of amide-linked small molecules was synthesized by using parallel synthesis strategy. Moreover, these new compounds were also evaluated for their inhibitory effects on nitric oxide (NO) by using tumor necrosis factor alpha (TNF-α)-induced inflammatory responses in chondrocytes. Among the tested compounds, N-(3-chloro-4-fluorophenyl)-2-hydroxybenzamide (HS-Ck) was the most potent inhibitor of NO production and inducible nitric oxide synthase (iNOS) expression in TNF-α-stimulated chondrocytes. In addition, our biological results indicated that HS-Ck might suppress the expression levels of iNOS and matrix metalloproteinases-13 (MMP-13) activities through downregulating the activation of nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT-3) transcriptional factors. Therefore, the parallel synthesis was successful used to develop a new class of potential anti-inflammatory agents as chondroprotective candidates for the treatment of osteoarthritis.

Introduction

Osteoarthritis (OA) is characterized by breakdown of collagen and aggrecan in the cartilage, which leads to chronic joint pain and disability in the middle aged and elderly patients [1, 2]. Many catabolic factors such as pro-inflammatory cytokines, matrix metalloproteinases (MMPs), and nitric oxide (NO) can lead to the progressive destruction of joints in OA.
The tumor necrosis factor alpha (TNF-α) is one of the most important catabolic cytokine in the pathogenesis of OA [7–9]. Through the activation of TNF-α-induced signaling transduction cascades, it can activate various transcriptional factors, including the nuclear factor kappa B (NF-κB) [10, 11] and the signal transducer and activator of transcription 3 (STAT-3) [12, 13], which play critical roles in the inflammation-mediated diseases. In addition, the accumulating studies report that the TNF-α is a vital cell signaling cytokine in the progression of cartilage degradation releasing several inflammatory cytokines, inflammatory mediator NO, and MMPs such as MMP-3 (stromelysin-1) and MMP-13 (collagenase-3) [14–16]. Furthermore, the degradation of type II collagen by enzymatic cleavages (MMPs) is an essential step in the loss of integrity of cartilage, especially for collagenase-3 (MMP-13) [17]. Hence, the modulation of these catabolic factors provides a principal target for the treatment of OA diseases in the development of anti-inflammatory drugs.

The “combinatorial chemistry” concept has been adopted as an efficient technique to synthesize, in parallel synthesis, more than one compound [18–20]. In addition, the parallel synthesis approach has been widely used as a powerful method in the drug development [21, 22]. Taking advantage of the concept of combinatorial chemistry, our previous studies have identified two potent amide-linked small molecules (HS-Cf and HS-Cm) from a mini-library chemical bank (containing more than 300 small molecules) by using TNF-α-induced inflammatory responses in chondrocytes as a screening tool (Fig 1) [23–25]. In our previous study, HS-Cf (NDMC077) has been found to have the chondroprotective effect that could prevent TNF-α-induced cartilage destruction through downregulating the interferon regulatory factor-1 (IRF-1) signaling [25]. Moreover, our recent reports showed that HS-Cm (NDMC101) has been found to be a potent immunomodulatory agent with anti-inflammatory activities in inflammation-related diseases [23, 24]. All encourage results and previous substantial efforts initiated quest for the development of anti-inflammatory agents, which can prevent TNF-α-induced cartilage damage in OA diseases.

In the present work, the parallel synthesis approach was used to prepare a diverse range of amide-linked small molecules through coupling different core structures of carboxylic acids with appropriate amines (Fig 2). These new compounds were also evaluated their anti-inflammatory effects on TNF-α-induced NO production in chondrocytes. On the basis of primary
screening results, HS-Ck was found to be a potent chondroprotective agent through suppressing TNF-α-induced NO production and iNOS expression in chondrocytes. In addition, the chondroprotective effects of HS-Ck on the activation of TNF-α-induced signaling transduction were further identified by reducing the MMP-13 expression and decreasing the transcriptional activation of NF-κB and STAT-3. In this study, HS-Ck can be considered as a potent structure for the development of chondroprotective agents to disrupt the TNF-α-mediated inflammatory responses in the pathogenesis of OA diseases.

Materials and Methods

Chemistry

Unless otherwise stated, all materials used were commercially available. Chemical reagents and solvents were purchased from ALDRICH and MERCK without further purification. Reactions requiring anhydrous conditions were performed in oven-dried glassware and cooled under nitrogen atmosphere. The parallel synthesis reactor was used using the BÜCHI SynCore® reactor. Melting points were determined by the BÜCHI B-545 melting point apparatus and are uncorrected. Analytical thin layer chromatography was performed with E. MERCK silica gel 60 F254. 1H Nuclear magnetic resonance (NMR) and 13C NMR spectra were recorded on AGILENT 400 MR DD2 (400 MHz). δ value is presented in parts per million (ppm) relative to TMS as an internal standard (0 ppm). Coupling constants (J) are expressed in Hz. Multiplicities were recorded as singlet (s), doublet (d), triplet (t), and double of doublet (dd). High resolution mass spectra (HRMS) were measured by FINNIGAN MAT-95XL (high resolution electron impact ionization, HREI) and FINNIGAN MAT-95S (high resolution electrospray ionization, HRESI). Spectral data are recorded as m/z values.

![Diagram of Parallel Synthesis of Amide-linked Small Molecules](image-url)
General Procedure for the Preparation of Compounds

To a solution of carboxylic acids (2 mmol) in methylene chloride (10 mL), solid 1-hydroxybenzotriazole monohydrate (0.27 g, 2 mmol) and N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.38 g, 2 mmol) were added. The mixture solutions were reacted with various anilines (4 mmol) and then stirred at room temperature for 3 days in parallel synthesis reactor. The reaction mixture was evaporated to dryness under reduced pressure and the residue was extraction with ethyl acetate, washed with 10% NaHCO₃, and H₂O. The organic phase was separated and dried with anhydrous MgSO₄, and dried in vacuo. The crude product was washed and purified by crystallization from hot ethanol and methylene chloride to obtain title compounds.

2-Oxo-N-(3-(trifluoromethyl)benzyl)-2H-pyran-5-carboxamide (Aa). The pure compound was obtained as orange powder (yield 41%). Mp: 157–158°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): δ ppm 4.69 (d, J = 6.4 Hz, 2H), 7.47 (d, J = 9.2 Hz, 1H), 7.60–7.70 (m, 4H), 7.75 (s, 1H), 8.17 (d, J = 14.4 Hz, 1H), 10.10 (t, J = 6.8 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 93.30, 101.28, 101.46, 103.29, 104.35, 110.95, 111.21, 122.80, 128.47, 129.37, 130.80, 133.25, 139.74, 148.52, 149.02, 149.16, 152.37, 160.42, 160.70, 161.52, 163.67; HRMS (ESI): calcd [M+H]+, 277.0521 (C₁₂H₁₁N₃O₃S⁺), found [M+H]+*, 240.0655 (C₁₄H₁₀NO₃⁺).

N-(4-Ethynylphenyl)-2-oxo-2H-pyran-5-carboxamide (Ah). The pure compound was obtained as orange powder (yield 41%). Mp: 157–158°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): δ ppm 4.23 (s, 1H), 7.51–7.59 (m, 5H), 7.63 (d, J = 9.2 Hz, 1H), 8.58 (d, J = 14 Hz, 1H), 11.13 (d, J = 13.6 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 116.37, 116.42, 120.06, 123.71, 126.69, 130.31, 135.75, 139.13, 141.86, 148.82, 152.49, 158.19, 192°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): δ ppm 7.70 (m, 4H), 7.93 (m, 2H), 10.61 (s, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 116.55, 116.79, 123.75, 124.63, 124.77, 128.07, 129.51, 130.42, 1135.76, 148.87, 152.60, 154.32, 156.82, 164.34; HRMS (ESI): calcd [M+]**, 297.0613 (C₁₄H₁₀F₂N₃O₃S⁺), found [M+]**, 296.0550 (C₁₄H₁₀F₂NO₃⁺).

N-(4-Chloro-2-fluorophenyl)nicotinamide (Bc). The pure compound was obtained as white powder (yield 36%). Mp: 191–192°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): δ ppm 6.20–6.98 (m, 2H), 7.39–7.49 (m, 3H), 7.86–7.93 (m, 2H), 10.61 (s, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 109.78, 109.99, 117.18, 117.32, 117.37, 117.55, 117.91, 119.01, 129.24,
11.62 (s, 1H); 13C NMR (100 MHz, DMSO-

found [M]+, 302.0732 (C15H14N2O3S+).

N-(3-Ethynylphenyl)-2-hydroxybenzamide (Ci). The pure compound was obtained as white powder (yield 75%). Mp: 127–128°C (EtOH); 1H NMR (400 MHz, DMSO-
d6): δ ppm 7.02 (m, 2H), 7.10 (tt, J = 9.2, 2.4 Hz, 1H), 7.35–7.47 (m, 3H), 7.51–7.55 (m, 1H), 7.59–7.64 (m, 2H), 7.71 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8 Hz, 1H), 10.44 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 110.11, 110.18, 110.30, 110.36, 121.69, 127.66, 128.99, 129.64, 134.79, 139.41, 141.46, 144.29,
The pure compound was obtained as white powder (yield 82%). Mp: 86–87°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.83 (d, J = 15.6 Hz, 1H), 7.40–7.47 (m, 4H), 7.55–7.65 (m, 4H), 7.87 (d, J = 8.4 Hz, 1H), 8.22 (s, 1H), 10.58 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 115.24, 115.28, 119.66, 119.69, 121.71, 122.76, 127.87, 128.22, 129.07, 130.02, 130.08, 134.52, 140.04, 140.97, 146.05, 167.61; HRMS (ESI) m/z calcd [M]+, 273.0871 (C16H12F3NO+), found [M]+, 291.0874 (C16H12F2NO+).

N-(3-(Trifluoromethyl)phenyl)cinnamamide (DF). The pure compound was obtained as orange powder (yield 90%). Mp: 133–134°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.81 (d, J = 16 Hz, 1H), 7.33–7.47 (m, 5H), 7.62–7.66 (m, 3H), 7.71 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8.4 Hz, 1H), 10.32 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 80.71, 83.44, 119.85, 121.95, 122.02, 124.11, 124.55, 126.65, 127.42, 127.84, 129.09, 129.36, 129.96, 134.61, 139.50, 140.65, 163.79; HRMS (ESI) m/z calcd [M]+, 247.0997 (C16H11NO2), found [M-H]+, 246.0924 (C16H12F2NO+).

N-(3-Ethynylphenyl)cinnamamide (Di). The pure compound was obtained as white powder (yield 89%). Mp: 133–134°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.77 (d, J = 15.6 Hz, 1H), 7.36–7.46 (m, 4H), 7.52–7.56 (m, 1H), 7.59–7.64 (m, 3H), 8.04 (dd, J = 7, 2.4 Hz, 1H), 10.42 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 116.96, 117.17, 119.12, 119.30, 119.47, 119.54, 120.57, 121.63, 127.85, 129.07, 130.03, 134.52, 136.51, 136.54, 140.85, 151.95, 154.36, 163.74; HRMS (ESI) m/z calcd [M]+, 385.0555 (C18H13F3O3S2+), found [M]+, 385.0559 (C18H15NO3S2+).

N-(3-Chloro-4-fluorophenyl)cinnamamide (Dk). The pure compound was obtained as white powder (yield 90%). Mp: 138–139°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.77 (d, J = 15.6 Hz, 1H), 7.36–7.46 (m, 4H), 7.52–7.56 (m, 1H), 7.59–7.64 (m, 3H), 8.04 (dd, J = 7, 2.4 Hz, 1H), 10.42 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 116.96, 117.17, 119.12, 119.30, 119.47, 119.54, 120.57, 121.63, 127.85, 129.07, 130.03, 134.52, 136.51, 136.54, 140.85, 151.95, 154.36, 163.74; HRMS (ESI) m/z calcd [M]+, 275.0513 (C15H12NOF2S2+), found [M]+, 275.0514 (C15H11F2S2O+).

(E)-N-(4-Chloro-2-fluorophenyl)-3-(2-chlorophenyl)acrylamide (Ec). The pure compound was obtained as white powder (yield 36%). Mp: 164–165°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.59 (d, J = 16 Hz, 1H), 7.12 (d, J = 16 Hz, 1H), 7.43–7.45 (m, 2H), 7.50–7.56 (m, 1H), 7.74–7.77 (m, 1H), 7.85–7.90 (m, 2H), 8.16 (t, J = 8.6 Hz, 1H), 10.15 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 116.05, 116.28, 122.32, 124.41, 124.64, 125.45, 125.57, 127.74, 127.79, 127.92, 128.26, 129.97, 130.13, 131.43, 131.72, 131.85, 132.39, 133.58, 136.11, 138.71, 163.53, 167.21; HRMS (ESI) m/z calcd [M]+, 309.0123 (C13H10Cl2FNO+), found [M-H]+, 308.0055 (C13H9Cl2FNO+).

(E)-N-(3-Chloro-4-fluorophenyl)-3-(2-chlorophenyl)acrylamide (Ek). The pure compound was obtained as white powder (yield 39%). Mp: 162–163°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 6.59 (d, J = 16 Hz, 1H), 6.84 (d, J = 15.6 Hz, 1H), 7.40–7.46 (m, 2H), 7.52–7.57 (m, 1H), 7.76–7.78 (m, 1H), 7.85–7.93 (m, 2H), 8.04–8.06 (m, 1H), 10.56 (s, 1H); 13C NMR (100 MHz, DMSO-d6): δ ppm 117.02, 117.23, 119.60, 120.71, 122.32, 124.77, 127.80, 127.99, 128.26, 129.98, 130.14, 131.44, 131.73, 131.85, 132.32, 133.55, 136.33, 138.71, 163.23, 167.20; HRMS (ESI) m/z calcd [M]+, 309.0123 (C13H10Cl2FNO+), found [M-H]+, 308.0051 (C13H9Cl2FNO+).

N-(4-Chloro-2-fluorophenyl)-3-phenylpropanamide (Fc). The pure compound was obtained as white powder (yield 79%). Mp: 110–111°C (EtOH); 1H NMR (400 MHz, DMSO-d6): δ ppm 2.69 (t, J = 8 Hz, 2H), 2.89 (t, J = 8 Hz, 2H), 7.15–7.27 (m, 6H), 7.45 (dd, J = 10.8,
3-Phenyl-N-(3-(Trifluoromethyl)phenyl)propanamide (FF). The pure compound was obtained as orange powder (yield 88%). Mp: 228–229°C (EtOH); 1H NMR (400 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 6.99 (s, 2H), 6.95 (d, J = 7.6 Hz, 2H), 2.91 (t, J = 8 Hz, 2H), 7.15–7.19 (m, 1H), 7.23–7.28 (m, 4H), 7.36 (d, J = 8 Hz, 1H), 7.52 (t, J = 8 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H), 8.08 (s, 1H), 10.25 (s, 1H); 13C NMR (100 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 108.11, 118.53, 124.39, 126.02, 126.48, 147.62, 147.75, 150.04, 150.17, 157.72; HRMS (EI): \( \text{calcd } [\text{M}]^+, 287.0711 \) (C\(_{14}\)H\(_{13}\)ClFNO\(_2\)), found [\( \text{M}]^+, 287.0711 \).

3-Phenyl-N-(4-(N-(thiazol-2-yl)sulfamoyl)phenyl)propanamide (Ff). The pure compound was obtained as orange powder (yield 72%). Mp: 206–207°C (EtOH); 1H NMR (400 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 4.72 (s, 2H), 6.95–7.01 (m, 3H), 7.27–7.33 (m, 2H), 7.77–7.83 (m, 1H), 10.42 (s, 1H); 13C NMR (100 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 67.07, 114.70, 119.31, 121.31, 126.73, 129.59, 138.84, 141.35, 157.77, 167.27; HRMS (EI): \( \text{calcd } [\text{M}]^+, 279.0467 \) (C\(_{14}\)H\(_{14}\)F\(_2\)NO\(_3\)S\(_2\)), found [\( \text{M}]^+, 279.0467 \).

\( \text{N-(3,4-Difluorophenyl)-2-phenoxyacetamide (Gb).} \) The pure compound was obtained as white powder (yield 83%). Mp: 110–111°C (EtOH); 1H NMR (400 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 6.95–7.02 (m, 2H), 7.33 (m, 2H), 7.51 (dd, J = 10.4, 2.4 Hz, 1H), 7.84 (t, J = 8.8 Hz, 1H), 9.97 (s, 1H); 13C NMR (100 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 108.68, 108.90, 114.72, 116.07, 116.10, 116.13, 116.16, 117.40, 117.58, 121.33, 129.58, 123.65, 135.39, 135.48, 144.27, 144.40, 146.68, 146.81, 147.62, 147.75, 150.04, 150.17, 157.72, 167.01; HRMS (EI): \( \text{calcd } [\text{M}]^+, 306.0674 \) (C\(_{14}\)H\(_{14}\)F\(_2\)NO\(_3\)S\(_2\)), found [\( \text{M}]^+, 306.0680 \).

\( \text{N-(4-Chloro-2-fluorophenyl)-2-phenoxyacetamide (Gc).} \) The pure compound was obtained as white powder (yield 68%). Mp: 90–91°C (EtOH); 1H NMR (400 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 6.95–6.99 (m, 3H), 7.26–7.33 (m, 2H), 7.78–7.83 (m, 1H), 10.30 (s, 1H); 13C NMR (100 MHz, DMSO-\textit{d}_6): \( \delta \) ppm 114.69, 115.81, 120.05, 120.09, 121.29, 123.28, 125.44, 129.28, 129.56, 136.15, 141.03, 158.13, 167.15; HRMS (EI): \( \text{calcd } [\text{M}]^+, 310.027 \) (C\(_{14}\)H\(_{13}\)ClFNO\(_3\)S\(_2\)), found [\( \text{M}]^+, 293.1034 \) (C\(_{13}\)H\(_{14}\)F\(_2\)NO\(_3\)S\(_2\)).
$J = 5.2 \text{ Hz}, 1H), 10.47 (s, 1H), 11.67 (s, 1H); ^{13}C\ NMR (100 \text{ MHz, DMSO-}d_{6}): \delta \text{ ppm } 23.27, 66.99, 114.63, 118.84, 121.26, 129.78, 134.75, 142.22, 156.55, 157.74, 167.34; \text{HRMS (EI) } m/z \text{: calcld } [M]^+ , 398.1049 (C_{19}H_{18}N_4O_4S^+), \text{found } [M]^+ , 398.1049 (C_{19}H_{18}N_4O_4S^+).

N-(3-Chloro-4-fluorophenyl)-2-phenoxyacetamide (Gk). The pure compound was obtained as white powder (yield 69%). Mp: 102–103°C (EtOH); $^1H\ NMR (400 \text{ MHz, DMSO-}d_6): \delta \text{ ppm } 4.69 (s, 2H), 6.95–7.01 (m, 3H), 7.29–7.33 (m, 2H), 7.38 (t, $J = 9.2 \text{ Hz}, 1H), 7.55–7.59 (m, 1H), 7.95 (dd, $J = 7, 2.8 \text{ Hz}, 1H), 10.28 (s, 1H); ^{13}C\ NMR (100 \text{ MHz, DMSO-}d_6): \delta \text{ ppm } 114.71, 116.85, 117.07, 119.03, 119.21, 120.09, 120.16, 121.22, 121.31, 129.56, 131.60, 135.63, 152.17, 154.59, 157.69, 167.01; \text{HRMS (EI) } m/z \text{: calcld } [M]^+ , 279.0462 (C_{14}H_{11}ClFNO_2^+), \text{found } [M]^+ , 279.0439 (C_{14}H_{11}ClFNO_2^+).

X-ray Crystallography

A single crystal of suitable size for X-ray diffractometry was selected under a microscope and mounted on the tip of a glass fibre, which was positioned on a copper pin. Crystallographic assay was performed as described in the reported protocol [26, 27]. The X-ray data for HS-Dc and HS-Gc were collected with a BRUKER Kappa CCD diffractometer, employing graphite-monochromated Mo-$\text{K} \alpha$ radiation at 200 K and the $\theta$–$2\theta$ scan mode. The space group for HS-Dc and HS-Gc were determined on the basis of systematic absolences and intensity statistics, and the structure of HS-Dc and HS-Gc were solved by direct methods using SIR92 or SIR97 and refined with SHELXL-97.

Ethics Statement

Human cartilage from OA patients who received total knee joint replacement were obtained aseptically with prior approval of the Institutional Review Board (IRB), Tri-Service General Hospital (Permit Number 1-102-05-091). This study was conducted under the guidelines of the Helsinki Declaration and approved by the Human Subjects Protection Offices (IRB) at the Tri-Service General Hospital. Because all participants provided their written consent to participate in this study, informed consent was obtained by the approving IRB (TSGHIRB No. 1-102-05-091). In this study, we chose the Taiwan Black Pig (approximate age 8–10 months) as breeding pigs from Taoyuan farm in Taiwan. All pigs were sacrificed for routine farm purposes. These breeding pigs were euthanized by electrocution. The isolation and culture of sampling chondrocytes were obtained from the hind leg joints of pigs (n = 16) for all experiments. The care of the pigs, and all procedures were performed according to institutional guidelines, and were approved by the Ethics Committee of Council of Agriculture, Executive Yuan, R.O.C. (Taiwan).

Isolation and Culture of Chondrocytes

The porcine cartilages were obtained from the hind leg joints of pigs. The preparation of chondrocytes from cartilage was performed according to our previous report [25]. Briefly, the extracted cartilages were firstly minced into small pieces and chondrocytes were isolated by enzymatic digestion of articular cartilage with 2 mg/mL protease in serum-free Dulbecco’s modified Eagle’s medium (DMEM)/antibiotics, the specimens were then digested overnight with 2 mg/mL collagenase I and 0.9 mg/mL hyaluronidase in DMEM containing 10% fetal bovine serum (FBS). The cells were collected, passed through a cell strainer (Becton Dickinson, Mountain View, CA, USA), and cultured in DMEM containing 10% FBS and antibiotics for 3–4 days before use.

Human cartilage from OA patients who received total knee joint replacement were obtained aseptically with prior approval of the Institutional Review Board, Tri-Service General Hospital.
The preparation of first passage chondrocytes from cartilage was performed according to the previous studies [28, 29]. In brief, the full thickness articular cartilage was removed from the underlying bone and cut into pieces of around 0.5 cm². After enzymatic digestion with 2 mg/mL Pronase (Calbiochem, La Jolla, CA) in serum-free DMEM/antibiotics (Gibco-BRL, Gaithersberg, MD) for 1 h at 37°C in 5% CO₂ atmosphere, the specimens were then digested with collagenase I at 0.25 mg/mL in DMEM medium containing 5% FBS for overnight. Finally, the cells in monolayer culture were suspended and cultured in DMEM medium containing 10% fetal bovine serum and antibiotics for 5–7 days before use.

**Cell Viability Assay**

The cytotoxic effects of synthesized compounds were evaluated by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay [30]. In brief, chondrocytes were seeded into 24-well culture plate at a density of 5×10⁴ per well and then were incubated in the presence or absence of tested compounds for 24 h. Then, 100 μL of MTT (5 mg/mL in H₂O) was added, and cells were incubated at 37°C for 6 h followed by the addition of 100 μL of DMSO. After incubation at 37°C for another 30 min, the content of dissolved reduced MTT crystals were measured with a plate reader (TECAN, Grodig, Austria).

**Measurement of NO concentrations**

The measurement of NO release was reflected by determination of its stable end product, nitrite, in supernatants [29, 30]. The Griess reaction was performed with the concentrations of nitrite measured by a spectrophotometer. In brief, an aliquot (100 μL) of culture supernatant was incubated with 50 μL of 0.1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% N-1-naphthyl-ethylenediamine dihydrochloride. After 10 min of incubation at room temperature, the absorbance was measured at 550 nm wavelength with a plate reader (Tecan, Grodig, Australia).

**Western Blotting Assay**

Enhanced chemiluminescence (ECL) Western Blotting (Amersham-Pharmacia, Arlington Heights, IL, USA) was performed as detailed in our previous reports [29, 30]. Briefly, equal amounts of whole cellular extracts were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with Tris-buffered saline with 1% Triton X-100 containing 5% nonfat milk for 1 h and then blotted with antibodies against specific proteins for another 2 h at room temperature. After washing with milk buffer, the filter was incubated with rabbit anti-goat IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase at a concentration of 1:5,000 for 30 min. The filter was incubated with the substrate and then exposed to X-ray film (GE Healthcare, Buckinghamshire, UK).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extract preparation and EMSA analysis were performed as detailed in our previous reports [29, 30]. The oligonucleotides containing NF-κB-binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and STAT-3-binding site (5′-CAG AAG GAG AAG CCC TTG CC-3′) were purchased from Promega and used as DNA probes. The DNA probes were radio-labeled with [γ-³²P]ATP using the T4 kinase (Promega). For the binding reaction, the radiolabeled probe was incubated with 4 μg of nuclear extracts. The binding buffer contained 10 mM
Tris–HCl (pH = 7.5), 50 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 1 mM MgCl₂, 4% glycerol, and 2 μg poly(dI-dC). The reaction mixture was left at room temperature to proceed with binding reaction for 20 min. The final reaction mixture was analyzed in a 6% non-denaturing polyacrylamide gel with 0.5xTris/borate/EDTA as an electrophoresis buffer.
Results and Discussion

Chemical Synthesis

In this study, the parallel synthesis was successful used to synthesize a diversity of amide-linked small molecules. In the present work, several carboxylic acids, including coumalic acid [31], salicylic acid [32], nicotinic acid [33], cinnamic acid [34], 2-chlorocinnamic acid [35], hydrocinamic acid [36], and 2-phenoxyacetic acid [37], have been found to have wide variety of biological activities. Therefore, these carboxylic acids were selected as our core structures for the preparation of targeted compounds. The synthetic procedures were carried out by coupling the carboxylic acids with the appropriate amines in the presence of 1-hydroxybenzotriazole (HOBt), N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC), and dichloromethane (DCM) at room temperature for 3 days (Fig 3). The byproducts were further separated and purified by employing their different physicochemical properties using recrystallization and chromatography procedures. The structures of all synthesized compounds are elucidated along with their spectroscopic characterizations in the experimental section. Furthermore, two synthesized structures (HS-Dc and HS-Gc) were confirmed by using the single-crystal X-ray crystallography (Fig 4).

Effects of all Synthesized Compounds on TNF-α-induced NO Production in Chondrocytes

In order to evaluate the effects of all synthesized compounds on anti-inflammatory activities, TNF-α-induced NO production in porcine chondrocytes are used as a screening model in this study. As shown in Table 1, the highest percent inhibition values of NO production were for three compounds 8 (HS-Ck), 11 (HS-Dc), and 6 (HS-Cb) at 10 μM (78.66 ± 0.21%, 50.24 ± 0.50%, and 43.16 ± 0.57%, respectively). Further, the most potent compound HS-Ck was found to dose-dependently suppress TNF-α-induced NO production in porcine chondrocytes with IC₅₀ value of 7.15 ± 2.25 μM. The inhibitory effects of HS-Ck on TNF-α-induced NO production in porcine chondrocytes were significantly suppressed at concentrations of 2.5, 5, and 7.5 μM (Fig 5A). To investigate whether HS-Ck did not affect the viability of porcine

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**Fig 4. X-ray structures of the synthesized compounds (HS-Dc and HS-Gc).**

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chondrocytes, MTT assay was used for identifying the cell survival rate. The observation of HS-Ck proves that its inhibitory activity was not due to its cytotoxic effect in porcine chondrocytes (Fig 5B).

In addition, the inhibitory effects of HS-Ck on TNF-α-induced NO production in human chondrocytes were significantly suppressed at concentrations of 2.5, 5, 7.5, and 10 μM (Fig 6A). HS-Ck was also found to dose-dependently decrease TNF-α-induced NO production in human chondrocytes with IC50 value of 7.15 ± 0.25 μM. Interestingly, the observation of HS-Ck proves that its inhibitory activity was also not due to its cytotoxic effect in human chondrocytes (Fig 6B).

Based on these primary results of TNF-α-induced NO releases, the SARs of all synthesized compounds are briefly summarized as follows. Amongst compounds bearing the carboxylic

| Compound | Name   | Inhibition of NO production (%) ± SD |
|----------|--------|-------------------------------------|
| 1        | HS-Aa  | 0 ± 1.02                            |
| 2        | HS-Ah  | 0 ± 1.14                            |
| 3        | HS-Bc  | 4.06 ± 0.96                         |
| 4        | HS-Bd  | 10.24 ± 0.90                        |
| 5        | HS-Bf  | 3.48 ± 0.97                         |
| 6        | HS-Ci  | 43.16 ± 0.57                        |
| 7        | HS-Ck  | 78.66 ± 0.21                        |
| 8        | HS-Da  | 35.94 ± 0.64                        |
| 9        | HS-Db  | 11.79 ± 0.88                        |
| 10       | HS-Dc  | 50.24 ± 0.50                        |
| 11       | HS-Dd  | 9.08 ± 0.91                         |
| 12       | HS-De  | 10.82 ± 0.89                        |
| 13       | HS-Ec  | 23.19 ± 0.77                        |
| 14       | HS-Ek  | 10.68 ± 0.89                        |
| 15       | HS-Ek  | 4.79 ± 0.95                         |
| 16       | HS-Fj  | 0.87 ± 0.99                         |
| 17       | HS-Fj  | 5.88 ± 0.82                         |
| 18       | HS-Gc  | 0 ± 1.12                            |
| 19       | HS-Gb  | 0 ± 1.16                            |
| 20       | HS-Gd  | 5.88 ± 0.82                         |
| 21       | HS-Gf  | 1.09 ± 0.99                         |
| 22       | HS-Gh  | 9.59 ± 0.91                         |
| 23       | HS-Gk  | 1.96 ± 0.98                         |

Porcine chondrocytes were pretreated with all synthesized compounds at 10 μM for 2 h, and then stimulated with TNF-α (5 ng/mL) for 24 h. The amount of nitrite with TNF-α-treated only group was set as 100.0%. Inhibition (%) = 100% — NO production (%) of compounds. NO concentration in medium was determined using the Griess reagent. All representative data were performed the means ± SD at least three times independently (n > 3).

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Table 1. Effects of all synthesized compounds on TNF-α-induced NO production in porcine chondrocytes.
acid moieties, we found that the salicylic acid skeleton showed relatively higher anti-inflammatory activities than the others. Amongst compounds bearing the di-substituted groups at the aniline moieties, we observed that these compounds showed relatively higher activities than the mono-substituted groups. On the basis of TNF-α-induced NO production results, we conclude that N-(3-chloro-4-fluorophenyl)-2-hydroxybenzamide (HS-Ck) is the most potent anti-inflammatory and/or immunomodulatory agent for further study. Thus, we chose HS-Ck that was the most potent among the synthesized series for the mechanistic investigation in TNF-α-induced inflammatory signaling pathway.
Effects of HS-Ck on TNF-α-induced Expression Levels of iNOS and MMP-13 in Porcine Chondrocytes

The accumulating evidences showed that the inflammatory cytokine TNF-α contribute to an increased NO production and the activation of inducible nitric oxide synthase (iNOS) pathway [38, 39]. Moreover, the iNOS-NO system has been demonstrated to be the essential factor in
OA pathogenesis [40–42]. Therefore, we further determine whether HS-Ck would suppress the TNF-α-induced expression levels of iNOS in porcine chondrocytes by using Western Blotting assay. As shown in Fig 7, the results showed that the expression levels of iNOS were significantly suppressed after treatment with HS-Ck at concentrations of 2.5, 5, 7.5 μM. Thus, these results revealed that HS-Ck could significantly decrease the TNF-α-induced iNOS expression in porcine chondrocytes.

In addition, the enzymatic cleavage of MMP-13 plays a key role in the pathogenesis of cartilage degradation [17]. Since type II collagen is preferentially cleaved by MMP-13, the accumulating studies showed that MMP-13 can be considered as a principle target for the treatment of OA [43–45]. In order to investigate the chondroprotective effects of HS-Ck, the suppressive effects of HS-Ck on TNF-α-induced pro-MMP-13 expression levels in porcine chondrocytes were further examined by using Western Blotting assay. This assay can analyze the pro-MMP-13 activity through determining the expression levels of TNF-α-induced pro-MMP-13 activity in the culture supernatants. As shown in Fig 8, the results showed that the expression levels of pro-MMP-13 were significantly suppressed after treatment with HS-Ck at concentrations of 5 and 7.5 μM. Altogether, the above results revealed that HS-Ck could significantly suppressive the TNF-α-induced expression levels of iNOS and MMP-13 in porcine chondrocytes.
Effects of HS-Ck on TNF-α-induced Activation of NF-κB and STAT-3 Transcriptional Factors in Porcine Chondrocytes

Since the activation of NF-κB and STAT-3 transcriptional factors play vital roles in inflammatory responses [10, 11], these transcriptional factors are also important in regulating iNOS and MMP-13 activities [14–16]. In addition, the inflammatory effects of TNF-α can induce different signaling pathway, including NF-κB and STAT-3 activation, which are responsible of increased NO production and MMP-13 activity. To evaluate the chondroprotective effects of HS-Ck on TNF-α-induced activation of NF-κB and STAT-3 transcriptional factors in porcine chondrocytes, we further investigated the suppressive effects of HS-Ck on TNF-α-induced transcriptional activation of NF-κB and STAT-3 by using electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions.

As shown in Fig 9, the expression levels of NF-κB and STAT-3 were increased by TNF-α treatment. However, such elevated levels were reduced by treatment with HS-Ck at 7.5 μM. Our results showed that the expression levels of NF-κB and STAT-3 were significantly
suppressed after treatment with HS-Ck at 7.5 μM. Thus, the mechanism of action of HS-Ck might reduce TNF-α-induced inflammatory responses through suppressing the activation of NF-κB and STAT-3 transcriptional factors in this study.

**Conclusion**

In a continuous interest in the development of anti-inflammatory agents, the parallel synthesis strategy was used for the development of a new class of chondroprotective agents in osteoarthritis therapeutics. In the present study, bioassay screening of amide-linked small molecules revealed that HS-Ck was the most potent inhibitor of NO production and iNOS expression in TNF-α-induced chondrocytes. In addition, our biological results indicated that HS-Ck a potent chondroprotective agent toward TNF-α-induced cartilage degradation. The proposed mechanism of this study is showed in Fig 10. The chondroprotective effects of HS-Ck might decrease iNOS-NO production and MMP-13 activity through suppressing the activation of NF-κB and STAT-3 transcriptional factors. Thus, HS-Ck could be considered as a new potent chondroprotective agents in OA therapeutics.
Supporting Information

S1 Fig. Spectroscopic characterizations (1H NMR, 13C NMR, and HRMS spectra) of HS-Ck.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CCL LJH JHL FCL HSH. Performed the experiments: CCL YL FCL CLC TCC. Analyzed the data: CCL FCL. Contributed reagents/materials/analysis tools: LJH JHL SBL LCL FCL HSH. Wrote the paper: CCL FCL HSH. Synthesized chemical compounds: CCL YL.

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Fig 10. Proposed mechanism of action for the chondroprotective effects of HS-Ck on TNF-α-induced cartilage degradation in porcine chondrocytes includes the suppressive effects on activation of NF-κB and STAT-3 transcriptional factors, iNOS expression, NO production, and MMP-13 activities.
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