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A salicylate-based small molecule HS-Cm exhibits immunomodulatory effects and inhibits dipeptidyl peptidase-IV activity in human T cells

Jun-Ting Liou [a,b], Hsu-Shan Huang [c], Meng-Lin Chiang [d], Chin-Sheng Lin [b], Shih-Ping Yang [b], Ling-Jun Ho [e], Jenn-Haung Lai [a,f,*]

[a] Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan, ROC
[b] Division of Cardiology, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, ROC
[c] School of Pharmacy, National Defense Medical Center, Taiwan, ROC
[d] Graduate Institute of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, ROC
[e] Institute of Cellular and System Medicine, National Health Research Institute, Zhunan, Taiwan, ROC
[f] Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, No. 5, Fusing St., Gueishan Township, Tao-Yuan County, Taiwan, ROC

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ABSTRACT

Activated T cells are key players in chronic inflammatory diseases, including atherosclerosis. Salicylates, like aspirin, display not only anti-inflammatory, anti-thrombotic, anti-atherosclerotic activities, but also immunomodulatory effects in T cells at high dosages. Here, we aimed to identify potent immunomodulators for T cells through cell-based screening from a mini-library of 300 salicylate-based small molecules, and elucidate the mechanisms. Human peripheral blood T cells were isolated from buffy coat. Phorbol 12-myristate 13-acetate plus ionomycin (P/I) was used to stimulate T cells. Cytokine production was measured by enzyme-linked immunosorbent assays. T cell activation markers were determined by flow cytometry. The activation of transcription factors and kinases was analyzed by western blotting, electrophoretic mobility shift assay, or kinase assay. Through library screening, we identified a small molecule named HS-Cm [$C_{13}H_9ClFNO_2$; *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide] that exhibited potent immunomodulatory effects on T cells with low cytotoxicity. In P/I-stimulated T cells, HS-Cm inhibited the production of interleukin-2, tumor necrosis factor-alpha, and interferon-gamma and suppressed the expression of surface activation markers CD25, CD69, and CD71, but not CD45RO. HS-Cm down-regulated DNA-binding activities of activator protein-1 and nuclear factor-kappa B, but not nuclear factor of activated T-cells, through inhibiting c-Jun N-terminal kinase/p38 and inhibitor of kappaB alpha (IκBα) kinase (IKK)/IκBα pathways, respectively. On the basis of structure-activity relationship, HS-Cm exerted considerable inhibition of dipeptidyl-peptidase IV/CD26 activity in T cells. Our results suggested that the small molecule HS-Cm exhibiting immunomodulatory effects on T cells may be useful for therapeutics in chronic inflammatory diseases, like atherosclerosis, diabetes and autoimmune arthritis.

1. Introduction

Non-resolving inflammation, though not the primary cause, contributes significantly to the pathogenesis of various chronic inflammatory diseases (Nathan and Ding, 2010). The difficulty in removing or identifying precise identity of the inflammatory stimulus in these diseases provides a rationale for the development of anti-inflammatory therapies, targeting specific immune effector cells, cytokines, signaling molecules or pathways (Tabas and Glass, 2013).

Atherosclerosis is one of the best-known examples of chronic inflammatory disease and remains a major cause of death in humans worldwide (Hansson, 2005). T cells have been identified in all stages of atherosclerotic lesions and are known to strongly influence disease severity (Hansson et al., 2006). Through cytokine production and interplay among different immune effector cells in atherosclerotic plaque, activated T cells contribute to amplified and sustained inflammation, lesion growth, and disease progression (Koltsova et al., 2012). Surprisingly, the activation of peripheral blood T cell is also demonstrated in patients with coronary artery disease, in particular acute coronary syndrome (Caligiuri et al., 1998; Methe et al., 2005). Thus, T cells may serve as targets for the novel and unconventional anti-atherosclerotic therapy

* Corresponding author at: Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, No. 5, Fusing St., Gueishan Township, Tao-Yuan County, Taiwan, ROC. Tel.: +886 2 8792 7135; fax: +886 2 8792 7136.
E-mail address: laiandho@gmail.com (J.-H. Lai).

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(Goronzy and Weyand, 2006). Immunomodulatory agents that inhibit T cell activation and subsequent cytokine production may provide anti-atherosclerotic effects. In addition to atherosclerosis, activation of T cells has been implicated in the pathogenesis of several chronic inflammatory disorders, such as diabetes, rheumatoid arthritis, chronic obstructive pulmonary disorder, and inflammatory bowel disease (Cosio et al., 2009; Hansson et al., 2006; Monteleone et al., 2011; Sell et al., 2012; Smolen and Steiner, 2003). It is therefore reasonable to target T cells in the treatment of these chronic inflammatory disorders.

Salicylate-based drugs, and in particular acetylsalicylic acid (best known as aspirin), are commonly employed for treating a wide variety of inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease. Aspirin is the most commonly used salicylate on account of its broad therapeutic indications including prevention of myocardial infarction and ischemic stroke, treatment of pain, fever, and inflammatory disorders (Awtry and Loscalzo, 2000). It is generally accepted that the main therapeutic effect of aspirin comes from inhibition of cyclooxygenase (COX) by acetylation and subsequent reduction of prostanoids biosynthesis. However, it has been established that the anti-inflammatory properties of aspirin involve additional mechanisms independent of COX inhibition (Tegeder et al., 2001). Salicylate, the active metabolite of aspirin, may play more important roles in the anti-atherosclerotic effects of aspirin (Jaichander et al., 2008). Moreover, aspirin has been shown to reduce atherosclerosis by inhibiting fractalkine expression in atherosclerotic plaques of murine models (Liu et al., 2010) providing more evidence for anti-atherosclerotic potential of salicylate.

The COX-independent pharmacological actions of aspirin and salicylates demonstrated in different models are mediated by the inhibition of several transcription factors, such as nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T-cells (NFAT), all essential signaling pathways for T-cell proliferation, survival, and cytokine production (Aceves et al., 2004; Kopp and Ghosh, 1994; Yin et al., 1998). Therefore, salicylates with more potent immunomodulatory effects may provide more therapeutic potential and benefit.

There is continuing interest in developing salicylate/salicylic acid-based small molecules potential in tuning cellular response for different therapeutic purposes (Kim et al., 2012). We previously reported a novel small molecule HS-Cf with potential therapeutic activity in the treatment of osteoarthritis (Liu et al., 2011). In the current study, we used cell-based screening of the same mini-library and identified another novel small molecule, designated HS-Cm [$C_{13}H_9ClFNO_2$; *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide], with unique immunomodulatory effects on human T cells. Surprisingly, HS-Cm preserved considerable inhibitory effect on the enzymatic activity of dipeptidyl peptidase IV (DPP4)/CD26. We also evaluated the molecular mechanisms of HS-Cm on T-cell inhibition.

2. Materials and methods

2.1. Chemicals and reagents

The 300 synthetic small molecules were structurally similar and consisted of core amino compounds (aniline) coupled with carboxylic acids (salicylic acid) via peptide (amide) bonding and finally synthesized with different modifications and conjugations. The structure of HS-Cm is depicted in Fig. 1 and the synthesis process is explained in Supplementary materials. All compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM. For each experiment, the stock solution was further diluted in culture medium to the desired concentration with a final DMSO concentration of 0.05% as indicated.

2.2. Isolation of human peripheral blood T cells

Human T lymphocytes were isolated and purified by negative selection from the buffy coat of whole blood obtained from the Taipei Blood Bank (Taipei, Taiwan) according to our previous report (Lai et al., 2001). After collection, T cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2 and were used for single experiments within 24 h.

2.3. Cell treatment and stimulation

Cells were pretreated with small molecules at various concentrations or 0.05% DMSO as a vehicle control for various time as indicated. For cell activation, T cells were stimulated with 5 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO, USA) and 1 μM ionomycin (Sigma; PMA+ionomycin: P/I). After stimulation, cell pellets or supernatants were collected at different time points for analysis.

2.4. Measurement of cytokine production by enzyme-linked immunosorbent assay (ELISA)

Human T cells were seeded in 24-well plate at a density of 1×10^6 cells/mL, followed by treatment with various compounds and stimulation with P/I as indicated in the figure legends. Concentrations of interleukin (IL)-2, interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α) in culture supernatants were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) and our previous report (Yang et al., 2004). The IC_{50} value (concentration of compound causing 50% inhibition of cytokine release) of each compound was calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

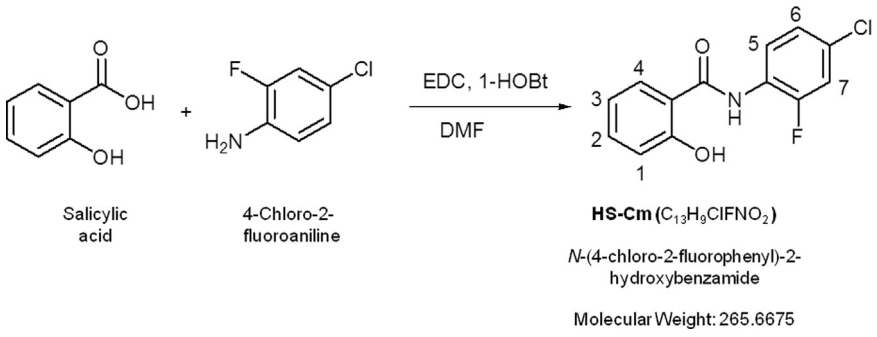

Fig. 1. Structure and chemical synthesis of HS-Cm. Chemical structures of HS-Cm [$C_{13}H_9ClFNO_2$; *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide], salicylic acid, 4-chloro-4-fluoronaniline. DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole monohydrate; *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).

2.5. Lactate dehydrogenase (LDH) cytotoxicity assays

Cytotoxicity of small molecule in human T cells (1×10^6 cells/mL in 24-well plate) was estimated by measuring the release of LDH into the culture medium. The LDH activity was quantified using a cytotoxicity detection kit (LDH; Roche, Indianapolis, IN, USA). Maximum LDH release was determined by lysing equal amounts of cells with 1% Triton X-100 and spontaneous LDH release (low control) was determined by incubating cells with medium alone. The percent cytotoxicity was calculated as:(experimental release − spontaneous release)/(maximum release–spontaneous release) × 100.

2.6. Flow cytometry analysis of cell-surface molecule expression

After treatment as indicated in the figure legend, cells (5×10^5 cells/mL) were washed, collected, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-IL-2 receptor alpha (anti-IL-2Rα or anti-CD25), anti-CD69, anti-CD71, anti-CD45RO, or FITC-conjugated isotype-matched monoclonal antibodies (Pharmingen) at 4 °C for 30 min. Cells were then washed with PBS and fixed with 0.5% paraformaldehyde in PBS. The expression of cell-surface molecules was determined using a flow cytometer (Becton Dickinson FACS Calibur) with CellQuest (Becton Dickinson) software. The mean fluorescence intensity from 10,000 cells was acquired, and the values were used to represent the expression levels of surface molecules.

2.7. Electrophoretic mobility shift assay (EMSA)

Human T cells (2×10^6 cells/mL) in 10-cm culture dishes and serum-starved (1% FBS) condition were treated as indicated in the figure legend. EMSA of nuclear extracts was performed as detailed in our previous report (Yang et al., 2003). Oligonucleotides encoding AP-1, NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or NFAT (Promega, Madison, WI, USA) were used as DNA probes. These DNA probes were radiolabeled with [γ-^{32}P] ATP by T4 kinase according to the manufacturer's instructions (Promega). Competition assays with 100-fold molar excess of unlabeled AP-1 or NF-κB probes were performed to verify the binding specificity. Unlabeled nonspecific oligonucleotides (Santa Cruz Biotechnology) were also included as a negative control.

2.8. Western blot analysis

Nuclear, cytoplasmic, and total lysate proteins were extracted from treated cells (2×10^6 cells/mL in 10-cm culture dishes) and quantified by Bradford Assay (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts of protein were subjected to western blotting, as described previously (Lai et al., 2001; Liou et al., 2008). Membranes were blotted with primary antibodies against c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, p65, p50, IκBα (Santa Cruz Biotechnology), β-actin (Chemicon, Temecula, CA, USA), IκBα kinase (IKK; IKKα or IKKβ), phosphorylated JNK (p-JNK), p-ERK, p-p38, or p-c-Jun (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP; Chemicon), positive immunoreactivity was detected with the ECL substrate (Amersham Bioscience) and exposed to BioMax Light Film (Kodak).

2.9. Immunoprecipitation kinase assay

A GST-IκBα-fusion protein purified from *Escherichia coli* transfected with pGEX-4T-2-IκBα was used as a substrate for IKKα and IKKβ (Ho et al., 2004). Rabbit polyclonal antibodies for IKKα (H-744, sc-7218) and goat polyclonal antibodies for IKKβ (T20, sc-7330)

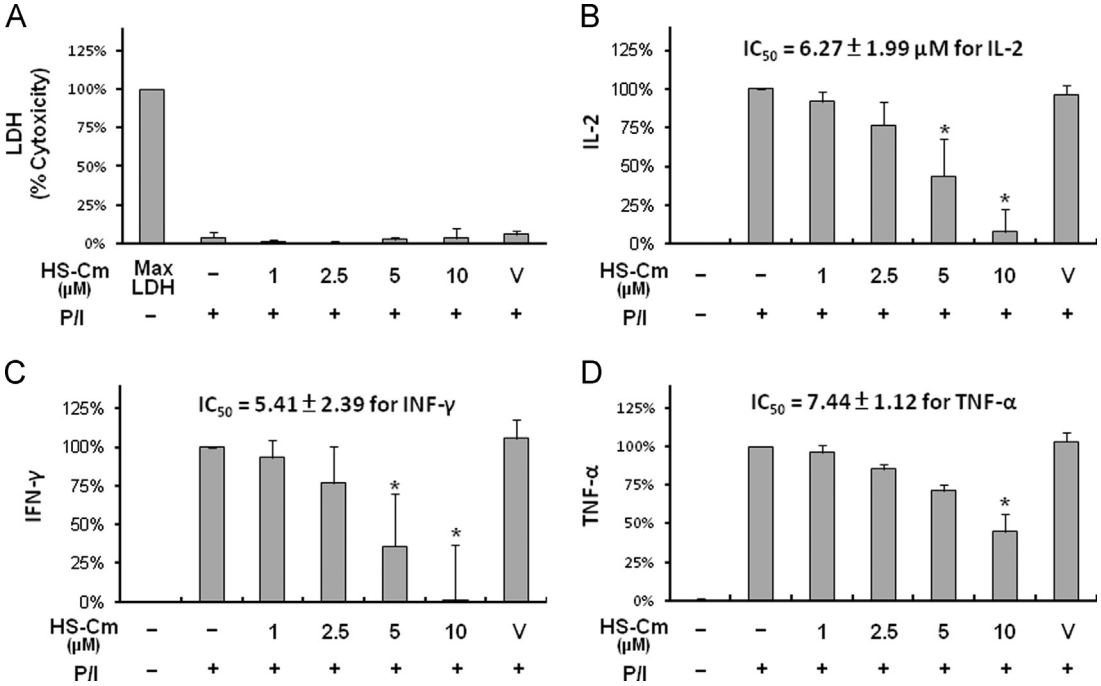

Fig. 2. HS-Cm inhibited cytokine production from stimulated T cells without significant cytotoxicity. (A) Human T cells were pretreated with indicated concentrations of HS-Cm or vehicle (V; DMSO, 0.05%) in duplicate or triplicate for 16 h and were then stimulated with P/I for another 24 h. Cell culture supernatants were collected, and LDH activities were measured. Maximum LDH (Max LDH) release was determined from equal amount of cells lysed with 1% Triton X-100, and spontaneous LDH release was determined from untreated cells. The percent cytotoxicity was calculated as:(experimental release − spontaneous release)/(maximum release–spontaneous release) × 100. Human T cells were pretreated with HS-Cm for 2 h (B, C) or 16 h (D). Following 24 h of P/I stimulation, IL-2, INF-γ, and TNF-α levels in the supernatants, indicating T cell activation, were measured by ELISA. Data are expressed as the percent cytokine produced compared with that in the control group without pretreatment (set at 100%) and are the average ± SD of 3–5 independent experiments conducted in triplicate; *$P < 0.05$. The 50% inhibitory concentration (IC_{50}) of HS-Cm (concentration of HS-Cm causing 50% inhibition of cytokine release) were estimated after 24 h of P/I stimulation and are presented on the tops of the panels.

were purchased from Santa Cruz Biotechnology. The immunoprecipitation kinase assay was performed as detailed in our previous report (Ho et al., 2004).

2.10. DPP4 peptidase activity in human T cells

Human T cells (1×10^6 cells/mL) in 6-well plates were pretreated with 10 μM HS-Cm, 0.05% DMSO, or 50 μM of the DPP4 inhibitor diprotin A (DP-A) for 1 h. CD26/DPP4 peptidase activity of treated cells was measured using the chromogenic substrate glycyl-prolyl-para-nitroanilide (Gly-Pro-pNa; Sigma) according to a previous report with modifications (Christopherson et al., 2003). A well-known DPP4 inhibitor, DP-A (50 μM dissolved in DMSO; final DMSO concentration of 0.4% in the culture medium; Sigma), was used as a positive control to evaluate the inhibition of peptidase activity. Data were obtained from two independent experiments in triplicate, and representative results are shown as the mean ± standard error of the mean (S.E.M.) of all tests.

2.11. Statistical analysis

All results were obtained from at least three independent experiments and are expressed as the mean ± SD unless otherwise specified. Data were analyzed with one-way ANOVA plus Dunnett's multiple comparison tests using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. HS-Cm exhibited superior immunomodulatory effects in human T cells

First, we performed cell-based screening of our salicylate-based small-molecule library for potential anti-inflammatory compounds. The inhibition of IL-2 release in activated human T cells served as an indicator of the immunomodulatory effect of each small molecule. As representative data shown in Fig. S1, one novel small molecule HS-Cm exhibited the most potent inhibitory effects of all the compounds on IL-2 production; this compound was subjected to further analysis.

3.2. HS-Cm inhibited pro-inflammatory cytokine production without significant cytotoxicity

The results from LDH assay showed that HS-Cm was not toxic to T cells at concentrations below 10 μM (Fig. 2A). Further analysis

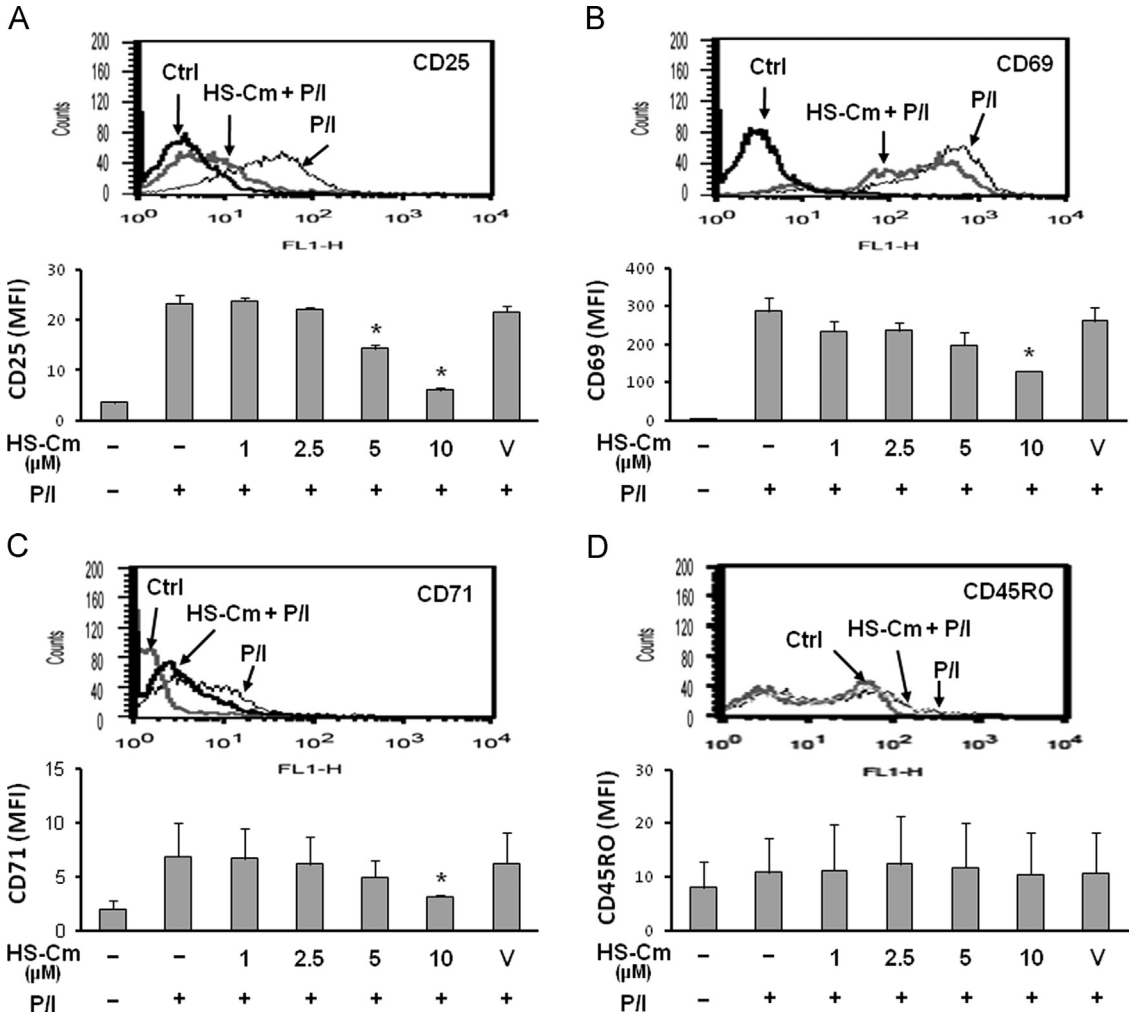

Fig. 3. HS-Cm inhibited the expression of CD25, CD69, and CD71, but not CD45RO. Human T cells were pretreated with indicated concentrations of HS-Cm (1–10 μM) or vehicle for 2 h (A, B) or 16 h (C, D) and were then stimulated with P/I for 24 h. Sorted cells were analyzed for expression of CD25, CD 69, CD71, or CD45RO by flow cytometry to determine T-cell activation status. Mean fluorescence intensities (MFIs) are presented as means ± SDs ($n=3$ independent experiments, except for CD45RO where $n=2$); *$P < 0.05$ vs. stimulated T cells without HS-Cm pretreatment. Representative histograms are shown for staining with specific antibodies for untreated control (ctrl) and for P/I-stimulated T cells with and without 10 μM HS-Cm pretreatment.

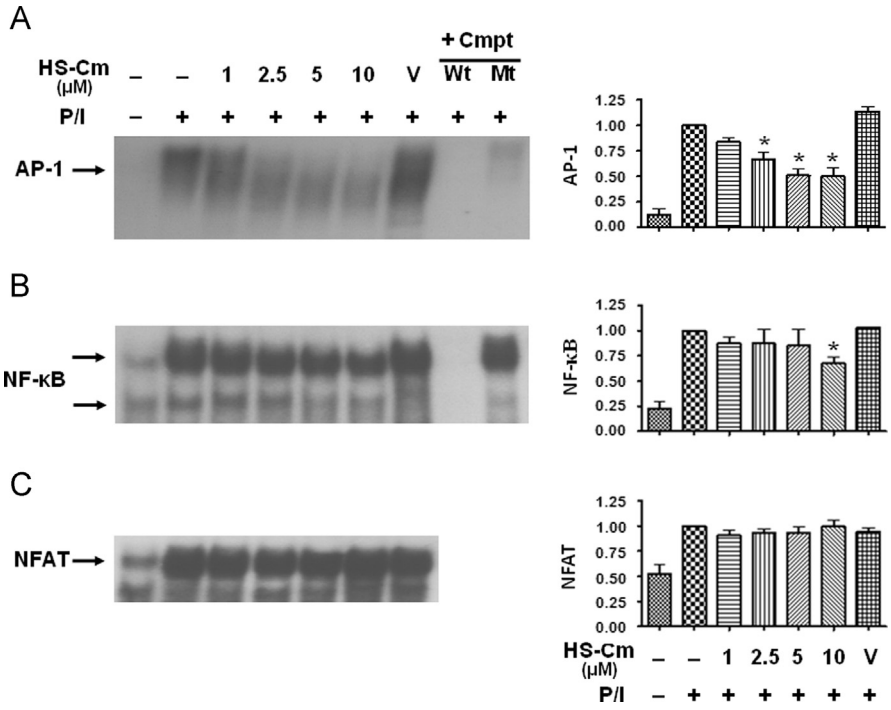

Fig. 4. HS-Cm suppressed the DNA-binding activities of AP-1 and NF-κB, but not NFAT. Human T cells in serum-starved (1% FBS) condition were pretreated with indicated concentrations of HS-Cm (0–10 μM) or vehicle for 2 h and were then stimulated with P/I for 2 h. The nuclear protein extracts were analyzed for DNA-binding activities of AP-1 (A), NF-κB (B), and NFAT (C) by EMSA. As described in Section 2, competition assays (Cmpt) were also performed using unlabeled wild-type (Wt) and mutant (Mt) oligonucleotide probes to identify the bands specific for individual transcription factors (competition assay data for NFAT were not shown). AP-1 and NF-κB bindings were specific because they could be competed out with an unlabeled, identical oligonucleotide, but not with an unrelated, nonspecific oligonucleotide. The representative results and statistics of densitometric intensities from at least three independent experiments were shown; $^{*}P < 0.05$ vs. vehicle control.

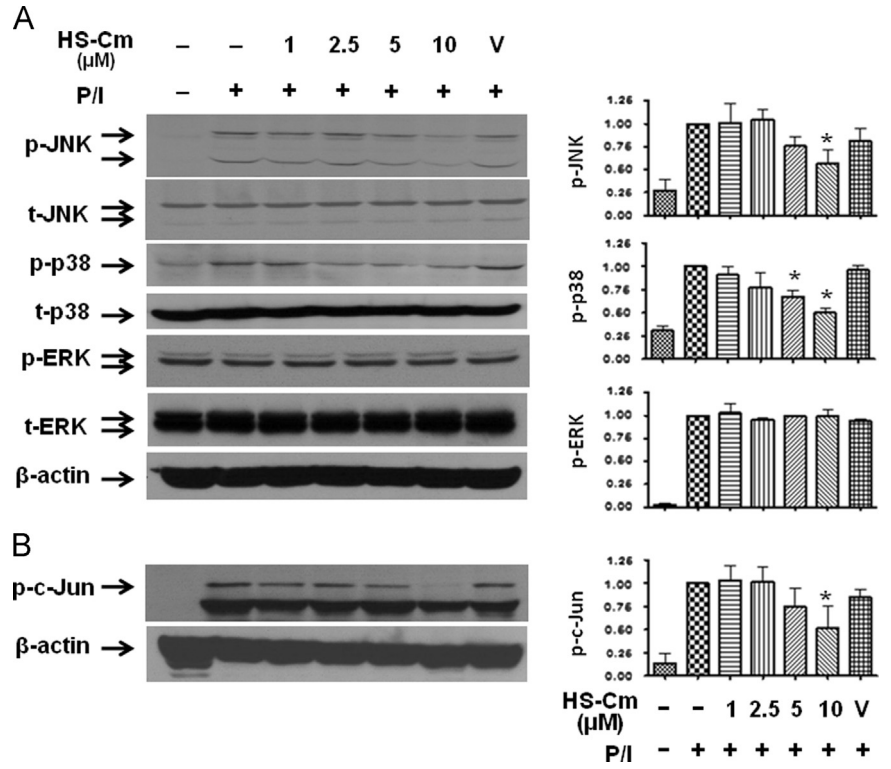

Fig. 5. HS-Cm downregulated AP-1 activity through inhibition of JNK/p38 MAPK activation. Human T cells were pretreated with indicated concentrations of HS-Cm (0–10 μM) or vehicle for 2 h and were then incubated with (+) or without (−) P/I for 15 min (A) or 2 h (B). The expression of target proteins was analyzed by western blotting. The prefixes p- and t- indicate phosphorylated (activated) protein and total protein, respectively. The representative results and statistics of densitometric intensities from at least three independent experiments were shown; $^{*}P < 0.05$ vs. vehicle control.

by ELISA showed that not only IL-2 but also IFN-γ and TNF-α productions were concentration-dependently inhibited by HS-Cm in P/I-stimulated T cells, with IC_{50} values of 6.27 ± 1.99, 5.41 ± 2.39, and 7.44 ± 1.12 μM, respectively (Fig. 2B–D).

3.3. HS-Cm inhibited the expression of T-cell activation markers

We next examined the surface expression of T-cell activation marker influenced by HS-Cm using flow cytometry. As Fig. 3A–C showed, the expression of CD25, CD69 and CD71, markedly induced by P/I stimulation, was significantly reduced by HS-Cm in a concentration-dependent manner. However, the slightly induced CD45RO was not suppressed by HS-Cm (Fig. 3D).

3.4. HS-Cm downregulated the DNA-binding activities of AP-1 and NF-κB, but not NFAT

To study the molecular mechanisms of the immunomodulatory effects of HS-Cm on T cells, we conducted EMSAs to examine the DNA binding activities of AP-1, NF-κB, and NFAT, three major transcription factors for T cell activation. The results showed that the activities of both AP-1 and NF-κB, but not NFAT, were concentration-dependently inhibited by HS-Cm (Fig. 4).

3.5. HS-Cm suppressed the activation of JNK and p38, but not ERK

AP-1 activation is mainly controlled by the activation of mitogen-activated protein kinases (MAPKs) signaling pathways, including JNK, ERK, and p38. Hence, MAPK activities in T cells were evaluated by western blotting. As Fig. 5A showed, HS-Cm treatment for 2 h inhibited the phosphorylation of JNK and p38, but not ERK, in T cells stimulated by P/I for 15 min. The expression of phosphorylated c-Jun, activated by phosphorylated JNK, was also suppressed by HS-Cm (Fig. 5B). These results suggested that the immunomodulatory effects of HS-Cm on T cells were partly attributable to the suppression of the JNK/p38 MAPK/AP-1 pathway.

3.6. HS-Cm suppressed NF-κB signaling through inhibition of IκBα kinase activity, IκBα degradation, and p65/p50 nuclear translocation

NF-κB stays inactive and retains in the cytoplasm before activation due to the binding by inhibitory protein IκBα.

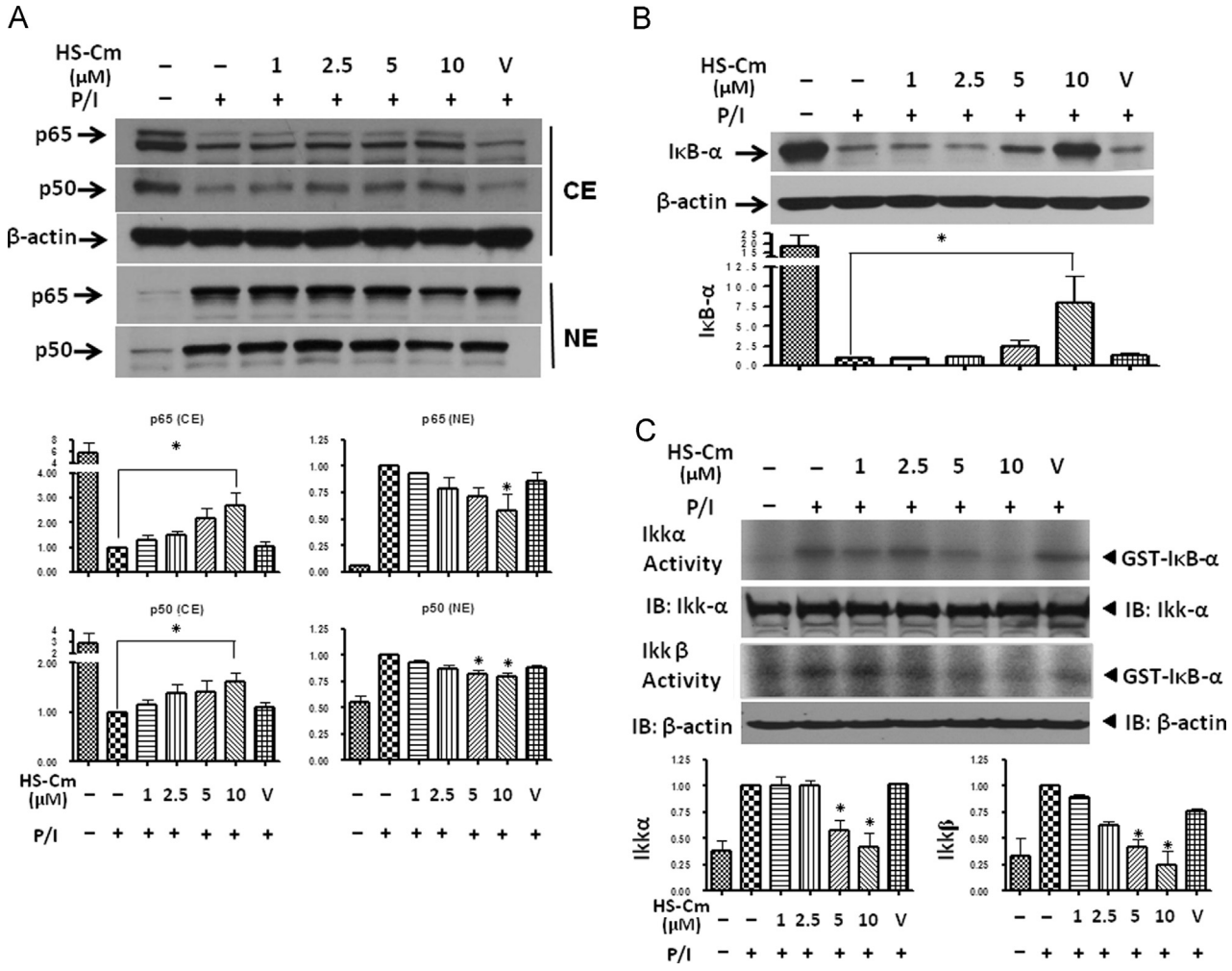

Fig. 6. HS-Cm inhibited the IKK-IκBα-NF-κB signaling pathway. Human T cells in serum starved (1% FBS) condition were pretreated with the indicated concentrations of HS-Cm (0–10 μM) or vehicle for 2 h and were then stimulated with P/I for 2 h (A) or 15 min (B, C). In (A), cytoplasmic extracts (CEs) and nuclear extracts (NEs) from collected cells were analyzed for the expression of NF-κB p50 and p65 subunits. In (B), total cell lysates were analyzed by western blotting for measurement of IκBα protein and β-actin expression as an internal control. In (C), IKKα and IKKβ kinase activities were analyzed by immunoprecipitation kinase assays. Total cell lysates were immunoprecipitated with anti-IKKα or anti-IKKβ antibodies. After sequential washing, GST-IκBα as a substrate and 10 mCi of [γ-^{32}P]ATP were added. After the kinase reaction, the reaction mixture was analyzed for IKKα and IKKβ activity by autoradiography. For determination of IKKα and β-actin protein levels, immunoblotting (IB) assays were performed. The representative results and statistics of densitometric intensities from at least three independent experiments were shown; *$P < 0.05$ vs. control.

The down-regulation of NF-κB DNA-binding activity by HS-Cm in activated T cells suggested the inhibition of upstream signaling molecules. Western blotting showed that HS-Cm reduced the nuclear translocation and reversely increased cytoplasmic sequestration of both p50 and p65, two subunits of NF-κB (Fig. 6A). Further analysis of total cell lysates showed that IκBα protein levels increased in cells treated with HS-Cm, suggesting that IκBα degradation was inhibited (Fig. 6B). Immunoprecipitation kinase assays were then employed to evaluate the activities of IκBα kinases (IKKs), which phosphorylate IκBα and bring its degradation. As Fig. 6C showed, HC-Cm effectively blocked kinase activities of both IKKα and IKKβ.

3.7. HS-Cm inhibited DPP4 activity on primary human T cells

In view of the correlation between DPP4 activity and T cell activation (Ohnuma et al., 2008) and the structural similarities of HS-Cm to DPP4 substrates and DPP4 inhibitors, we next analyzed the effects of HS-Cm on DPP4 activity in human T cells. The well-known DPP4 inhibitor diprotin A (50 μM) sufficiently suppressing DPP4 activity (Liu et al., 2009) was used as a positive control. The results showed that DPP4 activity in T cells was effectively suppressed by 10 μM HS-Cm (Fig. 7A), suggesting the potential of HS-Cm as a DPP4 inhibitor analog and immunomodulatory agent. For comparison, the chemical structures of HS-Cm (Fig. 7B), DPP4 substrate Gly-Pro-pNa (Fig. 7B) and DPP4 inhibitor diprotin A (Fig. 7C) are shown (Fig. 7D). The immunomodulatory effects and mechanisms of HS-Cm in human T cells are summarized in Fig. 8.

4. Discussion

T cells interact with other leukocytes via various cytokines, participate in the regulation of atherogenic responses, and contribute to atherosclerosis progression (Wigren et al., 2012). Therapeutic approaches to inhibit pro-atherogenic cytokines produced by activated T cells are clinically relevant in mitigating atherosclerosis (Ait-Oufella et al., 2011). To identify potent immunomodulator for T cells, in the current study we screened 300 synthetic salicylate-based small molecules using human peripheral blood T cells as a primary target and identified a novel small molecule HS-Cm exhibiting the most potent immunomodulatory effect but with very low toxicity to human T cells. HS-Cm effectively suppressed T-cell activation and inhibited the P/I-induced production of IL-2, IFN-γ, and TNF-α in T cells, potentially attenuating T cell-mediated inflammatory processes, including atherosclerosis.

T cell activation leads to cell differentiation and expression of various cell surface markers, clearly distinguishing activated and naïve T cells (Shipkova and Wieland, 2012). The COX-2 specific inhibition severely diminished early and late events of T cell activation, namely reduced CD25 and CD71 cell surface expression, IL-2,

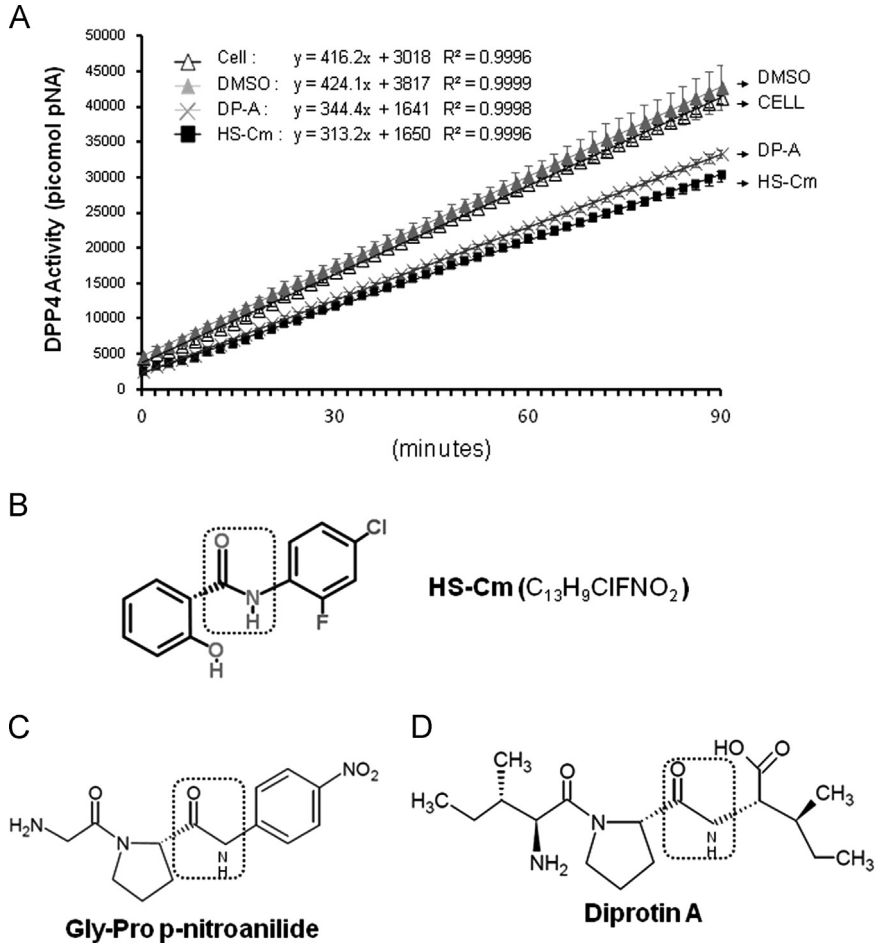

Fig. 7. HS-Cm exhibited considerable inhibitory effect on DPP4 activity in human T cells. (A) Human T cells were pretreated with 10 μM HS-Cm, 0.05% DMSO, or 50 μM of the DPP4 inhibitor diprotin A (DP-A) for 1 h. DPP4 activity of sorted cells was then analyzed in 96-well plates using the chromogenic substrate Gly-Pro-pNa. The pNA produced by DPP4 cleavage was measured at 405 nm every 2 min according to a pNA standard curve. The representative results are shown as the mean ± S.E.M. of all tests. Linear regression curves were calculated, and the equations are presented. (B) Structure of HS-Cm and (C) the chemical structures of Gly-Pro p-nitroanilide (DPP4 substrate) and diprotin A (DPP4 inhibitor).

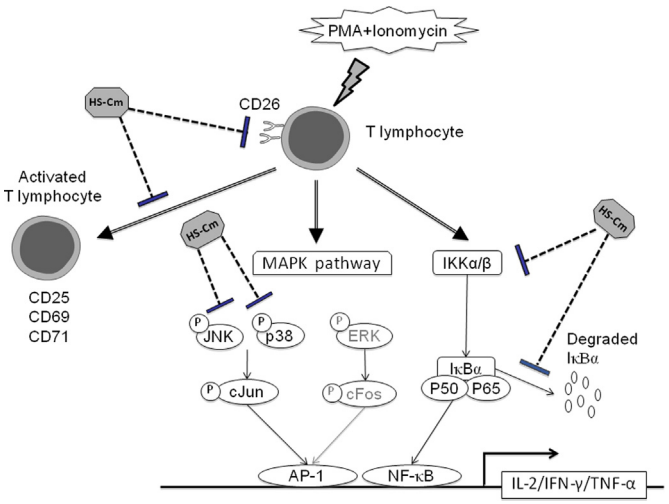

Fig. 8. The proposed mechanism for the immunomodulatory effect of HS-Cm in human T cells. HS-Cm inhibited T cell activation and cytokine production through suppressing phosphorylation of JNK and P38, and the kinase activities of both IKKα and IKKβ, leading to downregulation of AP-1 and NF-κB DNA binding activities respectively. HS-Cm also inhibited the enzymatic activity of DPP4/CD26.

TNF-α and IFN-γ production and cell proliferation, but not CD69 expression. COX-2 inhibition also inhibited the transcription from NF-κB and NFAT-dependent enhancers (Iniguez et al., 1999). Interestingly, in our study, P/I-induced up-regulations of CD25, CD69, and CD71 on human T cells were all effectively suppressed by HS-Cm. It implied the unique immunomodulatory mechanism of HC-Cm on T cells different from COX-2 inhibition. Besides, the expression of CD45RO, a surface marker of memory T cells, was not affected by HS-Cm. The differential effects on these activation markers implied that HS-Cm, though a significant inhibitor of early T-cell activation, probably exerted minor effects on T-cell differentiation.

NF-κB and AP-1, not only essential signaling pathways for T-cell activation and differentiation, but also are critical regulators of inflammatory responses, and both proteins are potential therapeutic targets for various inflammatory diseases, including atherosclerosis (Meijer et al., 2012; Pamukcu et al., 2011). The marked suppression of AP-1 and NF-κB DNA-binding activities in P/I-stimulated human T cells by HS-Cm suggested the potent anti-inflammatory properties of HS-Cm. AP-1 activity is mainly regulated by MAPKs, including JNK, ERK, and p38 (Shaulian and Karin, 2002). ERK regulates cell proliferation, differentiation, and migration (Qi and Elion, 2005); however, JNK and p38 are associated with cell responses to stress, apoptosis, and inflammation (Kaminska, 2005). Our data demonstrated that HS-Cm suppressed JNK/p38 but not ERK activation in stimulated T cells, providing additional evidence for the unique anti-inflammatory effects of HC-Cm. In contrast to the effects on NF-κB and AP-1, the NFAT signaling pathway appeared to be unaffected by HS-Cm.

The hypoglycemic effect of salicylates has been observed for more than 100 years and further supported by two recently published randomized clinical trials (Goldfine et al., 2013a, 2013b; Pedersen and Febbraio, 2010). These findings provide more evidence linking inflammation and diabetes mellitus. HS-Cm is a salicylate-based small molecule and composed of two aromatic rings conjugated with amide-bonding in chemical structure, which is similar to the substrate and inhibitors of DPP4 (Fig. 7B–D). In light of structure-activity relationship of amide-bonding structures, we evaluated the effect of HS-Cm on the DPP activity in T cells using well-known DPP4 inhibitor diprotin-A (DP-A) as positive control. Interestingly, considerable inhibition of total DPP4 enzymatic activity was shown in HS-Cm treated T cells; with the level comparable to DP-A treated T cells. These findings offered a new scope for future studies on the hypoglycemic mechanisms of salicylate-based small molecules, in addition to anti-inflammatory activities.

DPP4, identical to T-cell activation antigen CD26, is involved in T-cell activation, costimulation, and immune regulation (Ohnuma et al., 2008). DPP4 (CD26) inhibitors have become promising drugs for treating inflammatory diseases due to their ability to inhibit T-cell proliferation and cytokine production (Yazbeck et al., 2009). The beneficial effects of DPP4 inhibitors on cardiovascular disease beyond glucose control are attracting more attention lately (Zhong et al., 2013). The inhibition of CD26 activity by HS-Cm implicated that immunomodulatory effects of HC-Cm on human T cells probably involves more complex mechanisms and may also provide cardiovascular benefits.

In conclusion, through cell-based compound screening of a mini-library of 300 salicylate-based small molecules, HS-Cm was identified as a candidate compound with potent immunomodulatory effects in activated T cells. HS-Cm exhibited low cytotoxicity, but significantly reduced production of IL-2, IFN-γ, and TNF-α in activated T cells. HS-Cm also suppressed the expression of cell surface activation markers on T cells, including CD25, CD67, and CD71, but not CD45RO. Further analysis showed that the DNA-binding activities of AP-1 and NF-κB, but not NFAT, were down-regulated by HS-Cm through suppression of the JNK/p38 MAPK and IKK/IκBα signaling pathways respectively (see Fig. S2 for an illustration of the proposed mechanism). In addition, HS-Cm markedly inhibited DPP4/CD26 activity in T cells. These results demonstrated the potential of HS-Cm as an anti-inflammatory, immunomodulatory, and anti-atherogenic agent targeting T cell activation and as a DPP4 inhibitor analog.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2014.01.049.

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