European Journal of Pharmacology 726 (2014) 124–132

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

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

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ARTICLE INFO

Article history:
Received 9 August 2013
Received in revised form
22 January 2014
Accepted 24 January 2014
Available online 31 January 2014

Keywords:
Salicylate
T lymphocyte
Atherosclerosis
Diabetes
Arthritis
Dipeptidyl-peptidase IV inhibitor

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

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http://dx.doi.org/10.1016/j.ejphar.2014.01.049

(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).

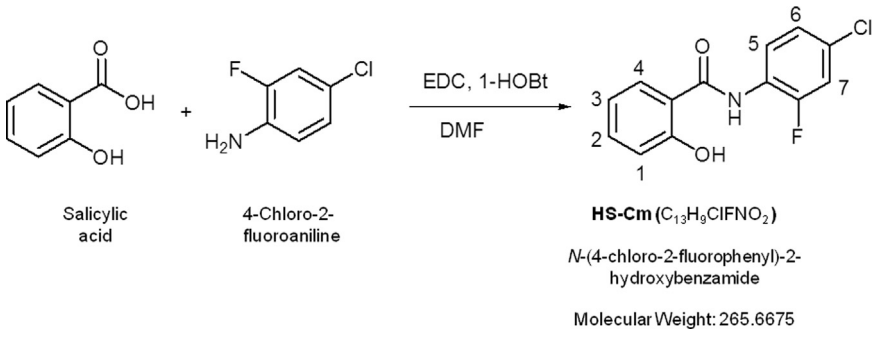

Fig. 1. Structure and chemical synthesis of HS-Cm. Chemical structures of HS-Cm [$C_{13}H_9ClFNO_2$; *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide], salicylic acid, 4-chloro-4-fluoronaniline. DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole monohydrate; *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide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