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Simple modifications to methimazole that enhance its inhibitory effect on tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression by human endothelial cells

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ABSTRACT

The expression of vascular cell adhesion molecule-1 (VCAM-1) on the vascular endothelium can be increased by pro-inflammatory cytokines [e.g. tumor necrosis factor-α (TNF-α)]. VCAM-1 contributes to leukocyte adhesion to, and emigration from, the vasculature which is a key aspect of pathological inflammation. As such, a promising therapeutic approach for pathological inflammation is to inhibit the expression of VCAM-1. Methimazole [3-methyl-1, 3 imidazole-2 thione (MMI)] is routinely used for the treatment of Graves' disease and patients treated with MMI have decreased levels of circulating VCAM-1. In this study we used cultured human umbilical vein endothelial cells (HUVEC) to investigate the effect of MMI structural modifications on TNF-α induced VCAM-1 expression. We found that addition of a phenyl ring at the 4-nitrogen of MMI yields a compound that is significantly more potent than MMI at inhibiting 24 h TNF-α-induced VCAM-1 protein expression. Addition of a para methoxy to the appended phenyl group increases the inhibition while substitution of a thiazole ring for an imidazole ring in the phenyl derivatives yields no clear difference in inhibition. Addition of the phenyl ring to MMI appears to increase toxicity as does substitution of a thiazole ring for an imidazole ring in the phenyl MMI derivatives. Each of the compounds reduced TNF-α-induced VCAM-1 mRNA expression and had a functional inhibitory effect, i.e. each inhibited monocytic cell adhesion to 24 h TNF-α-activated HUVEC under fluid flow conditions. Combined, these studies provide important insights into the design of MMI-related anti-inflammatory compounds.

1. Introduction

Leukocyte adhesion to the vascular endothelium plays a crucial role in inflammation and is mediated, in part, by endothelial cell adhesion molecules [ECAMs; e.g., VCAM-1, ICAM-1, and E-selectin] (Luscinskas and Gimbrone, 1996). Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) up-regulate ECAM expression [e.g. TNF-α induces ECAM expression on cultured endothelial cells (Bevilacqua, 1993)]. While elevated ECAM expression is important for a normal inflammatory response, the ECAMs can be aberrantly expressed and contribute to pathological inflammation (Luscinskas and Gimbrone, 1996). For example, VCAM-1 has been implicated in many inflammatory diseases such as atherosclerosis, and arthritis (Carter et al., 2002; Cybulsky et al., 2001). Thus, compounds which inhibit cytokine-induced VCAM-1 expression could aid in treating pathological inflammation. Methimazole [3-methyl-1, 3 imidazole-2 thione (MMI)] is routinely used clinically for the treatment of Graves' disease (Giuliani et al., 2010) and patients treated with MMI have decreased levels of circulating VCAM-1 (Wenisch et al., 1995).

Our group has extensively studied a phenyl derivative of MMI termed C10 (**1a** in this manuscript; 4-phenyl-3-methyl-1, 3 imidazole-2-thione). Adding the aromatic ring (phenyl group) to MMI potentially enhances cell membrane transit and thus increases

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activity (Seydel, 2002). Previous studies have revealed that **1a**, compared to MMI, exhibits a significantly greater suppression of abnormal MHC gene expression in vitro (Giuliani et al., 2010). We have found that **1a** is effective in various in vitro and in vivo models of disease or disease processes [e.g. (Benavides et al., 2010; McCall et al., 2010; Schwartz et al., 2009)]. Most germane to the present study, we previously reported that **1a** can reduce TNF-α-induced VCAM-1 expression in human endothelial cells (Dagia et al., 2004). In the previous study, the effect of MMI on VCAM-1 expression was not determined. Thus, the first goal of the present study was to investigate the hypothesis that **1a** is more active than MMI in inhibiting TNF-α-induced VCAM-1 expression by human endothelial cells.

Our second goal was to determine if small modifications to **1a** could significantly enhance the ability to inhibit TNF-α-induced VCAM-1 expression. We focused on two modifications. First, we wished to see if a simple modification to the phenyl ring of **1a** might enhance or decrease the activity of this class of compounds. We chose to use an electron donating group, methoxy, as an initial substitution. The strongly electron donating properties of this group should have some effect on the activity if the phenyl ring is involved in significant non-covalent interactions with the biological target. Second, the heterocyclic ring of **1a** is an imidazole. Thiazoles (an imidazole with an N substituted for an S) are important anti-inflammatory and immune-suppressive agents (Emami and Foroumadi, 2006; Nishikaku et al., 1994; Pattan et al., 2009; Siddiqui et al., 2009). In addition, previous studies have suggested that thiazoles may exhibit higher efficacy than imidazoles in rat models of inflammation with edema (Unangst et al., 1994). For these reasons, we probed the hypothesis that thiazole phenyl-MMI analogs are more active than imidazole phenyl-MMI analogs in reducing TNF-α-induced VCAM-1 expression.

2. Materials and methods

2.1. Materials

The reagents for human umbilical vein endothelial cell culture and ELISA were described previously (Dagia and Goetz, 2003). 16% para-formaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA). The assay buffer was Hanks buffered saline solution with Ca^{2+} and Mg^{2+} ($HBSS^{+}$) supplemented with 5% FBS. MMI was obtained from Sigma (St. Louis, MO). **1a** and **1b** were prepared according to reported procedures (Kohn et al., 2011; Theoclitou et al., 2002). Compounds **2a** and **2b** were prepared by the method reported by Gan et al. (2010). They were prepared as 200 mM stock solution in 100% DMSO. Recombinant human TNF-α was obtained from R&D Systems (Minneapolis, MN). MTS was purchased from Promega (Madison, WI). The buffer for the adhesion assay was Dulbecco's phosphate buffered solution (DPBS) with Ca^{2+} and Mg^{2+} (Life Technologies, Carlsbad, CA) supplemented with 0.1% BSA (Sigma).

2.2. Antibodies

Anti-CD106 (Anti-VCAM-1, clone: 1.G11B1) was purchased from Ancell (Bayport, MN). Human IgG was purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgG (heavy and light chain specific F $(ab)'_2$ fragment, peroxidase conjugate) was purchased from Calbiochem (La Jolla, CA).

2.3. Cell culture and treatment of HUVEC

HUVEC (Lonza, Walkersville, MD) and U937 monocytic cells (American Type Culture Collection, Manassas, VA) were cultured as previously described (Dagia et al., 2004; Dagia and Goetz, 2003). HUVEC were grown to 95–100% confluence prior to use in an assay. The cells were activated with TNF-α at a 10 ng/ml concentration, and treated with compound in the carrier (0.1% DMSO for ELISA, adhesion assays and real time PCR and 0.25% DMSO for MTS) for a 24 h period unless otherwise noted.

2.4. ELISA

The activated and treated HUVEC were washed, fixed in 1% paraformaldehyde for 20 min at 4 °C, washed, and incubated with 5% FBS in $HBSS^{+}$. Following that, the HUVEC were washed and incubated with primary antibody (e.g., anti-VCAM-1) at 10 μg/ml for 20 min at 4 °C. The HUVEC were then washed and incubated with peroxidase conjugated anti-mouse IgG at 1:100 dilution for 20 min at 4 °C. Subsequently, the HUVEC were subjected to 5–6 washes with the $HBSS^{+}$. Thereupon, HUVEC were treated with OPD dissolved in phosphate citrate buffer containing sodium perborate. After 10 and 20 min incubations in the dark, the Optical Density (OD) values were read at 450 nm on a Multiskan MCC Spectrophotometer (Fisher Scientific, Dubuque, IA).

2.5. Cell viability (MTS) assay

20 μl of MTS/PMS solution was added to the wells containing the HUVEC as per the supplier's protocol. Subsequently, the plate was incubated at 37 °C for 1 h in a humidified 5% CO_2 atmosphere. OD was read at 490 nm on the Multiscan MCC Spectrophotometer.

2.6. Parallel plate flow chamber assay

Flow adhesion assays were conducted using a parallel plate flow chamber (Glycotech, Rockville, MD) mounted on a Nikon TE 300 inverted microscope equipped with a CCD camera. HUVEC were plated in sterile 6.5 mm FlexiPerm gaskets (Sarstdet, Numbrecht, Germany) on 35 mm tissue culture dishes (Corning, Corning, NY). After reaching confluence, the HUVEC were treated for 24 h with TNF-α (10 ng/ml) and compounds **1a**, **1b**, **2a**, **2b** (70 μM) or DMSO (carrier control) at 37 °C and 5% CO_2. The HUVEC were loaded into the flow chamber, the chamber placed on the microscope stage and a suspension of U937 cells (5×10^5 cells/ml) perfused over the HUVEC for 2 min at a wall shear stress of 1.8 dynes/cm^2 using a syringe pump (Harvard Apparatus, Cambridge, MA). The interaction of the U937 cells with HUVEC was recorded for offline analysis as previously described (Dagia and Goetz, 2003). The number of adhering cells included all cells attaching from the free fluid stream.

2.7. Real-time PCR

Total RNA was isolated from HUVEC (RNeasy Kit, Qiagen, Valencia, CA, USA), treated with DNase (RNase-Free DNAse Kit, Qiagen), and quantified with NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed using human VCAM-1 (Hs0036-5485_m1) and Actin, beta (VIC, Hs99999903_m1) Taqman Gene Expression Assays (Applied Biosystems) and amplified using the StepOnePlus Real-Time PCR system (Applied Biosystems). Fold changes in gene expression were calculated using the ΔΔCt method (Livak and Schmittgen, 2001).