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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.

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Keywords

Methimazole; Inflammation; Endothelial cell; Adhesion; Leukocyte

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 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 antiinflammatory 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. (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 $\mu\text{g}/\text{ml}$ 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 μ l 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% CO₂ 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 (Sarstedt, Numbrecht, Germany) on 35 mm tissue culture dishes (Corning, Corning, NY). After reaching confluence, the HUVEC were treated for 24 hours with TNF- α (10 ng/ml) and compounds **1a**, **1b**, **2a**, **2b** (70 μ M) or DMSO (carrier control) at 37°C and 5% CO₂. 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 minutes at a wall shear stress of 1.8 dynes/cm² 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 (Hs00365485_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 $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001).

2.8 Statistics

One-way analysis of variance (ANOVA) was performed to assess the statistical significance of the differences observed in the ELISA and the cell viability assays. If compounds exhibited significant differences, Tukey's test was used for multiple pair wise comparisons.

3. Results

3.1 Modifications to MMI yield **1a** and related compounds used in the present study

The parent structure for this work is the compound Methimazole [3-methyl-1, 3 imidazole-2 thione (MMI)] which is used clinically for the treatment of autoimmune disease (Cooper, 1984) (Fig. 1). As previously reported (Kohn et al., 2011) addition of a phenyl ring onto the 4-position of the imidazole ring of MMI yields phenyl methimazole [4-phenyl-3-methyl-1, 3 imidazole-2-thione] or C10 (compound **1a** in this report; Fig. 1).

For this project we wished to examine two specific alterations to the core structure of **1a**. The first was a simple substitution of the aromatic ring. The unsubstituted benzene ring of

1a was replaced with a 4-methoxyphenyl to provide **1b**. The goal was simply to identify portions of the molecule that might be useful to modify in a larger structure activity relationship study. A second alteration of the core structure of **1a** was the change of the unsubstituted N–H of the imidazole-2-thione ring to an S providing a thiazole-2-thione ring. Such a substitution might be expected to alter interactions with the pharmacological target if interactions of the somewhat acidic N–H are important.

3.2 Addition of a phenyl ring to methimazole significantly increases its ability to inhibit TNF- α -induced VCAM-1 expression by human endothelial cells

Dagia et al. have shown that **1a** significantly reduces TNF- α -induced VCAM-1 expression by human endothelial cells while having limited effect on other ECAMs (Dagia et al., 2004). Interestingly, a comparison between **1a** and MMI was not made in this previous work. Thus, as a first step in the present study, we compared, at a range of concentrations, the effect of **1a** to that of MMI in terms of the ability to inhibit TNF- α -induced VCAM-1 expression. We have previously shown that TNF- α induces VCAM-1 mRNA and protein expression in HUVEC (Dagia and Goetz, 2003; Deosarkar et al., 2011). We chose to initially focus this study on TNF- α induced VCAM-1 protein expression (as opposed to VCAM-1 mRNA expression) since VCAM-1 protein expression is more functionally relevant than VCAM-1 mRNA. Previous work (Bevilacqua, 1993) has established that VCAM-1 expression reaches its peak at ~24 h post-TNF- α activation and our preliminary data demonstrated that treatment of HUVEC with 10 ng/ml TNF- α gave maximal levels of VCAM-1 protein expression (i.e. the level of VCAM-1 protein expression resulting from 10 ng/ml TNF- α was similar to the level of VCAM-1 protein expression that resulted from treatment with 5, 25 and 50 ng/ml of TNF- α). Thus, we chose to treat the HUVEC for 24 hours with 10 ng/ml for this study.

As shown in Figure 2A and 2B an ELISA reveals that **1a** significantly reduced TNF- α -induced VCAM-1 protein expression on HUVEC at concentrations where MMI had little, if any, effect. For example at 50 μ M, **1a** inhibited over 40% of the VCAM-1 protein expression elicited by TNF- α while MMI had no inhibitory effect on VCAM-1 expression (Figures 2A and 2B). We determined the IC₅₀, the concentration of compound needed to inhibit 50% of the VCAM-1 expression, for both **1a** and MMI using linear regression. This was done for multiple experiments and the resulting IC₅₀ values averaged to give the results presented in Figure 2C. As shown in Figure 2C, the IC₅₀ for MMI (1135 μ M) was over 17 times higher than that of **1a** (66 μ M). Thus, the addition of the phenyl ring onto MMI appears to significantly improve its potency in terms of inhibiting TNF- α -induced VCAM-1 protein expression.

3.3 Addition of a para methoxy to the appended phenyl ring increases inhibition of 24 h TNF- α -induced VCAM-1 expression by human endothelial cells

We next sought to determine if some relatively simple modifications to **1a** would further enhance the potency of the compound. Specifically we made a methoxy derivative of **1a** (termed **1b**), which is described in section 3.1 and depicted in Figure 1, and a thiazole derivative of **1a** (**2a**; Fig. 1), and a thiazole derivative of **1b** (**2b**; Fig. 1). For each compound, we used ELISA and performed dose response experiments to determine the effect of the

compounds on 24 h TNF- α -induced VCAM-1 protein expression. The results are presented in Figure 3. This was done multiple times and the resulting IC₅₀ values, determined from each replicate, averaged to give the IC₅₀ value for each compound (Figure 4). The IC₅₀ for **1a**, **1b**, **2a** and **2b** were 66 μ M, 25 μ M, 57 μ M, and 36 μ M, respectively (Figure 4). Each of the **1a** derivatives has activity that is more similar to **1a** than MMI, i.e. they are all at least 19 times more potent than MMI which had an IC₅₀ of 1135 μ M. Figure 4 reveals that substituting the imidazole ring for a thiazole ring does not have a significant effect on activity (compare **1a** to **2a** and **1b** to **2b**). However, addition of a para methoxy group to the appended phenyl does significantly increase activity (compare **1a** to **1b** and **2a** to **2b**).

During the final steps of the ELISA assay [after the addition of the secondary (detection) antibody] we observed that some HUVEC were washed away around the perimeters of the wells treated with **1b** and **2b** at 100 μ M. Hence, for **1b** and **2b**, only data up to 50 μ M were used to obtain the IC₅₀. This observation led us to investigate the effect of the compounds on cell viability.

3.4 As measured by a MTS assay, the phenyl derivatives are more toxic than MMI and the thiazole derivatives appear to be more toxic than the imidazole derivatives

To gain insight into the effect of the compounds on cell viability, we performed MTS assays. The MTS assay measures metabolic activity which often correlates with the number of viable cells. For each compound, we performed an MTS assay for a range of concentrations (Figure 5). This allowed us to estimate the concentration of compound needed to reduce the MTS signal by 50% of the control value, i.e. the TC₅₀. These experiments were done multiple times and the resulting TC₅₀ values, determined from each replicate using linear regression, averaged to give the TC₅₀ value for each compound (Figure 6). The TC₅₀ for MMI, **1a**, **1b**, **2a** and **2b** was 2450 μ M, 1240 μ M, 1010 μ M, 470 μ M, and 520 μ M respectively (Figure 6). Figure 6 reveals that MMI is significantly less toxic than any of the phenyl derivatives used in this study and that the thiazole derivatives are more toxic than their imidazole counterparts (**2a** vs **1a** and **2b** vs **1b**).

Several noteworthy observations were made during these assays. While performing the MTS assay for **2a**, it was observed that at the higher concentrations (1000 μ M and 500 μ M) the “solution” contained “fuzzy structures” that were occupying a significant portion of the wells. At a concentration of 1000 μ M of **2b** or **2a** small round bodies that appeared to be disintegrated cells/precipitates were seen throughout the wells. Therefore, only the MTS data for concentrations up to 250 μ M for **2a** and up to 500 μ M for **2b** were used to determine their TC₅₀.

3.5 Toxic to inhibitory index of imidazole and thiazole derivatives of MMI

To gain insight into the effectiveness of the compounds relative to their potential toxic effects, we determined the ratio of TC₅₀ to IC₅₀. We term this ratio the “toxic to inhibitory index (TII)”. The results are presented in Figure 7 where it is shown that the TII for MMI, **1a**, **1b**, **2a**, and **2b** are 2, 19, 40, 8, and 14 respectively. Figure 7 reveals that all the phenyl derivatives of MMI appeared to exhibit a greater TII than MMI and that **1b** appears to have the maximal TII.

3.6 MMI and each of the derivatives inhibit monocytic cell adhesion to 24 h TNF- α -activated human endothelial cells under fluid shear conditions

We sought to determine if each compound would have an inhibitory effect in a functional assay. During pathological inflammation, VCAM-1 helps mediate the adhesion of monocytes to the endothelium in the fluid dynamic environment of the circulation (Luscinskas and Gimbrone, 1996). Thus, we tested the effect of the compounds on monocytic (U937) cell adhesion to 24 h TNF- α -activated HUVEC in an *in vitro* flow adhesion assay that mimics conditions present *in vivo* (Gopalan et al., 1996). As shown in Figure 8, unactivated HUVEC supported very little U937 adhesion. In contrast, treatment of HUVEC with TNF- α for 24 hours dramatically increased U937 adhesion. Incubation of the HUVEC with a function blocking mAb to VCAM-1, prior to the adhesion assay but after the TNF- α 24 h treatment, revealed that the majority, but not all, of the adhesion appeared to be mediated by VCAM-1. Each of the compounds caused a significant reduction in adhesion when added to the HUVEC during the TNF- α treatment (Figure 8) and thus each demonstrates a significant inhibitory effect on function.

3.7 MMI and each of the derivatives significantly reduce 24 h TNF- α -induced VCAM-1 mRNA expression by human endothelial cells

We have previously shown, using Northern blot analysis, that compound **1a** inhibits 24 h TNF- α -induced VCAM-1 mRNA expression in human aortic endothelial cells (Dagia et al., 2004). To expand on this previous finding and to begin to explore the mechanism by which these compounds inhibit induced VCAM-1 protein expression, we used real time PCR to determine the compounds' effects on induced VCAM-1 mRNA expression. HUVEC were treated for 24 h with TNF- α in the presence of one of the compounds or DMSO (carrier control). Subsequently, VCAM-1 transcripts were analyzed by PCR. As shown in Figure 9, each of the compounds significantly reduced 24 h TNF- α -induced VCAM-1 mRNA expression. This result, combined with our previous studies with **1a** (Dagia et al., 2004), suggests that the compounds diminish TNF- α -induced VCAM-1 protein expression by inhibiting VCAM-1 mRNA expression. [Note that in these assays the MMI concentration (1200 μ m) was significantly greater than the concentration of the other compounds (70 μ m).]

4. Discussion

Our studies reveal that addition of a phenyl group to MMI dramatically increases the ability to inhibit TNF- α -induced VCAM-1 protein expression by human endothelial cells. Indeed, all four of the compounds tested, **1a**, **1b**, **2a** and **2b** had an IC₅₀ value that was over an order of magnitude lower than the IC₅₀ achieved for MMI (Figure 4 compared to Figure 2C). To our knowledge the ability of **1a** and MMI to inhibit TNF- α -induced VCAM-1 expression has not previously been compared. However, others have observed the greater potency of **1a**, relative to MMI. For example, **1a** was reported to exhibit a 70–100 fold greater, compared to MMI, suppression of abnormal MHC gene expression in thyroid epithelial cells (Giuliani et al., 2010).

It is important to note that in parallel with the finding of increased activity, the toxic effects of the compounds on human endothelial cells appeared to also increase with the addition of

the phenyl ring. Specifically, the TC₅₀ value for MMI was two to five times higher than that observed for the phenyl appended compounds (Figure 6). Hence, it would appear that addition of the phenyl group augments the desired activity but also potentially increases unwanted toxic side effects. That said, it is true that all drugs have a desired, therapeutic, effect as well as a toxic effect(s) at some level. Insight into the potential usefulness of a compound can be gained by comparing the TC₅₀ values to the IC₅₀ values – a ratio we termed the toxic to inhibitory index (TII) (Figure 7). The phenyl appended compounds all have a higher TII (anywhere from 4 to 20 times higher) than MMI demonstrating that, while addition of the phenyl group appears to increase toxicity relative to MMI (Figure 6), the activity increases to a greater extent (Figure 4) suggesting that addition of the phenyl group does move the compound toward a structure that will have lower adverse side effects for the same therapeutic effect (Figure 7).

Interestingly, the data demonstrated that addition of a methoxy group to **1a** or **2a** significantly increased the activity by ~2.6 and ~1.5 fold (Figure 4) as determined by calculating a ratio of the IC₅₀ for **1a** vs **1b** and **2a** vs **2b**, respectively. At the same time, there was not a significant difference in the toxicity with the addition of the methoxy group (Figure 6). Not surprisingly given these results, the TII data revealed that addition of the methoxy group appears to increase the TII by ~2 fold (compare TIIs of **1b** and **1a** as well as **2a** and **2b** in Figure 7). Hence, addition of the methoxy group to the appended phenyl would appear to be a step towards a more optimal compound and suggests that the phenyl ring may be involved in non-covalent interactions with the biological target. That said, it is important to note the observation we mentioned in the results section. Specifically, we observed that for both **1b** and **2b** at higher concentrations (e.g. 100 µM) some of the HUVEC were observed to detach from the tissue culture dishes during the washing steps. This was not observed with **1a** or **2a**. Loss of endothelial cells *in vivo* would obviously be of significant concern since a denuded endothelial cell layer can cause a plethora of problems some of which can be quite severe (Butcher and Nerem, 2007). Thus, while addition of the methoxy group did appear to be advantageous based on the TII results, the fact that **1b** and **2b** appeared to induce detachment of HUVEC, albeit at higher concentrations, is of concern when considering these compounds.

Many thiazole-based compounds are approved drugs (e.g. meloxicam) (Martin et al., 2012) or have been shown to be anti-inflammatory immune-suppressive agents (Nishikaku et al., 1994; Pattan et al., 2009). In addition, previous studies have demonstrated that thiazole-based compounds may exhibit higher efficacy than matched imidazole-based compounds in rat models of inflammation with edema (Unangst et al., 1994). These observations motivated us to make thiazole analogs of **1a** and **1b**. Interestingly, the thiazole derivatives appeared to be no more potent than the imidazole derivatives (Figure 4) but did appear to be more toxic (Figure 6). It is unclear at this time why the thiazoles appear to be more toxic than the imidazoles and additional investigation is certainly warranted. Such future studies could include making additional imidazole/thiazole pairs and comparing them in the MTS assay.

To determine if the ability to reduce VCAM-1 protein expression translates into an inhibitory effect on function, we tested each compound in an *in vitro* dynamic cell adhesion assay that mimics flow conditions present *in vivo*. As shown in Figure 8, each of the

compounds significantly inhibited U937 (a monocytic cell line) adhesion to 24 h TNF- α -activated HUVEC. Somewhat surprisingly, MMI had a significant inhibitory effect on adhesion (Figure 8) despite the fact that, at the concentration of MMI used in the adhesion assay (70 μ m), there is little, if any, inhibition of VCAM-1 protein expression (Figure 2A). The finding with MMI underscores the fact that the U937 cells adhere to the HUVEC via VCAM-1-dependent as well as VCAM-1-independent mechanisms (Dagia et al., 2004). It is well established that TNF- α increases the expression of ICAM-1 and induces the expression of E-selectin both of which are functional endothelial cell adhesion molecules (Bevilacqua, 1993). Treatment of the TNF- α -activated HUVEC with a mAb to VCAM-1 caused a significant decrease in adhesion, but clearly did not eliminate all of the adhesion (Figure 8), strongly suggesting the presence of a VCAM-1-independent mechanism(s) for adhesion. Thus, the inhibition on adhesion observed with MMI (Figure 8) could be due to inhibition of a VCAM-1-independent adhesion mechanism.

To begin to explore the mechanism by which the compounds inhibit induced VCAM-1 protein expression, we probed VCAM-1 expression at the mRNA level using real time PCR. This analysis revealed that all of the compounds inhibit 24 h TNF- α -induced VCAM-1 mRNA expression (Figure 9). (Note that MMI was used at 1,200 μ m and the other compounds were used at 70 μ m in this assay.) This finding is in line with our previous result with **1a** where we observed, via Northern analysis, that **1a** inhibited 24 h TNF- α -induced VCAM-1 expression by human aortic endothelial cells (Dagia et al., 2004). In addition, we have previously reported that **1a** acts to inhibit signal transduction (Courreges et al., 2012; Dagia et al., 2004; McCall et al., 2010; Schwartz et al., 2009) and our finding that the compounds inhibit TNF- α -induced VCAM-1 mRNA expression is consistent with the hypothesis that the compounds inhibit VCAM-1 via blockade of a signal transduction mechanism(s).

In conclusion, we have found that addition of a phenyl ring at the 4-position of MMI yields a compound that is significantly more potent than MMI at inhibiting 24 h TNF- α -induced VCAM-1 protein expression by HUVEC. 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 increases 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 results provide important insights into the design of MMI-related anti-inflammatory compounds.

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References

- Benavides U, Gonzalez-Murguiondo M, Harii N, Lewis CJ, Sakhalkar HS, Deosarkar SP, Kurjiaka DT, Dagia NM, Goetz DJ, Kohn LD. Phenyl methimazole suppresses dextran sulfate sodium-induced murine colitis. *Eur J Pharmacol.* 2010; 643:129–138. [PubMed: 20550948]
- Bevilacqua MP. Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol.* 1993; 11:767–804. [PubMed: 8476577]
- Butcher JT, Nerem RM. Valvular endothelial cells and the mechanoregulation of valvular pathology. *Philos Trans R Soc B-Biol Sci.* 2007; 362:1445–1457.
- Carter RA, Campbell IK, O'Donnel KL, Wicks IP. Vascular cell adhesion molecule-1 (VCAM-1) blockade in collagen-induced arthritis reduces joint involvement and alters B cell trafficking. *Clin Exp Immunol.* 2002; 128:44–51. [PubMed: 11982589]
- Cooper DS. Antithyroid drugs. *N Engl J Med.* 1984; 311:1353–62. [PubMed: 6387489]
- Courreges MC, Kantake N, Goetz DJ, Schwartz FL, McCall KD. Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization. *Mol Basel Switz.* 2012; 17:12365–12377.
- Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest.* 2001; 107:1255–62. [PubMed: 11375415]
- Dagia NM, Goetz DJ. A proteasome inhibitor reduces concurrent, sequential and long term IL-1b and TNF-a induced endothelial cell adhesion molecule expression and adhesion. *Am J Phys.* 2003; 285:C813–C822.
- Dagia NM, Harii N, Meli AE, Sun X, Lewis CJ, Kohn LD, Goetz DJ. Phenyl methimazole inhibits TNF-a induced VCAM-1 expression in an IFN regulatory factor-1-dependent manner and reduces monocytic cell adhesion to endothelial cells. *J Immunol.* 2004; 173:2041–2049. [PubMed: 15265939]
- Deosarkar SP, Bhatt P, Lewis CJ, Goetz DJ. A quantitative real-time PCR approach for the detection and characterization of endothelial cells in whole blood. *Ann Biomed Eng.* 2011; 39:2627–2636. DOI: 10.1007/s10439-011-0354-x [PubMed: 21796502]
- Emami S, Foroumadi A. Synthesis of 4-(4-methylsulfonylphenyl)-3-phenyl-2(3H)-thiazole thione derivatives as new potential COX-2 inhibitors. *Chin J Chem.* 2006; 24:791–794.
- Gan SF, Wan JP, Pan YJ, Sun CR. Water-Mediated Multicomponent Reaction: A Facile and Efficient Synthesis of Multisubstituted Thiazolidine-2-thiones. *Synlett.* 2010:973–975.
- Giuliani C, Bucci I, Montani V, Singer DS, Monaco F, Kohn LD, Napolitano G. Regulation of major histocompatibility complex gene expression in thyroid epithelial cells by methimazole and phenylmethimazole. *J Endocrinol.* 2010; 204:57–66. [PubMed: 19837722]
- Gopalan, PK.; Jones, DA.; McIntire, LV.; Smith, CW. Cell adhesion under hydrodynamic flow conditions. In: Coligan, JE.; Kruisbeek, AM.; Margulies, DH.; Shevach, EM.; Strober, W., editors. *Current Protocols in Immunology.* J. Wiley; New York: 1996. p. 7.29.1-7.29.23.
- Kohn LD, Goetz DJ, Benavides-Peralta U, Gonzalez-Murguiondo M, Harii N, Lewis CJ, Napolitano G, Dagia ND. Methods for the amelioration of episodes of acute or chronic ulcerative colitis. *US Patent.* 2011; 7:928,132.
- Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2⁻CT Method. *Methods.* 2001; 25:402–408. DOI: 10.1006/meth.2001.1262 [PubMed: 11846609]
- Luscinskas FW, Gimbrone MA. Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. *Annu Rev Med.* 1996; 47:413–421. [PubMed: 8712792]
- Martin AI, Nieto-Bona MP, Castellero E, Fernandez-Galaz C, Lopez-Menduina M, Gomez-Sanmiguel AB, Gomez-Moreira C, Villanua MA, Lopez-Calderon A. Effect of cyclooxygenase-2 inhibition by meloxicam, on atrogen-1 and myogenic regulatory factors in skeletal muscle of rats injected with endotoxin. *J Physiol Pharmacol Off J Pol Physiol Soc.* 2012; 63:649–659.
- McCall KD, Holliday D, Dickerson E, Wallace B, Schwartz AL, Schwartz C, Lewis CJ, Kohn LD, Schwartz FL. Phenylmethimazole blocks palmitate-mediated induction of inflammatory cytokine

- pathways in 3T3L1 adipocytes and RAW 264.7 macrophages. *J Endocrinol.* 2010; 207:343–353. [PubMed: 20813836]
- Nishikaku F, Aono S, Koga Y. Protective Effects of D-Penicillamine and a Thiazole Derivative, Sm-8849, on Pristane-Induced Arthritis in Mice. *Int J Immunopharmacol.* 1994; 16:91–100. [PubMed: 7514159]
- Pattan SR, Hullolikar RL, Dighe NS, Ingalagi BN, Hole MB, Gaware VM, Chavan PA. Synthesis and Evaluation of some New Phenyl Thiazole Derivatives for their Anti-Inflammatory Activities. *J Pharm Sci Res.* 2009; 1:96–102.
- Schwartz AL, Malgor R, Dickerson E, Weeraratna AT, Slominski A, Wortsman J, Harii N, Kohn AD, Moon RT, Schwartz FL, Goetz DJ, Kohn LD, McCall KD. Phenylmethimazole decreases Toll-like receptor 3 and noncanonical Wnt5a expression in pancreatic cancer and melanoma together with tumor cell growth and migration. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2009; 15:4114–4122.
- Seydel, JK. *Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling.* Vol. 15. Wiley-VCH Verlag GmbH & Co KGaA; 2002. *Drug-Membrane Interaction and Pharmacokinetics of Drugs*; p. 141-215.
- Siddiqui N, Arshad MF, Ahsan W, Alam MS. Thiazoles: A valuable insight into the recent advances and biological activities. *Int J Pharm Sc Iences Drug Res.* 2009; 1:136–143.
- Theoclitou ME, Delaet NGJ, Robinson LA. Rapid parallel synthesis of combinatorial libraries of substituted 3-thio-1,2,4-triazoles and 2-thioimidazoles. *J Comb Chem.* 2002; 4:315–319. [PubMed: 12099849]
- Unangst PC, Connor DT, Cetenko WA, Sorenson RJ, Kostlan CR, Sircar JC, Wright CD, Schrier DJ, Dyer RD. Synthesis and Biological Evaluation of 5-[[3,5-Bis(1,1-Dimethylethyl)-4-Hydroxyphenyl]methylene]oxazoles, -Thiazoles, and -Imidazoles – Novel Dual 5-Lipoxygenase and Cyclooxygenase Inhibitors with Antiinflammatory Activity. *J Med Chem.* 1994; 37:322–328. [PubMed: 8295221]
- Wenisch C, Myskiw D, Gessl A, Graninger W. Circulating selectins, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in hyperthyroidism. *J Clin Endocrinol Metab.* 1995; 80:2122–6. [PubMed: 7541802]

Fig. 1.

Compounds used in the present study. Methimazole (MMI) is used clinically for the treatment of autoimmune disease and is chemically defined as 3-methyl-1,3 imidazole-2 thione (The number 1 next to the nitrogen indicates the 1-nitrogen). Addition of a phenyl group to the 4th position of the imidazole ring of MMI yields phenyl methimazole (C10 – termed **1a** in this paper). Addition of a methoxy group, at the para position of the phenyl ring of **1a**, yields **1b**. Substitution of the imidazole ring for a thiazole ring in **1a** yields **2a**. Addition of a methoxy group, at the para position of the phenyl ring of **2a**, yields **2b**. Note that **2b** is the thiazole analog of **1b**.

Fig. 2.

1a is significantly more potent than MMI in terms of the ability to inhibit 24 hr. TNF- α induction of VCAM-1 protein expression. (A and B) Confluent HUVEC monolayers were treated with TNF- α in the presence of varying concentrations of MMI or **1a**. After 24 hours, the level of VCAM-1 protein present on the HUVEC was determined via ELISA as described in the methods. A ratio between the determined values and the value obtained when HUVEC were treated with carrier alone (DMSO) and TNF- α was multiplied by 100% to give the % activity. The % activity is plotted versus the concentration of MMI (A) or **1a** (B). (C) The IC₅₀ for MMI and **1a** was determined from at least three separate experiments for each compound. The results for each compound were averaged to arrive at the IC₅₀ for that compound in terms of the ability to inhibit 24 h TNF- α induction of VCAM-1 protein expression. Error bar is S.E.M.; * $P < 0.05$.

Fig. 3.

Modification of **1b** affords additional compounds that inhibit 24 h TNF- α -induced VCAM-1 protein expression. Confluent HUVEC monolayers were treated with TNF- α in the presence of varying concentrations of (A) **1b**, (B) **2a**, or (C) **2b**. After 24 hours, the level of VCAM-1 protein present on the HUVEC was determined via ELISA as described in the methods. A ratio between the determined values and the value obtained when HUVEC were treated with carrier alone (DMSO) and TNF- α was multiplied by 100% to give the % activity. The % activity is plotted versus the concentration of compound used in the treatment. Error bar is S.E.M.

Fig. 4.

A graph comparing the average IC₅₀ obtained for the various compounds. The IC₅₀ for the compounds was determined from at least three separate experiments for each compound. The results for each compound were averaged to arrive at the IC₅₀ for that compound in terms of the ability to inhibit 24 h TNF- α induction of VCAM-1 protein expression. There was no significant difference between the imidazole and thiazole paired compounds, specifically there was no difference between **1a** and **2a** nor between **1b** to **2b**. Addition of a para methoxy group to the appended phenyl does significantly increase activity; specifically **1b** activity is significantly greater than **1a**'s activity and **2b**'s activity is significantly greater than **2a**'s activity. The values presented are mean $\pm$ S.E.M. of at least three independent experiments. * P < 0.05.

Fig. 5.

The effect of the compounds on MTS signal. Confluent HUVEC monolayers were treated with varying concentrations of (A) MMI, (B) **1a**, (C) **1b**, (D) **2a** or (E) **2b**. After 24 hours, an MTS assay was performed. A ratio between the determined values and the value obtained when HUVEC were treated with carrier alone (DMSO) was multiplied by 100% to give the % MTS signal. The % MTS signal is plotted versus the concentration of compound used in the assay. Error bar is S.E.M.

Fig. 6.

TC₅₀ for the compounds as assessed by an MTS assay. The concentration of compound that resulted in 50% decrease (TC₅₀) in the MTS HUVEC signal is shown. MMI is significantly less toxic than any of the phenyl derivatives (* $P < 0.05$) and the thiazole derivatives are more toxic than the imidazole counterparts (**2a** vs. **1a**, # $P < 0.05$; **2b** vs. **1b**, % $P < 0.05$). The values presented are mean $\pm$ S.E.M. of three independent experiments.

Fig. 7.

Comparison of the compounds' toxic to inhibitory indices (TII). TII is calculated by taking the ratio of the TC_{50} to IC_{50} for each compound. The phenyl derivatives of MMI exhibited a greater TII than MMI. The values presented above are ratio $\pm$ S.E.M. as determined by propagation of error analysis.

Fig. 8.

The effect of the compounds on monocytic (U937) cell adhesion to HUVEC in an *in vitro* adhesion assay that mimics fluid dynamic conditions present *in vivo*. U937 monocytic cells were perfused over unactivated HUVEC or 24 h TNF- α -activated HUVEC in an *in vitro* flow chamber assay. To gain insight into the role of VCAM-1 in the adhesion, the HUVEC were pre-treated with a function blocking mAb to VCAM-1 immediately prior to the adhesion assay. In certain cases, the TNF- α activation was carried out in the presence of one of the compounds or DMSO (carrier control). Each compound caused a significant reduction in U937 adhesion compared to treatment with DMSO alone (* $P < 0.05$) – bracket indicates comparison of each bar under bracket to DMSO bar. Wall shear stress = 1.8 dynes/cm². The values presented are mean $\pm$ S.E.M. of $n = 3$. All compounds were used at 70 μ M and the mAb concentration was 20 μ g/ml.

Fig. 9.

Each compound inhibits 24 h TNF- α -induced VCAM-1 mRNA expression by HUVEC. HUVEC were treated with TNF- α for 24 hours in the presence of the various compounds or carrier control (DMSO). Subsequently, RNA was collected, cDNA generated, and real-time PCR was performed. The % inhibition was determined relative to the carrier control using the $\Delta\Delta C_t$ method. Each compound caused a significant reduction in VCAM-1 mRNA transcripts compared to treatment with DMSO alone (* $P < 0.05$) – bracket indicates comparison of each bar under bracket to DMSO bar. All compounds were used at 70 μM except MMI, which was used at 1,200 μM . The values presented are an average of $n = 3$ experiments.