Small Organic Molecule Inhibition of Tumor Necrosis Factor-α Induced Vascular Cell Adhesion Molecule-1 Expression by Endothelial Cells

A thesis presented to

the faculty of

Russ College of Engineering and Technology of Ohio University

In partial fulfillment

of the requirements for the degree

Master of Science in Biomedical Engineering

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August 2013

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This thesis titled
Small Organic Molecule Inhibition of Tumor Necrosis Factor-α Induced Vascular Cell Adhesion Molecule-1 Expression by Endothelial Cells

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ABSTRACT

ALAPATI, ANUJA, M.S., August 2013, Biomedical Engineering

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Director of thesis: Douglas J. Goetz

Suppressing aberrant endothelial cell adhesion molecule (ECAM) expression, which plays a critical role in leukocyte adhesion and thus pathological inflammation, is a promising therapeutic approach to treat inflammatory disease. The ECAMs, in particular vascular cell adhesion molecule-1 (VCAM-1), are up regulated by pro-inflammatory cytokines, e.g. tumor necrosis factor-α (TNF-α), during inflammation. VCAM-1 is considered a crucial molecule for inhibition as it is involved in many inflammatory diseases including atherosclerosis, arthritis, and colitis. Several studies report that subtle modifications in a compound’s structure can significantly increase the compound’s activity and toxicity. In this study, we compared the ability of several small organic compounds to inhibit TNF-α induced VCAM-1 expression by human umbilical vein endothelial cells (HUVECs) using an enzyme-linked immunosorbent assay (ELISA). In parallel, the compounds’ effects on HUVEC viability was studied using an MTS assay. The MTS assay is a measure of metabolic activity and thus can correlate with cell viability. After obtaining the inhibition and toxicity levels of the compounds, the “therapeutic indices” were determined. The therapeutic index gives insight into the compound’s potency relative to its toxicity.
Combined, these studies provide insights into the structure-activity relationships for small organic compound inhibition of TNF-α induced VCAM-1 expression by HUVECs.
ACKNOWLEDGEMENTS

I would like to express deepest gratitude to my academic advisor, Dr. Douglas Goetz, for his continuous support in the pursuit of my MS studies. I very much appreciate his motivation, enthusiasm, immense knowledge and guidance that helped me in the research and writing of this thesis. I would also like to thank Dr. Stephen Bergmeier for providing the compounds used in this research. My sincere thanks extend to the rest of my thesis committee members Dr. Monica Burdick, Dr. Fabian Benencia for their encouragement, valuable insights and mentorship offered during my study and research. Additionally, I am thankful to Dr. Sudhir Deosarkar and Cecilia Benencia for their mentorship and training in the laboratory. I also thank my fellow lab mates Chunyian Qi, John O’ Brien, Venkatesh Shirure, and Grady Carlson for their assistance and alliance in the course of accomplishing my thesis studies over the past two years.
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CHAPTER 1: INTRODUCTION

1.1 Pathological inflammation, ECAMs, and VCAM-1

Leukocyte adhesion to the endothelium plays a crucial role in pathological inflammation [1]. This adhesion, which occurs in the fluid dynamic environment of the circulation, is mediated in part by endothelial cell adhesion molecules (ECAMs; e.g., VCAM-1, ICAM-1, and E-selectin) [1]. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) up-regulate ECAM expression [2]. For instance, TNF-α has been shown to induce VCAM-1 expression on human umbilical vein endothelial cells (HUVECs) in vitro [3]. While these elevated levels are important for a normal inflammatory response, the ECAMs can be overexpressed and functional during pathological inflammation [1, 4]. These elevated ECAMs can contribute to disease progression [5]. For example, VCAM-1 expression is a critical factor in many inflammatory diseases such as atherosclerosis, arthritis, neuro-inflammatory, and cancer [3, 5-8]. Therefore, compounds which inhibit cytokine induced ECAM expression could aid in treating the inflammatory diseases by interrupting leukocyte adhesion.

1.2 Methimazole

Methimazole (MMI) is the classic treatment in Europe for Graves’ disease, a hyperthyroid auto-immune disease [15]. It is thought that MMI, a thioamide compound, reduces thyroid disease by inhibition of IFN-γ induced major histocompatibility complex (MHC; e.g., Class-I molecules) gene expression [15, 16]. MMI has also shown efficacy in animal models of autoimmune diseases including systemic lupus erythematosus, uveitis,
and diabetes [9-11]. Upon treatment of Graves’ disease patients with MMI, decreased levels of circulating ECAMs which include VCAM-1 have been observed [12].

1.3 Small organic compounds related to MMI may hold promise as inhibitors of VCAM-1 expression

1.3.1 Phenyl methimazole

Subtle changes to the structure of a drug can immensely affect the drug’s biological activity [18]. Addition of aromatic rings, such as phenyl groups, may allow the compound to cross the cell membrane more easily [19]. Phenyl methimazole (C10) is a phenyl structural derivative of MMI [5, 13]. Previous work revealed that C10 exhibits 70-100 fold greater suppression of abnormal MHC gene expression than MMI in thyroid epithelial cells [15]. C10 was shown to be effective in in vitro models of diabetes and in vivo murine model of ulcerative colitis [13, 14]. It has also been shown that C10 can reduce the pro-inflammatory cytokine, specifically TNF-α, induced VCAM-1 expression in human aortic endothelial cells through IFN regulatory factors [5]. In the latter study the effect of MMI on VCAM-1 expression was not determined. Thus, a goal of the present study was to determine the effect of MMI on TNF-α induced VCAM-1 expression.

1.3.2 Structural derivatives of C10; Imidazole vs. Thiazole

We also sought to explore the structure space around C10. Specifically, several studies have shown that addition of activating groups like methoxy or alkyl in the drug structure increases the activity of the drug [3, 8, 20-23]. In addition, C10 is an imidazole, which is a closely related heterocyclic analog of thiazole [24]. The commercial
significance of thiazole derivatives as anti-inflammatory and immune-suppressive agents is well known [25-35]. Thiazole derivatives are also highly selective to protein kinase activity than imidazole derivatives, which indicates a prominent pharmaceutical significance [36, 37]. In addition, previous studies have shown that thiazoles exhibit higher efficacy than imidazole 5-LOX, COX inhibitors in rat models of inflammation with edema [38]. Thus, we sought to investigate thiazole analogs of C10.

Combined, an effort to identify compounds more effective than MMI motivated us to study C10 and structural derivatives of C10, as well as thiazole analogs, on TNF-α induced VCAM-1 expression on HUVECs.
CHAPTER 2: SPECIFIC AIMS

Hypothesis: Derivatives of MMI, C10 and its structural derivatives, can show greater efficacy in the reduction of TNF-α induced VCAM-1 expression on HUVECs than MMI.

The following specific aims will be performed in order to evaluate the validity of the proposed hypothesis.

The research aims to identify structural derivatives of 3-methyl-1, 3 imidazole-2 thione (MMI) that exhibit greater efficiency than MMI in inhibiting TNF-α induced VCAM-1 expression and to compare their toxicity.

The 50 % inhibition concentration (IC$_{50}$) for MMI and its derivatives will be determined for TNF-α induced VCAM-1 expression on human umbilical vein endothelial cell (HUVECs) using an enzyme linked immunosorbent assay (ELISA). In addition, we will determine the concentration of compound needed to reduce 50% of the signal in an MTS assay (the TC$_{50}$). The MTS signal correlates with metabolic activity / cell viability.

a. IC$_{50}$/ TC$_{50}$ values for MMI versus phenyl methimazole (C10) will be determined.

b. IC$_{50}$/ TC$_{50}$ values for C10 versus 4- (4-methoxyphenyl)-3-methyl-1, 3-imadizole-2-thione (COB-111: a methoxy derivative of C10) will be determined.

c. IC$_{50}$/ TC$_{50}$ values for thiazole derivatives of C10 and COB-111 will be obtained.

Specifically, we will determine the IC$_{50}$/ TC$_{50}$ for
1. 4-phenyl-3-methyl-1, 3 thiazole-2-thione (COB-140: a thiazole derivative of C10).

2. 4- (4-methoxyphenyl)-3-methyl-1, 3 thiazole-2-thione (DRB-2: a thiazole derivative of COB-111).

3. 4-phenyl-3-propyl-1, 3 thiazole-2-thione (COB-141: a thiazole derivative of C10 with propyl vs. a methyl group on the 3-nitrogen).
CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Materials

The reagents for HUVECs culture and ELISA were described previously [5]. 16% para-formaldehyde was obtained from Electron Microscopy Sciences (# 15710, Hatfield, PA). The assay buffer was Hanks buffered saline solution with Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS\(^{+}\)) supplemented with 5% FBS. MMI, C10, COB-111, COB-140, COB-141, and DRB-2 were synthesized by Dr. Stephen Bergmeier in the Department of Chemistry and Biochemistry at Ohio University. 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).

3.2 Antibodies

Anti-CD106 (Anti-VCAM-1) 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). Anti E-selectin, anti ICAM-1, and anti Class-1 were obtained from commercial sources.

3.3 Cell Culture and treatment of HUVECs

HUVECs were cultured as previously described [5]. For the ELISA and cell viability assays, HUVECs were grown until they reached 95-100% confluency. The cells were activated with TNF-α at 10 ng/ml concentration, and treated with compound in the carrier
(0.1% DMSO for ELISA and 0.25% DMSO for MTS) for 24 hour period unless otherwise noted.

3.4 ELISA

The activated and treated HUVECs were washed, fixed in 1% paraformaldehyde for 20 min at 4°C, washed, and incubated with 5% FBS in HBSS+. Following that, the HUVECs were washed and incubated with primary antibody (e.g., anti-VCAM-1) at 10 µg/ml for 20 min at 4°C. The HUVECs were then washed and incubated with peroxidase conjugated anti-mouse IgG at 1:100 dilution for 20 min at 4°C. Subsequently, the HUVECs were subjected to 5-6 washes with the HBSS+. Thereupon, HUVECs 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, # RS-232 C, Dubuque, IA).

3.5 Cell viability assay

20 µl of MTS/PMS solution was added to the wells containing the HUVECs as per the supplier’s protocol. Subsequently, the plate was incubated at 37°C for 1 hour in humidified 5% CO2 atmosphere. OD was read at 490 nm on the Multiskan MCC Spectrophotometer.

3.6 Statistics

One-way analysis of variance (ANOVA) was performed to assess the statistical differences in ELISA and cell viability assays. If compounds exhibited significant differences, Dunnett’s test was used for multiple pair wise comparisons. Statistical
differences of VCAM-1 expression (4 & 24 hr.) ± pre-incubation (0.5, 1 & 2 hr.) were also assessed using ANOVA analysis. Student’s t-test was used to assess statistical differences for compounds’ effect on adhesion molecules expression with its control in ELISA assays. Values of p < 0.01 were considered significant for the assays.
CHAPTER 4: EXPERIMENTAL RESULTS

4.1 Modifications to the parent compound MMI produce C10 and related derivatives and analogs

Methimazole is the parent compound and has a methyl group at the 3- nitrogen on an imidazole ring and a double bonded sulphur element (thione) on the 2-carbon. MMI is chemically defined as 3-methyl-1, 3 imidazole-2 thione (MMI) (Fig. 1A). Dr. Stephen C. Bergmeier’s laboratory at Ohio University has generated compounds structurally similar to methimazole. His laboratory provided the compounds used for this study.
Figure 1: C10 and its related derivatives were formed by structural modifications of parent compound MMI. Addition of a phenyl group to the 4th position of the imidazole ring of parent compound MMI (A) yields phenyl methimazole (B). Inclusion of methoxy group on the phenyl ring of C10 produces COB-111 (C). Alteration of the imidazole ring to a thiazole ring of C10 results in COB-140 (D). Substituting the methyl group for a propyl group on the 3-nitrogen on COB-140 generates COB-141 (E). Addition of a methoxy group on the phenyl ring of COB-140 in the para position gives DRB-2 (F). Note that DRB-2 is the thiazole analog of COB-111. Note: The compounds were provided by Dr. Stephen C. Bergmeier.

Addition of a phenyl ring onto the 4-position of the imidazole ring of MMI yields phenyl methimazole (C10) (Fig. 1B). C10 is chemically defined as 4-phenyl-3-methyl-1, 3 imidazole-2-thione. Addition of a para methoxy group onto the phenyl ring of C10 produces COB-111 that is chemically defined as 4- (4-methoxyphenyl)-3-methyl-1, 3-imadizole-2-thione (Fig. 1C). Alteration of the imidazole ring of C10 to a thiazole ring produces COB-140 that is chemically defined as 4-phenyl-1, 3 thiazole-2-thione (Fig. 1D). Substitution of the methyl group for a propyl group at the 3-nitrogen on the thiazole
ring of COB-140 yields COB-141. The propyl (CH$_3$-CH$_2$-CH$_2$) group is believed to add more hydrophobic nature to COB-141 [21]. COB-141 is chemically defined as 4-phenyl-3-propyl-1, 3 thiazole-2-thione. Inclusion of a methoxy group on the phenyl ring of COB-140 at the para position yields DRB-2 that is chemically defined as 4- (4-methoxyphenyl)-3-methyl-1, 3 thiazole-2-thione (Fig. 1 F). Note that DRB-2 is the thiazole analog of COB-111.

4.2 Preliminary screen of the effect of COB-111 on ECAMs led us to focus on VCAM-1

Dagia et al. have shown that C10 exhibits significant inhibitory effects on TNF-α induced VCAM-1 expression by human aortic endothelial cells (HAEC) [5]. We analyzed COB-111’s effect on TNF-α induced ECAMs and Class-1 expression. Specifically, we investigated COB-111’s effect on ICAM-1, VCAM-1, E-selectin, and Class-1 expression upon treatment of HUVECs for 4 hr. with TNF-α. Human IgG was used as a negative control for the primary antibodies and the concentration of COB-111 was 100 μM. This analysis showed that COB-111 exhibited no inhibitory effects on E-selectin, ICAM-1 and Class-1, while it showed statistically significant reduction in VCAM-1 expression (Fig. 2A).
Figure 2: COB-111 significantly reduces the 24 hr. TNF-α induced VCAM-1 expression, but showed no effect on 4 hr. TNF-α induced ICAM-1, E-selectin, and Class-1 expression. The data shows the COB-111 effect on ECAMs (E-selectin, VCAM-1, and ICAM-1) and Class-1 molecules upon 4 hr. TNF-α stimulation of HUVECs (A). COB-111 does not show any effect on E-selectin, ICAM-1 and Class-1, but reduces the augmented VCAM-1 expression due to 4 hr. treatment with TNF-α (A). IgG is used as a negative control for the antibodies (A & B). COB-111 effect on VCAM-1 expression upon 24 hr. TNF-α stimulation and 4hr. TNF-α stimulation ± pre-incubation (PI) with COB-111 is shown (B). COB-111 dramatically reduces the induced VCAM-1 expression upon 24 hr. TNF-α stimulation. The pre-incubation studies with COB-111 did not show any additional effect on VCAM-1 reduction (B). The values presented above are mean ± SD of triplicate wells (A & B). *p<0.001; **p<0.0001; ANOVA, Student’s t-test.

We next checked to see if the pre-incubation (PI) of HUVECs for 0.5, 1, and 2 hr. prior to treatment with TNF-α with 100 μM COB-111 could augment the drug action on VCAM-1 expression. Simultaneously, COB-111’s effect on 24 hr. TNF-α activation was also investigated. PI of HUVECs with COB-111 did not augment inhibition. Since the PI studies showed no significant effect, only 2 hr. PI data is included in the graph (Fig. 2B far right 4 hr. data). COB-111 did cause a significant reduction in VCAM-1 expression
post-24 hr. TNF-α treatment. This inhibitory effect was greater (50%) than that observed with 4 hr. TNF-α activation. We carried out studies to determine if PI for 1 hr. with COB-111, in a dose dependent manner, would further reduce VCAM-1 expression post-24 hr. TNF-α activation. We did not observe any difference with PI compared to no PI. We also varied DMSO conc. (0.1%, 0.25%, and 0.5%) and observed no difference in the inhibitory effect of COB-111 (data not shown).

4.3 C10 and its structural derivatives greatly reduces TNF-α induced VCAM-1 expression

ELISA experiments were conducted to determine the concentration of compounds needed to inhibit 50% of the VCAM-1 expression after 24 hr. incubation with TNF-α. The IC\textsubscript{50} values were calculated by using the linear regression curves (Fig. 4) obtained from ELISA data (Fig. 3). This analysis yielded a MMI IC\textsubscript{50} value of 1135 µM ±182 (0.25% DMSO). C10 showed ~90% higher reduction in VCAM-1 than MMI. Specifically, the IC\textsubscript{50} for C10 was 66 µM ±4. COB-111 exhibited ~50 % more reduction in VCAM-1 compared to C10.
Figure 3: C10 and its related derivatives show greater inhibition of 24 hr. TNF-α induced VCAM-1 expression compared to MMI. The data shown in (A) represent the percentage reduction in the VCAM-1 expression normalized to the DMSO+TNF-α treated HUVECs. There is a reduction in the TNF-α induced VCAM-1 expression with increasing concentrations of compounds. The values presented above are mean ± SEM. The graphs presented are representative of at least three independent experiments. Dashes (−) indicate data not taken due to solubility issues or the cells being washed off as described in the text. *p<0.01; **p<0.001; ANOVA, Dunnett’s test.
Figure 4: C10 and its related derivatives show greater inhibition of 24 hr. TNF-α induced VCAM-1 expression compared to MMI. The linear regressions for the corresponding ELISA data are shown above. The values presented above are mean ± SEM. Corresponding R2 values for the compounds are presented in the graphs. The results presented are representative of at least three independent experiments.

IC$_{50}$ for COB-111 was 25µM ±25. During the washing of the HUVECs, in the ELISA assay prior to addition of OPD, cells were observed to be washed away around the corners of the wells treated with COB-111 at 100 µM. Hence, for COB-111 only data up to 50 µM were used to obtain the IC$_{50}$. COB-140, a thiazole derivative of C10, exhibited an IC$_{50}$ of 57 µM ±2. COB-141 had an IC$_{50}$ as 31 µM ±4. DRB-2, a thiazole
version of COB-111, was found to exhibit an IC$_{50}$ at 36 µM±12. Similar to COB-111, cells were observed to be washed away around the corners of the wells treated with 100 µM of DRB-2 during the final washing steps. Thus, the 100 µM data for DRB-2 was not used in the IC$_{50}$ analysis. COB-141 had solubility issues at 100 µM and thus no data was collected at this concentration. The IC 50 data of the imidazole and thiazole compounds are compared in Fig. 4.

![Figure 5](image)

Figure 5: A graph comparing the IC$_{50}$ data obtained from ELISA is shown above. The concentrations of the drugs that yield ~50% decrease (IC$_{50}$) in TNF-α induced VCAM-1 expression are shown in the figure. The structural derivatives of MMI, individually, are compared with MMI in 4A & the C10 derivatives are compared, individually, with their thiazole analogs in 4B. C10, COB-140 had a ~90-95% greater inhibition of TNF-α induced VCAM-1 expression compared to MMI. COB-111, COB-141, DRB-2 exhibited a ~40-50% more reduction in TNF-α induced VCAM-1 expression when compared to C10. The values presented above are mean ± SEM of at least three independent experiments. ** p<0.001, * p<0.01; ANOVA, Dunnett’s test.
4.4 C10 and its structural derivatives are reasonably non-toxic to HUVECs

MTS assays were performed to evaluate the concentration of compounds needed to reduce MTS signals by 50% on HUVECs (Fig. 6). The MTS signal correlates with metabolic/cell activity. The TC$_{50}$ values were determined by using the linear regression curves obtained from MTS data (Fig. 7). TC$_{50}$ for MMI was 2446 µM ±215 (Note: MMI is diluted in 1% DMSO to obtain solubility). The TC$_{50}$ was reduced by 50% for C10 compared to MMI. TC$_{50}$ for C10 was 1236 µM ±121. COB-111 showed a TC$_{50}$ of 1013 µM ± 184. COB-111 exhibited similar toxicity as C10. Further, we compared the imidazole group compounds with the thiazole compounds. TC$_{50}$ for COB-140 was 467 µM ± 74.

While performing the MTS assay for COB-140, it was noted that at the higher concentrations (1000 µM and 500 µM) the “solution” contained “fuzzy structures” that were occupying a significant portion of the wells. To investigate this issue, we performed follow up experiments with COB-140. Fuzzy structures and long rod like structures were observed when COB-140 was diluted to 1000 µM and 500 µM (0.25% DMSO) in complete growth medium (CGM). A few rod like structures were observed in growth media without serum and PBS at 1000 µM and 500 µM. We suspect that at high concentrations, COB-140 may be affecting the proteins in CGM. Therefore, only the MTS data for concentrations up to and less than 250 µM were graphed and used to determine the TC$_{50}$ for COB-140. COB-141 had a TC$_{50}$ of 339 µM ±164. “Solubility” issues were observed at 1000 µM during the MTS assay for COB-141. COB-141 was not immediately soluble in 0.25% DMSO at high concentrations (>1000µM). It needed to be
vortexed thoroughly several times, incubated at 37°C for 20-30 min, or allowed to sit at room temperature for at least 1 hr. for COB-141 to be dissolved in CGM. Hence, only COB-141 data up to 500 µM were used to obtain the TC_{50}. DRB-2 analysis yielded a TC_{50} value of 521 µM±53. The cells were not clearly observed, under microscope, for 1000 µM DRB-2 and COB-111. The small round bodies that appeared like disintegrated cells/ precipitates were seen spread throughout the well at this concentration of DRB-2 and COB-111.

Figure 6: C10 and its related derivatives show higher cytotoxicity on 24hr. compound treated HUVECs compared to MMI. The data shown represents the percent reduction in HUVECs MTS with treatment of compounds normalized to HUVECs treated with the DMSO control. There is a decrease in HUVECs MTS value with increasing compound concentration. The graphs presented above are mean ± SEM of three independent experiments. Dashes (−) indicate data not taken due to issues as described in the text. * \( p<0.01; \) ANOVA, Dunnett’s test.
Figure 7: C10 and its related derivatives show higher cytotoxicity on 24hr. compound treated HUVECs compared to MMI. The linear regression lines for the corresponding MTS data are shown. The values presented above are mean ± SEM. Corresponding $R^2$ values for the compounds are presented in the graphs. The results presented are representative of at least three independent experiments.

The TC$_{50}$ data of imidazole and thiazole drugs are compared as shown in Fig. 8.
Figure 8: A bar chart comparing the TC$_{50}$ data of compounds obtained from the MTS assays is shown above. The concentration of compound that resulted in 50% decrease (TC$_{50}$) of MTS signal on HUVECs is shown in the figure. MMI has the lowest toxicity. C10 and COB-111 showed ~50% higher toxicity compared to MMI. COB-140, DRB-2, and COB-141 displayed ~70-90% increase in toxicity when compared to MMI. The values presented above are mean ± SEM of at least three independent experiments. The compounds are compared individually to one another for statistical analysis. *p<0.01; ANOVA, Dunnett’s test.

4.5 Therapeutic index of imidazole and thiazole derivatives of MMI

Therapeutic index (TI) was determined by calculating the ratio of TC$_{50}$ to IC$_{50}$. The TI for MMI is 2. The TI for C10 is 19 and for COB-111 is 40. TI of COB-141 is 11. TI for COB-140 is 8 and for DRB-2 is 14. TI data of imidazole and thiazole compounds was compared in Fig. 9.
Figure 9: Comparison of the compounds’ therapeutic indecies (TI). TI is calculated by taking the ratio of the TC$_{50}$ to IC$_{50}$ for each compound. C10 and its related derivatives showed higher TI than the parent compound MMI. The values presented above are ratio ± error. The error bars were obtained using propagation of error analysis.
CHAPTER 5: DISCUSSION

Our studies reveal that C10 and its structural derivatives, formed by subtle modifications of the parent compound MMI, work more effectively than MMI. Specifically, we found that C10 and its derivatives dramatically inhibit TNF-α induced VCAM-1 expression on HUVECs compared to MMI. Studies on medicinal structure-activity relationships affirm that subtle structural modifications can immensely affect the biological activity. The augmented effectiveness in the suppression of VCAM-1 is likely due to the effects of subtle structural alterations of the derivatives.

Preliminary studies of COB-111, a methoxy derivative of C10, on 4 hr. TNF-α induced ECAM expression using HUVECs revealed that COB-111 shows a modest effect on VCAM-1 reduction (Fig. 2A, column 8 vs. 9) and showed no effect on ICAM-1, E-selectin and Class-I inhibition (Fig. 2A, column 5 vs. 6, 11 vs. 12, 14 vs. 15). The 24 hr. data showed that COB-111 exhibits a higher reduction in VCAM-1 expression than at the 4 hr. time point (Fig. 2A, column 8 vs. 9, compared to Fig. 2B column 5 vs. 6). Altering DMSO concentration or performing pre-incubation with COB-111 prior to TNF-α treatment showed no additional effect on VCAM-1 reduction (Fig. 2B, column 13 vs. 14, 15 vs. 16).

Others have reported that inclusion of phenyl groups on compounds, e.g., globular adiponectin, flavonoids, and nitrobenzene compounds, yield an increased biological activity [40-42]. When C10 (phenyl methimazole) is tested in parallel with its parent
compound MMI (imidazole), C10 showed a dramatically increased (~95%) reduction in VCAM-1 expression compared to MMI (Fig. 5A).

COB-111 when tested alongside C10, as stated before, exhibited 50% higher reduction in VCAM-1 (Fig. 5B) than C10. This is likely due to addition of methoxy group to the phenyl ring. The two compounds also tended to show almost similar cytotoxic levels (Fig. 8). When high shear stress is applied during washings in the ELISA, the HUVECs showed decreased adhesion upon treatment with higher concentration of COB-111. A 40-50% increase in cytotoxicity levels were observed with C10 compared to MMI (Fig. 8).

Previous research affirms that thiazoles (e.g., urazole, aryl phenyl hetrocycyl sulphide derivatives) are more selective to protein kinase activity than imidazoles (e.g., flutrimazole) [33, 34, 37]. However, fewer, if any, studies have been performed on imidazole vs. thiazole efficiency on VCAM-1 reduction. In our studies, COB-140 (thiazole analog of C10) showed similar IC$_{50}$ as C10 and DRB-2 (thiazole analog of COB-111) IC$_{50}$ is close to COB-111 (Fig. 3). Thiazole substitutions for imidazole appear to retain similar activity for reduction of VCAM-1 expression. Similar to COB-111, HUVECs treated with DRB-2 showed reduced adhesive strength suggesting this quality is due to inclusion of a para methoxy group on the phenyl ring. Also, extension of the alkyl group (e.g., alkyl chain) on the thiazole analog of C10 (COB-141) had the same activity as COB-111 (Fig. 5) but an increase in solubility issues consistent with earlier reports [20]. Thiazoles with high protein kinase selectivity would be expected to be less toxic to cells [36]. However, we found that thiazole derivatives appeared to show higher
cytotoxic levels than imidazoles on HUVECs (Fig. 6). COB-140 appeared to induce aggregation that needs to be further investigated.

Previous research suggest that MMI could work for hyperthyroidism by reducing MHC gene expression and also, in part, by suppressing the ECAMs as the Graves’ diseases patients treated with MMI showed reduced circulating ECAMs [14, 15]. Comparatively, MMI is a less active than C10 in in vitro models of auto-immune disease (Graves') [15] and, based on results here, pathological inflammation. In this study, structural derivatives of C10 such as COB-111, DRB-2 and COB-141 appeared to be more effective than C10, while COB-140 showed similar activity as C10, on reduction of TNF-α induced VCAM-1 expression on HUVECs. Thus, the structural derivatives of C10 have potential for treatment of pathological inflammation. Further studies into the capability of C10 derivatives as anti-inflammatory agents are ongoing.

In conclusion, we found that i) addition of a phenyl ring, and methoxy-phenyl group on the 4-carbon of MMI enhances inhibition of TNF-α induced VCAM-1 expression compared to MMI, and ii) substitution of a thiazole ring for an imidazole ring retained activity levels of reduction of TNF-α induced VCAM-1 expression, but iii) substitution of a thiazole ring for the imidazole ring appears to increase the toxicity to HUVECs. In addition, we found evidence that inclusion of a para methoxy group on the 4-phenyl ring of MMI derivatives leads to decreased adhesive strength of HUVECs and solubility issues arise at lower concentration with addition of a propyl chain on 3-nitrogen of phenyl MMI derivatives. Combined, the aforementioned results suggest that C10 structural derivatives hold promise as potential therapeutics for pathological
inflammation particularly diseases involving TNF-α induced VCAM-1 (e.g., ulcerative colitis, arthritis and atherosclerosis).
CHAPTER 6: FUTURE AIMS

6.1 Study the effect of thiazole analog of MMI on the TNF-α induced VCAM-1 expression in vitro

By performing ELISA and MTS assays on the 3-methyl-1, 3 thiazole-2 thione (thiazole analog of MMI), we can obtain IC\textsubscript{50}, TC\textsubscript{50} data for this compound. These data would further substantiate or refute our conclusion that substitution of thiazole for an imidazole ring has little effect on the activity level, but increases the toxicity level.

6.2 Study the imidazole and thiazole compounds effect on protein kinase activity

High selectivity of compounds to protein kinase activity signifies pharmaceutical importance, as such compounds are expected to be less toxic to human cells and have been shown to cause fewer side effects on drug usage [37]. As mentioned earlier, thiazole derivatives are shown to be more selective to protein kinase activity than imidazole [37, 38]. Based on these studies, one would expect that the thiazole derivatives would exhibit a lower toxicity to HUVECs. However, the thiazole derivatives studied in this thesis appeared to exhibit higher toxicity than imidazoles. Hence, performing the protein kinase activity studies will provide better insights on the efficiency/toxicity of imidazole and thiazole derivatives of C10.

6.3 In vivo study of C10 and its structural derivatives on reduction of TNF-α induced VCAM-1 expression in a murine model

Mice (e.g., C57B1/6J) can be used to test the reduction of adhesion molecules in vivo [43]. Based on our in vitro studies, C10 and its structural derivatives can cause
significant reduction in TNF-α induced VCAM-1 expression. Hence, it would be
insightful to test the compounds in a murine model. Mice could be injected with TNF-α
(24 hr. treatment) [43]. Simultaneously, the mice could be dosed with a single or multiple
bolus of the compounds used in this study. The mice could be sacrificed, the organs
harvested and the tissue sectioned to perform immune-histochemical staining for VCAM-1
expression.
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