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NOVEL INHIBITORS OF CELL ADHESION MOLECULE EXPRESSION

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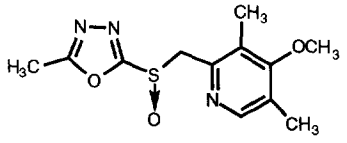
Abstract: 2-Alkylsulfinyl-1,3,4-oxadiazoles have been identified as novel inhibitors of cell adhesion molecule expression on endothelial cells. These compounds have been shown to inhibit the up-regulation of ELAM-1 and VCAM-1 on activated human umbilical vein endothelial cells (HUVECs) as measured by ELISA. Copyright © 1996 Elsevier Science Ltd

Cell adhesion molecules (CAM's) play an integral part in the recruitment of leukocytes in the pathogenesis of inflammation.[1] Exposure of the endothelium to proinflammatory cytokines and other inflammatory stimuli leads to the up-regulation of several adhesion molecules. These include; endothelial leukocyte adhesion molecule-1 (ELAM-1), intracellular adhesion molecule-1 (ICAM-1), and vasculature cell adhesion molecule-1 (VCAM-1). The interaction between the CAMs on the surface of the endothelium and their complementary counter parts on the leukocytes results in the extravasation of leukocytes to the site of injury. Although this is a normal part of the host-defense mechanism, unmodulated recruitment of leukocytes can exacerbate the injury resulting in tissue damage. Agents that could intervene at this step of the inflammation cascade by inhibiting the up-regulation of the adhesion molecules on the endothelium/activation of the leukocyte integrins may prove to be useful anti-inflammatory drugs.[2,3]



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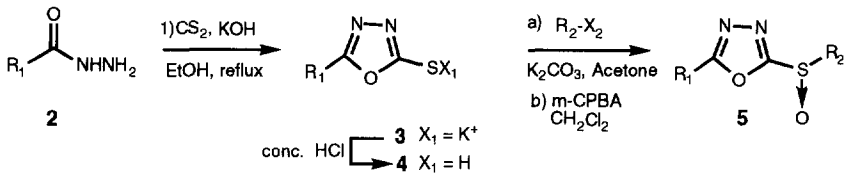
We initiated a program to identify small molecules that could prevent the cell-adhesion process by inhibiting the expression of CAM's on human umbilical vein endothelial cells (HUVECs). An ELISA-based system was set up to detect CAM expression on IL-1β activated HUVECs. Using this system, **1** was identified as an inhibitor of ELAM-1 and VCAM-1 expression with an IC_{50} of 10 μM and 8 μM, respectively. In vivo studies showed that **1** also inhibited neutrophil infiltration in a mouse skin immune-complex dermatitis model, a modified reversed passive Arthus reaction (RPA),[4] but only when **1** was given intradermally at the site of the immune-complex. Studies using rats indicated that **1** was metabolically unstable with an extremely short half-life.[5] We wish to communicate the results of our study to prepare analogs of **1** with improved potency and metabolic stability.

Chemistry, Results , and Discussion

It was established that the 2-alkylsulfinyl substituted 1,3,4-oxadiazole was required for biological activity.[6] A general synthetic route is outlined in Scheme 1. The C-5 substituted 2-mercapto-1,3,4-oxadiazoles were readily prepared by condensing appropriate acid hydrazides (**2**) with CS_2 in the presence of powdered KOH in refluxing EtOH. The reaction was essentially complete within 12-16 h. The 1,3,4-oxadiazoles could be isolated and used in the next step as their potassium salts (**3**), however, in general they were acidified using conc HCl to give the thiols (**4**).[7] The oxadiazole-2-thiols (**4**) were reacted with appropriate alkyl halides in the presence of K_2CO_3 in refluxing acetone to give the thioethers. Treatment of the thioethers with mCPBA gave the sulfinyl compounds (**5**) of interest.

Scheme 1

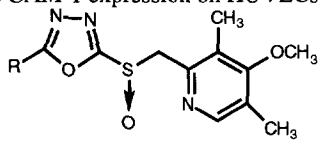


Replacement of the C-5 methyl of the oxadiazole moiety with an ethyl (compound **6**) or *n*-propyl group (compound **7**) gave compounds that were comparable to **1** in inhibiting ELAM-1/VCAM-1 expression (ELISA) (IC_{50}'s in Table 1).[8,9] There was a significant decrease in activity when the alkyl substituent was an *n*-butyl group or longer. Compound **8**, containing the *n*-butyl group at C-5, showed only a 60% inhibition of ELAM-1 expression at 30 μM, while compound **9** containing the *n*-pentyl group displayed a 45% inhibition at a 30 μM dose. Similarly, large lipophilic or sterically hindering groups at C-5 of the oxadiazole also gave compounds that were less active than **1** or completely inactive up to a 60 μM dose (compounds not shown).

Substitution at C-5 of the 1,3,4-oxadiazole with several aryl and heteroaryl groups resulted in varying degrees of activity. Several compounds containing large aromatic groups at C-5 (not shown) had detrimental effects on the morphology of the endothelial cell monolayer.[9] In contrast, many other C-5 substituted aryl and heteroaryl oxadiazoles proved to be extremely potent inhibitors of adhesion molecule expression. Replacement with a simple phenyl group at the C-5 of the 1,3,4-oxidiazole, compound **10**, resulted in a slight enhancement in potency against ELAM-1/VCAM-1 expression (IC_{50} of 7/6 μM vs. an IC_{50} of 10/8 μM for **1**). However, the introduction of electron withdrawing fluoro or trifluoromethyl groups on the phenyl at the C-5 of the oxadiazole gave compounds **11-14** that were less potent than **10** . The 3-fluorophenyl compound **11** and 4-fluorophenyl compound **12** were on the order of ~2-fold less active than **10**. The difference was even more dramatic when a trifluoromethyl group was introduced to the phenyl substituent. Both the C-3 and C-4 trifluromethylphenyl containing oxadiazoles, compounds **13** and **14**, respectively, displayed IC_{50}s that were close to the 30 μM testing dose. A number of compounds (not shown) containing electron donating groups on the phenyl ring at C-5 were found to be either chemically unstable or were toxic to the HUVECs. Compound **15**, which contains a 2-hydroxy group on the phenyl ring at C-5 of the 1,3,4-oxadiazole, was chemically stable and active with an IC_{50} comparable to **1**. Interestingly,

electron-rich 2-thienyl and 2-pyrrolyl groups at C-5 of the oxadiazole, compounds **16** and **17**, respectively, were nearly 2-fold better in inhibiting ELAM-1/VCAM-1 expression than **1**.

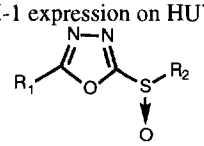
Table 1. Inhibition of ELAM-1 and VCAM-1 expression on HUVECs by C-5 variants.

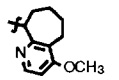


	R	IC$_{50}$ values (μM) and inhibition (%) of CAM expression (ELISA)[a] ELAM-1[b,c]	VCAM-1[b,c]
1	CH_3	10 μM	8 μM
6	CH_3CH_2	12 μM	10 μM
7	$CH_3(CH_2)_2$	10 μM	8 μM
8	$CH_3(CH_2)_3$	60%	85%
9	$CH_3(CH_2)_4$	45%	70%
10	C_6H_5	7 μM	6 μM
11	3-FC_6H_4	15 μM	12 μM
12	4-FC_6H_4	14 μM	11 μM
13	3-$CF_3C_6H_4$	70%	85%
14	4-$CF_3C_6H_4$	50%	70%
15	2-HOC_6H_4	10 μM	8 μM
16	2-thienyl	5 μM	3 μM
17	2-(N-CH_3)pyrrolyl	6 μM	4 μM

[a]HUVECs were stimulated using IL-1β; [b]% inhibition of expression of ELAM-1 or VCAM-1 @ 30 μM or the IC$_{50}$ (μM); [c]average of two assays performed in duplicate with a mean standard error less than 10%.

Table 2. Inhibition of ELAM-1 and VCAM-1 expression on HUVECs C-5 and S-variants.



	R_1	R_2	IC$_{50}$ values (μM) and inhibition (%) of CAM expression (ELISA)[a] ELAM-1[b,c]	VCAM-1[b,c]
18	CH_3	2-$CH_2C_5H_4N$	60%	80%
19	CH_3	$CH_2C_6H_5$	20%	35%
20	CH_3		0%	0%
21	CH_3	2,6-$(CH_3)_2C_6H_3$	0%	0%
22	2-thienyl	$CH_2C_6H_5$	7 μM	6 μM
23	2-pyridyl	$CH_2C_6H_5$	5 μM	4 μM
24	3-pyridyl	$CH_2C_6H_5$	8 μM	6 μM
25	4-pyridyl	$CH_2C_6H_5$	4 μM	3 μM
26	2-HOC_6H_4	$CH_2C_6H_5$	8 μM	6 μM

[a]HUVECs were stimulated using IL-1β; [b]% inhibition of expression of ELAM-1 or VCAM-1 @ 30 μM or the IC$_{50}$ (μM); [c]average of two assays performed in duplicate with a mean standard error less than 10%.

The requirement of the 3,5-dimethyl-4-methoxypyrid-2-ylmethylene group[10a] on the sulfoxide for biological activity was also examined (Table 2). In certain cases, we found that 2-pyridylmethylene or a simple benzyl group could also serve as surrogates for the 3,5-dimethyl-4-methoxypyrid-2-ylmethylene group. Compound **18** containing the 2-pyridylmethylene group in place of the 3,5-dimethyl-4-methoxypyrid-2-ylmethylene group was less active than **1**, only a 60 % inhibition in the ELAM-1 expression was seen at a 30 μM dose. Whereas a complete inhibition of ELAM-1/VCAM-1 expression was observed with **1** at the 30 μM dose. Only a 20% inhibition of ELAM-1 expression was detected at the 30 μM dose when the replacement was a benzyl group (compound **19**). Presumably, the basic nature of the nitrogen of the 2-pyridylmethylene group contributes to the better solubility of these compounds for biological testing. Compounds **22** and **26** containing a benzyl group, on the other hand, showed comparable activity against ELAM-1/VCAM-1 expression as compared with their respective counterparts **16** and **15**, which contain the 3,5-dimethyl-4-methoxypyrid-2-ylmethylene group.

In the case of the 2-benzylsulfinyl-5-(pyridyl)-1,3,4-oxadiazole series of compounds **23**, **24**, and **25**, the 5-(2-pyridyl)oxadiazole compound **23** and 5-(4-pyridyl)oxadiazole compound **25** were of nearly equal potency and greater than 2-fold better in inhibiting ELAM-1/VCAM-1 expression than **1**. The 5-(3-pyridyl)oxadiazole compound **24** was slightly less active than **23** and **25** but a little better than **1** against ELAM-1/VCAM-1. It is not clear on how much of the biological activity can be contributed to the electronic nature of the aryl (heteroaryl) group at C-5 of the oxadiazoles. The electron deficient nature of 2- and 4-pyridyl groups of **23** and **25**, respectively, and electron releasing groups like phenyl, 2-thienyl and 2-pyrrolyl of compounds **10**, **16**, and **17**, respectively, showed similar enhancement in biological activity as compared to **1**.[11]

Very large or sterically hindering groups on the 1,3,4-oxadiazol-2-ylsulfinyl sulfur tended to decrease the biological activity of these compounds, in both the HUVEC assay and the dermatitis model. For instance, **20** substituted with the bridged 4-methoxy-6,7,8,9-tetrahydro-5H-cyclohepta[b]pyrid-9-yl[10b] group and compound **21** containing the 2,6-dimethylphenylsulfinyl group showed no inhibition of ELAM-1/VCAM-1 expression at a 30 μM dose (no inhibition up to a 60 μM dose). We had anticipated that these groups should shield the C-2 position of the 1,3,4-oxadiazole from nucleophiles,[5] and therefore furnish compounds that were metabolically more stable. In fact, of the compounds presented herein, only compounds **20** and **21** displayed a slight improvement in metabolic stability versus **1** in an in vitro liver homogenate system.[12]

Table 3. Inhibition of neutrophil recruitment in a mouse skin immune complex model.

Compound	% Inhibition of Neutrophil Recruitment[a,b] (12.5 μg compound per site)
1	55
10	25
15	20
18	40
22	40
23	80
24	50
25	55

[a]Myeloperoxidase activity was used to quantitate the neutrophil count; [b]average of seven samples with a mean standard error less than 15%.

Table 3 briefly highlights the neutrophil recruitment inhibition data for the mouse skin immune-complex inflammation model (an ELAM-1 dependent model). An injection of 12.5 μg of **1** intradermally at the site of the immune-complex inhibited neutrophil infiltration to the site by 55% as compared with the untreated skin site.[13] Compounds containing straight chain alkyl groups longer than CH_3 at C-5 of the oxadiazole (**6**, **7**, **8**, and **9**) showed little or no inhibition in this model. Only compound **23** gave a better inhibition than **1**. Furthermore, the compounds that were more potent than **1** in the HUVEC assay did not necessarily show greater potency in this immune-complex model. This may be due to several factors: one factor may be the in vivo stability of these compounds; second is their solubility in the tissue site.

In summary, we have identified a novel series of 2-alkylsulfinyl-1,3,4-oxadiazoles that inhibit the up-regulation of adhesion molecules on endothelial cells. We have been able to prepare several compounds, compounds **16**, **17**, **23**, and **25**, that are ~2-fold more potent against ELAM-1/VCAM-1 (ELISA) than our lead compound **1**. Unfortunately, none of these compounds were metabolically more stable than **1**. Some of these compounds have shown anti-inflammatory effects in a mouse dermatitis model but only in a local administration.

References and Notes

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5. In rats, **1** is metabolized within minutes, predominately to 3,5-dimethyl-4-methoxypyrid-2-ylmethyl methylsulfoxide. This metabolite is thought to result from an initial attack of glutathione at the C-2 of the 1,3,4-oxadiazole of **1**. Related metabolites are also found in the metabolism of Omeprazole(Astra Hässle AB) [2(-4-methoxy-3,5-dimethyl-2-pyridyl)methylsulfinyl-5-methoxy-1H-benzimidazole], a H^+/K^+ ATPase inhibitor. For this case the attack of glutathione takes place at the C-2 of the benzimidazole. For a possible mechanism see Weidolf, L.; Karlsson, K.-E.; Nilsson, I. *Drug Metab. Dispos.* **1992**, *20*, 262.

6. Replacement of the 1,3,4-oxadiazole with other heterocycles or replacement of the sulfoxide group of 1,3,4-oxadiazole with a carbonyl or a sulfone resulted in compounds that were inactive in the HUVEC assay.

7. For 2-mercapto-1,3,4-oxadiazole synthesis see: (a) Hoggarth, E. *J. Chem. Soc.* **1952**, 4811. (b) Lacasse, G.; Muchowski, J. M. *Can. J. Chem.* **1972**, *50*, 3082.

8. (a) The ELISA for the determination of presence's of ELAM-1 and VCAM-1 is as follows: 96-well plates were coated with 2% gelatin (1 h). The excess gelatin was aspirated from the wells. HUVE cells were seeded at 20,000 cells/well in a 200 μL volume. The test compounds were dissolved in DMSO at a stock concentration of 50 mM. When the monolayer reached confluence (24-28h after seeding), the test compounds were added to the wells at a final concentrations of 30 μM, 10 μM, 3 μM, and 1 μM. Each

compound was added in duplicate. The plates were incubated for 4 h at 37 °C. Then, IL-1β was added at a final concentration of 2 ng/mL and the plates were incubated for 4 h at 37 °C. The supernatant was removed from the plates by decanting and the wells were washed 3X with 1% BSA-Dulbecco's PBS. Primary antibody [7A9 for ELAM-1 (Otsuka Pharm.) and 2G7 for VCAM-1 (Otsuka Pharm.)] at a concentration of 2.5 μg/mL in a 50 μL volume was added to each well. The plates were incubated at 4 °C for 45 min to 1 h. The wells were washed 3X with 1% BSA Dulbecco's PBS. Secondary antibody (F (Ab')2 fragment biotinylated goat antimouse IgG-IgM (Jackson ImmunoResearch) at 50 μL/well at a final dilution of 1:1,000 was added and incubated for 45 min to 1 h at 4 °C. The wells were washed 3X with 1% BSA-Dulbecco's PBS. Streptavidin-peroxidase (Sigma) at 50 μL/well with a final dilution of 1:30,000 was added to each well and the plates were incubated at 4°C for 30 min. Substrate solution (OPD tablets, citric acid buffer and hydrogen peroxide solution) was added at a concentration of 100 μL/well. The plates were incubated for 15 min at room temperature on plate shaker and the reaction was stopped with 12.5% sulfuric acid. Plates were read for absorbance at 492 nm and the reading for the duplicate averaged.

9. (a) Morphological changes to the monolayer was determined by light microscopy. Toxicity was determined by crystal violet and MTT staining assays in the presence of the compounds at a 30 μM dose.

10. (a) The 2-chloromethyl-3,5-dimethyl-4-methoxypyridine starting material can be prepared according to the procedure outlined in GB patent 2134523 and references ourtlined in Brändström, A.; Lindberg, P.; Bergman, N.; Alminger, T.; Ankner, K.; Junggren, U.; Lamm, B.; Nordberg, P.; Erickson, M.; Grundevik, I.; Hagin, I.; Hoffmann, K.; Johansson, S.; Larssom, S.; Lofberg, I.; Ohlson, K.; Bjorn, P.; Skånberg, I.; Tekenbergs-Hjelte, L. *Acta Chem. Scand.* **1989**, *43*, 536. (b) 9-chloro-4-methoxy-6,7,8,9-tetrahydro-5H-cyclohepta[b]pyridine precursor was prepared according to the procedure of Yamada, S.-I.; Goto, T.; Narita, S.-I. *Chem. Pharm. Bull.* **1994**, *42*, 718.

11. A selective alkylation (nucleophilic attack on the C-2 of the 1,3,4-oxadiazole) of a receptor or an enzyme in the signal transduction pathway is suspected. This may explain the decrease in activity for those compounds that appear to be more stable to attack at C-2 of the oxadiazole.

12. Mazel, P. In *Fundamentals of Drug metabolism and Drug Disposition*; La du, B. W., Mandel, H. G., Way, E. L., Eds.; Robert E. Krieger: Florida, 1971; pp 527-545.

13. Procedure conditions: C57BL/6 mice were shaven and an IV solution injection of 100 μL of BSA at 10 mg/mL in saline was administered. This was followed immediately by an ID 25 μL injection of anti-BSA (0.5 mg/mL solution) along with the test compound or vehicle on a predetermined site on the backside of the mice. Four hours post injection, the animals were sacrificed and the skins removed and assayed for myeloperoxidase (MPO) activity as a measure of neutrophils, according to the procedure of Mulligan, M. S.; Ward, P. A. *J. Imm.* **1992**, *149*, 331. Each compound was tested on a group of seven mice.

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