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Synthetic Analogues of TNP-470 and Ovalicin Reveal a Common Molecular Basis for Inhibition of Angiogenesis and Immunosuppression†

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Abstract—TNP-470 (**1**), a synthetic derivative of the natural product fumagillin (**2**), potently inhibits angiogenesis in vivo and the growth of endothelial cell cultures in vitro. The structurally related natural product ovalicin (**3**) also inhibits angiogenesis but possesses potent immunosuppressive activity. The recent finding that all three drugs bind and inhibit the same target, methionine aminopeptidase 2 (MetAP2), raised the question of whether TNP-470 is also immunosuppressive and whether inhibition of MetAP2 underlies both activities of ovalicin. To address these questions, we synthesized a series of analogues of TNP-470 and ovalicin and tested them for their abilities to inhibit the proliferation of either endothelial cell or mixed lymphocyte cultures. TNP-470 and its analogues were found to possess both immunosuppressive and anti-angiogenic activities. A strong correlation was observed between the ability of compounds to inhibit bovine and human endothelial cell growth and their ability to inhibit the mouse mixed lymphocyte reaction (MLR), implying that the two activities share a common molecular basis, i.e., inhibition of MetAP2. Interestingly, ovalicin and several other compounds behaved differently in the human MLR than in either the mouse MLR or human endothelial cell proliferation assays, pointing to possible species-specific and cell type-specific differences in the metabolism or uptake of these compounds.

Introduction

The growth of solid tumors and metastases is dependent on angiogenesis, the formation of new blood vessels.[1,2] Because blood vessel formation in adults occurs under limited circumstances, inhibition of angiogenesis is emerging as a novel form of cancer chemotherapy, which may be free from side effects associated with conventional cytotoxic drug treatment. Such drugs may also prove useful for treating other angiogenesis-dependent diseases, such as diabetic retinopathy and rheumatoid arthritis. TNP-470 (**1**), a derivative of the fungal metabolite fumagillin (**2**), was one of the first anti-angiogenic drugs to enter human clinical trials.[3,4] Though inactive against most tumor cell lines in vitro, TNP-470 inhibits the growth of tumors implanted in mice indirectly by blocking the proliferation of vascular endothelial cells and the consequent growth of new blood vessels.[3,5] Based on its striking structural similarity to fumagillin and TNP-470, the natural product ovalicin (**3**) was tested and found to be a potent angiogenesis inhibitor.[6] Ovalicin, however, was initially discovered as a potent immunosuppressive drug. It has been shown to inhibit antibody responses, reduce spleen weight, and prolong skin graft survival in mice and to inhibit the development of experimental allergic encephalomyelitis in rats.[7,8] In addition, ovalicin was shown to inhibit the proliferation of mouse and human mixed lymphocyte cultures, a cellular assay measuring allo-antigen-stimulated T cell proliferation.[9,10] The structural similarity between ovalicin and TNP-470 begs the

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†In honor of Professor Stuart Schreiber for his pioneering contributions to the interface between chemistry and biology.

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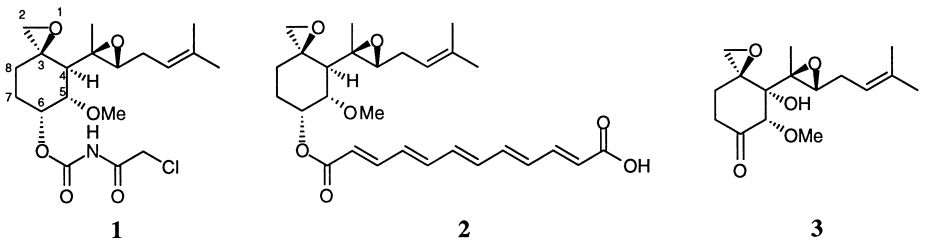


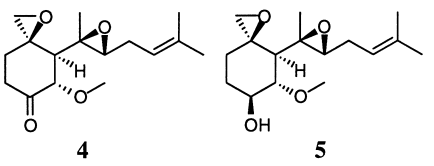
Figure 1.

speculation that TNP-470 may also possess immunosuppressive activity, which could have implications for its use in the clinic. Paradoxically, TNP-470 has been shown to enhance, rather than inhibit, T-cell dependent B lymphocyte proliferation in vitro and in mice.[11,12] Recently, we and others showed that TNP-470, fumagillin, and ovalicin all covalently bind and inactivate a common target, MetAP2.[13,14] Using a series of drug analogues, we found a strong correlation between the ability of compounds to inhibit the growth of cultured endothelial cells and to inhibit MetAP2 activity.[13] However, it remained unknown whether MetAP2 inhibition also mediates the immunosuppressive activity of ovalicin. As both TNP-470 and ovalicin appear to share the same molecular target, it is even more puzzling that the aforementioned difference exists between the apparent immunostimulatory activity of TNP-470 and the immunosuppressive activity of ovalicin. In this study, we examine the relationship between anti-angiogenic and immunosuppressive properties of these compounds by testing a variety of TNP-470 and ovalicin derivatives for their effects on mouse and human mixed lymphocyte cultures. We report that TNP-470 possesses potent antiproliferative activity against both mouse and human mixed lymphocyte cultures. We found a strong correlation between the activities of all analogues tested in the endothelial cell proliferation assay and the MLR, implying that there is a common molecular basis for the anti-angiogenic and immunosuppressive activities of this class of drugs. Surprisingly, we also found that the activity of several compounds diverged greatly between the mouse and human MLR. Inhibition of human endothelial cell growth correlated better with inhibition of the mouse MLR than the human MLR, indicating that there may be cell-type specific differences in availability and metabolism of TNP-470, ovalicin and their analogues that vary between species.

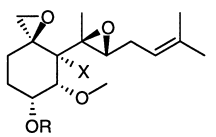
Results

To explore the relationship between inhibition of MetAP2, the anti-angiogenic and immunosuppressive properties of TNP-470 and ovalicin, we synthesized a series of analogues with a wide spectrum of potencies for determining the degree of correlation between those activities. In addition, as TNP-470 (**1**), fumagillin (**2**), and ovalicin (**3**) have all been shown to covalently inactivate MetAP2, we were interested in evaluating the relative contributions of the electrophilic chloroacetyl and epoxide groups of TNP-470 (**1**) to the activity. Thus, compounds bearing a variety of substituents at the C-6 position of the cyclohexyl ring were prepared with either a hydrogen (fumagillin series) or an hydroxyl group (ovalicin series) at the C-4 position of the ring (Scheme 1). In addition, several analogues were synthesized in which the spiro (C-3) epoxide was opened with thiomethoxide (Scheme 2). Compounds in the fumagillin series were prepared according to published procedures;[15–17] those in the ovalicin series were prepared analogously as described in the Experimental section.

We have shown previously that these compounds are active to varying degrees in inhibition of both MetAP2 enzymatic activity in vitro and endothelial cell proliferation in cell culture.[13] In this study, we measured

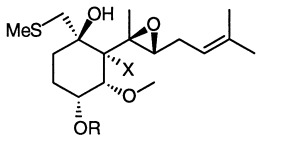


4 **5**



	X	R
6	OH	$CONHCOCH_2Cl$
7	OH	$CO(CH_2)_3Cl$
8	H	$CO(CH_2)_3Cl$
9	OH	H
10	H	H

Scheme 1.



	X	R
11	OH	H
12	H	H
13	OH	$CONHCOCH_2Cl$
14	H	$CONHCOCH_2Cl$

Scheme 2.

the activity of compounds **1**–**14** in the mouse MLR (Table 1). TNP-470 and fumagillin were found to be potent inhibitors of mouse MLR, indicating that they possess intrinsic immunosuppressive activity. Interestingly, the fumagillin analogue (**4**) in which the sidechain at C-6 was removed and replaced with a keto group as in ovalicin is the most potent analogue, rivaling ovalicin for inhibition of mouse MLR. A similar trend was observed previously for endothelial cell inhibition,[13] suggesting that the chloroacetyl group of TNP-470 is dispensable for its biological activity. Overall, there is a strong correlation between compounds that are active in the mouse MLR and compounds that are active at inhibiting bovine aortic endothelial cell (BAEC) proliferation (Fig. 2), though compounds are in general approximately one order of magnitude less potent in the MLR. In both assays, compounds within either the ovalicin or the fumagillin series follow a predictable trend in activity depending on the substituent at the C-6 position, with hydroxyl, chlorobutoyl, chloroacetylcarbamoyl, and carbonyl being the order of ascending activity. In contrast, all compounds in which the C-3 epoxide had been opened were much less active than their corresponding analogues with intact epoxides. An intact spiro epoxide moiety also appears essential for strong inhibition of MetAP2 by these compounds, which suggests that the epoxide rather than the chloroacetyl group mediates covalent inactivation of the enzyme (Table 1). These results imply that the inhibition of endothelial cell growth and the inhibition of lymphocyte proliferation by these compounds shares a common molecular basis, which is likely to be the inhibition of MetAP2.

As ovalicin has undergone and TNP-470 is currently undergoing human clinical trials, a subset of their synthetic analogues were also examined in the MLR performed with primary human peripheral blood mononuclear cells (PBMC, Table 1). Surprisingly, a number of compounds differed significantly in potency from the analogous mouse MLR and human endothelial cell proliferation assays. Most striking was the immunosuppressive parent drug ovalicin, which was over 1000-fold less active at inhibiting the proliferation of human cells compared to mouse cells. In contrast, ovalicin exhibited little toxicity in rodents but was found to be severely neurotoxic in higher animals. The opposing trends of the immunosuppressive activity and neurotoxicity may account for the failure of ovalicin to have become a useful immunosuppressive drug. Epimerization of the C-6 hydroxyl group of **10** generates **5**, which was not only more active against the MLR for both species but over 20-fold more potent in the human than in the mouse MLR. The potency of **5** in the mouse MLR is similar to that ($IC_{50} = 34$ nM) of a known immunosuppressive natural product, FR65814, which is structurally identical to **5** except the 5-methoxy

Table 1. IC_{50} values for TNP-470 and ovalicin analogues in various assays[a]

Compd	mouse MLR (nM)	human MLR (nM)	HUV-EC-C (nM)	BAEC (nM)[b]	MetAP2 (nM)[b]
1	1.63 ± 0.56	1.42 ± 0.40	0.00849 ± 0.00099	0.037 ± 0.0024	1.0 ± 0.3
2	2.32 ± 0.79	0.219 ± 0.072	0.0616 ± 0.0088	ND	ND
3	0.219 ± 0.011	601 ± 91	0.00253 ± 0.00051	0.018 ± 0.0059	0.4 ± 0.2
4	0.128 ± 0.033	0.292 ± 0.051	0.00202 ± 0.00045	0.013 ± 0.0015	6 ± 2
5	9.69 ± 0.54	0.409 ± 0.388	ND	ND	ND
6	7.23 ± 0.46	5.25 ± 5.78	0.0296 ± 0.0231	0.46 ± 0.26	2.0 ± 0.8
7	21.1 ± 9.2	ND	ND	0.31 ± 0.066	0.1 ± 0.03
8	43.8 ± 12.4	ND	ND	0.12 ± 0.01	3.5 ± 1.8
9	59.9 ± 15.1	89.8 ± 22.9	0.187 ± 0.111	2.2 ± 1.4	4 ± 1
10	199 ± 75	88.6 ± 26.3	1.07 ± 0.33	9.5 ± 4.6	8 ± 2
11	108 ± 20	> 10000	23.7 ± 5.0	65 ± 34	400 ± 200
12	> 5000	> 100000	ND	2800 ± 2300	5000 ± 2000
13	810 ± 230	ND	ND	40 ± 4	400 ± 200
14	877 ± 158	ND	ND	110 ± 18	3000 ± 1000

[a]Values represent the mean ± SD for representative experiments performed in triplicate.
[b]Taken from a previous report[13] and listed for comparison. MetAP2 assays were done using recombinant human protein.

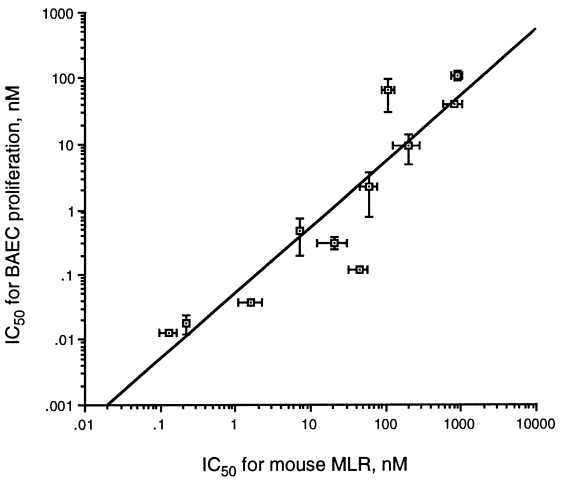


Figure 2.

substituent is replaced by a hydroxyl group.[18] Despite these exceptions, there still appears to be a correlation between modifications that enhance or diminish activity in the mouse MLR and those that do in the human MLR. The C-3 epoxide group, for example, appears to be essential for activity in the human MLR as well.

In order to determine whether the differences observed between mouse and human lymphocyte proliferation were due entirely to species differences, we tested the same subset of compounds for their ability to inhibit the growth of HUV-EC-C cells, a human umbilical vein endothelial cell-derived line which is sensitive to TNP-470 (Table 1). We found a stronger correlation between the activity of compounds in this assay and the BAEC inhibition assay or the mouse MLR than the human MLR. Ovalicin (**3**), for example, is among the most potent inhibitors of HUV-EC-C cell growth. These results suggest that there is something unique about human lymphocytes that makes them respond differently to a subgroup of ovalicin and fumagillin analogues.

Discussion

The striking structural similarity between TNP-470 and ovalicin and the recent finding that both bind to the common molecular target MetAP2 appears to contradict reports that TNP-470 has immunostimulatory activity while ovalicin is immunosuppressive. In this study, we measured the effect of both TNP-470 and ovalicin in the MLR and found that TNP-470 does possess potent immunosuppressive activity. Though previous reports have indicated that lymphocyte proliferation is in fact potentiated by TNP-470, those experimental conditions differed from ours in several respects.[11,12] Most notably, PHA was used as a mitogen, whereas our experiments used allogenic stimulation. Using PHA as a mitogen, we have also observed modest increases in human PBMC proliferation caused by ovalicin (B. T. and J. L., unpublished data). Mitogenic stimulus-dependent differences in the effect of TNP-470 on lymphocyte proliferation have been observed by others as well.[19] As PHA-induced mitogenesis is not mediated by a single receptor but by nonspecific crosslinking of many cell-surface molecules, it is possible that PHA treatment may trigger pathways that are not sensitive to inhibition by TNP-470 or its analogues. Those pathways may not be activated during T cell receptor-mediated signaling such as that provided by allogenic stimulus in the MLR.

Among the analogues we synthesized from fumagillin and ovalicin, compound **4** in which the side-chain containing chloroacetyl group at C-6 of TNP-470 was replaced with a carbonyl group is the most potent inhibitor of angiogenesis and the MLR. The chloroacetyl group in TNP-470 is therefore not essential for either the anti-angiogenic or the immunosuppressive activity of TNP-470. In contrast, opening of the spiro epoxide group led to a dramatic decrease in the activity of TNP-470, suggesting a key role of the epoxide group in the binding of these drugs to MetAP2.

The activities of TNP-470 and ovalicin analogues in the mouse MLR correlates well ($r^2 = 0.83$) with their activities for the inhibition of BAEC proliferation, though approximately 10-fold higher concentrations of drugs were required to elicit the same degree of inhibition in the MLR compared with the BAEC assay. The reason for this discrepancy is unknown, but the high degree of correlation argues for a common underlying molecular mechanism for both processes. This contention is consistent with previous observations that inhibition of lymphocyte proliferation by ovalicin and inhibition of endothelial cell proliferation by TNP-470 both result from cytostatic growth arrest in the G1 phase of the cell cycle.[9,10,20–22] Structural features important for high potency in both cellular assays are also key to the inhibition of MetAP2 (see Table 1), suggesting that inhibition of this enzyme mediates both the anti-angiogenic and immunosuppressive activities of the drugs. We note that the correlation between inhibition of MetAP2 enzymatic activity and the anti-angiogenic or the immunosuppressive activities is significant, but not perfect. For example, **14** is sevenfold more potent than **13** for MetAP2 inhibition, but is of similar potency for inhibition of mouse MLR. While compounds **4** and **10** are equally potent for inhibition of MetAP2, they differ by up to 1500-fold in potency in the cellular assays. One possible explanation is that those compounds have different rates of cellular uptake and metabolism. The

functional significance of these deviations and the regulation of cell growth by MetAP2 is the subject of current investigation.

Interestingly, we also observed significant differences in the activity of several compounds between the human and mouse MLR. The most striking decrease in activity toward human cells was seen for ovalicin (**3**), even though this compound went as far as human clinical trials, a fate undoubtedly based at least to some degree on the initial demonstration of its potent immunosuppressive activity in rodents. Though such observations might indicate species–specific differences in a drug's molecular target which confer differential binding specificities for analogous drugs, this does not appear to be so in the case of TNP-470 or ovalicin, as inhibition of human endothelial cell proliferation correlates better with inhibition of the mouse MLR than with inhibition of the human MLR (see Table 1). In contrast to ovalicin, compound **5** with an inverted stereochemistry at C-6, inhibits human MLR more potently than the mouse MLR. It is possible that there are cell type-specific differences in either the uptake, compartmentalization or metabolism of these drugs that are particular to humans.

The activity of TNP-470 in the MLR warrants reconsideration of its possible immunosuppressive properties in vivo. Immunosuppressive side effects, however, have not been reported for TNP-470 in either animal models or human clinical trials.[4] The apparent lack of immunosuppressive activity of TNP-470 in vivo may be attributed to a combination of a lower sensitivity of lymphocytes to the drug and rapid clearance of TNP-470 from the serum.[23] We have shown that human lymphocytes are over 100-fold less sensitive to TNP-470 than human endothelial cells (Table 1). In addition, it has been demonstrated that TNP-470 has a short serum half-life of less than 1 h.[23] The lack of continuous exposure to TNP-470 may be sufficient to block endothelial cell proliferation without significantly affecting lymphocytes, offering an effective therapeutic window in which beneficial anti-tumor activity can be observed clinically without undesirable immunosuppressive side effects. These observations also demonstrate that despite an apparently common mode of action, it may be possible to find clinically useful angiogenesis-inhibitory compounds which possess little immunomodulatory activity.

Experimental

Solvents were reagent grade and dried prior to use in most cases. Moisture-sensitive reactions were carried out in a flame-dried apparatus under a N_2 atmosphere. Column chromatographic separations were performed with TSI Chemical silica gel 60 (230–400 mesh). The products were dried under high vacuum overnight or over P_2O_5 at ambient temperature. TLC was performed with Merck silica gel 60 F_{254} precoated plates and products were detected with UV light, I_2, 10% H_2SO_4 in MeOH, $KMnO_4$ solution (2.0 g $KMnO_4$, 4.0 g Na_2CO_3, 100 mL H_2O) or *p*-anisaldehyde solution (3.7 mL *p*-anisaldehyde, 1.5 mL AcOH, 5 mL H_2SO_4, 135 mL EtOH). IR spectroscopy was performed with a Perkin-Elmer FT IR spectrometer. NMR spectra were acquired using a Varian VXR-500 spectrometer (^{1}H, 500 MHz), in $CDCl_3$; chemical shifts are reported as ppm downfield from TMS internal standard. MS was performed with Finnegan MATT-8200 spectrometer. Compounds **1**, **5**, **8**, **12**, and **14** were prepared as described.[15–17]

(3*S*,4*R*,5*R*,6*R*)-4-[(1′*S*,2′*S*)-1′,2′-Epoxy-1′,5′-dimethyl-4′-hexenyl]-5-methoxy-6-O-(*N*-chloroacetyl)carbamoyl-1-oxaspiro[2.5]octane-4,6-diol (6). To a stirred solution of **9** (35 mg, 0.12 mmol) in 3 mL of CH_2Cl_2 was added chloroacetyl isocyanate (56 mg, 40 mL, 0.47 mmol) at 0 °C. The reaction mixture was stirred for 1.5 h at room temperature, then diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was chromatographed on silica gel (ether:hexane, 1:2 used as the eluent) to give 39 mg (77.7%) of the product as colorless oil. IR (neat) cm^{-1}: 3466, 3282, 2964, 2935, 1753, 1719, 1497, 1221, 1197, 1101, 1076; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.16 (1H, S), 5.57 (1H, q, J = 3.4 Hz), 5.16 (1H, t, J = 7.3 Hz), 4.48 (2H, s), 3.65 (1H, d, J = 3.9 Hz), 3.48 (3H, s), 3.09 (1H, s), 2.99 (1H, t, J = 6.8 Hz), 2.97 (1H, d, J = 4.4 Hz), 2.54 (1H, d, J = 4.4 Hz), 2.50–2.36 (2H, m), 2.18–2.10 (1H, m), 2.06–1.90 (2H, series of m), 1.73 (3H, s), 1.65 (3H, s), 1.33 (3H, s), 1.08 (1H, m); MS (FAB) m/z 440.2 ($M + Na^+$, 100).

(3*S*,4*R*,5*R*,6*R*)-4-[(1′*S*,2′*S*)-1′,2′-Epoxy-1′,5′-dimethyl-4′-hexenyl]-5-methoxy-6-*O*-(4″-chlorobutyryl)-1-oxaspiro[2.5]-octane-4,6-diol (7). 4-Chlorobutyryl chloride (18 mg, 14 mL, 0.13 mmol) was added to a magnetically stirred solution of **9** (25 mg, 0.084 mmol) and DMAP (15 mg) in CH_2Cl_2 (4 mL) at 0 °C. After stirring for overnight, the reaction was diluted with $CHCl_3$ and which was washed with saturated aqueous NH_4Cl solution. The organic phase was dried, filtered, and evaporated. Chromatography of the residue on silica gel (eluted with 25% ethyl acetate in hexanes) resulted in 31 mg (91.5%) of the product as a colorless oil. IR (neat) cm^{-1}: 3514, 2935, 1728, 1443, 1376, 1245, 1202, 1173, 1144, 1101, 1004, 956, 927; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.60 (1H, dd, J = 3.4 and 7.8 Hz), 5.18 (1H, t, J = 7.5 Hz), 3.63 (1H, d, J = 3.9 Hz), 3.61 (2H, t, J = 6.5 Hz), 3.46 (3H, s), 3.02 (1H, t, J = 6.5 Hz), 2.96 (1H, d, J = 4.4 Hz), 2.87

(1H, s), 2.55 (1H, d, $J=6.8$ Hz), 2.53 (1H, d, $J=7.3$ Hz), 2.51 (1H, d, $J=4.4$ Hz), 2.43–2.33 (2H, series of m), 2.18–2.07 (3H, series of m), 1.80–1.84 (2H, series of m), 1.74 (3H, s), 1.65 (3H, s), 1.34 (3H, s), 1.14 (1H, dt, $J=4.4$, 13.7 Hz); MS(FAB) m/z: 425.1 ($M+Na^+$, 100).

(3*S*,4*R*,5*R*,6*R*)-4-[(1′*S*,2′*S*)-1′,2′-Epoxy-1′,5′-dimethyl-4′-hexenyl]-5-methoxy-1-oxaspiro[2.5]octane-4,6-diol (9). To a stirred solution of ovalicin (**3**, 100 mg, 0.34 mmol) in 5 mL of 1,4-dioxane was added sodium borohydride (25.5 mg, 0.67 mmol) at 0 °C. The reaction mixture was stirred for 30 min at room temperature, after which the reaction was quenched by adding saturated aqueous NH_4Cl and extracted with $CHCl_3$. The combined organic extracts were dried, filtered, and evaporated, and the residue was chromatographed on silica gel (elution with 50% ether in hexane). There was 95 mg (93.7%) of the desired product as a colorless oil. IR (neat) cm^{-1}: 3442, 2925, 1439, 1415, 1381, 1201, 1137, 1108, 1030, 981, 923, 801; ^1H NMR (500 MHz, $CDCl_3$) δ 5.16 (1H, t, $J=7.5$ Hz), 4.45–4.38 (1H, m), 4.02 (1H, d, $J=9.3$ Hz), 3.57 (1H, s), 3.50 (1H, t, $J=3.4$ Hz), 3.50 (3H, s), 2.95 (1H, d, $J=4.4$ Hz), 2.87 (1H, t, $J=6.35$ Hz), 2.52–2.50 (1H, m), 2.54 (1H, d, $J=4.4$ Hz), 2.44–2.35 (1H, m), 2.18–2.10 (1H, m), 2.07–2.00 (1H, 2m), 1.84–1.75 (1H, m), 1.73 (3H, s), 1.65 (3H, s), 1.33 (3H, s), 1.01–0.94 (1H, 2m); MS(FAB) m/z 321.4 ($M+Na^+$, 100).

(1*R*,2*S*,3*R*,4*R*)-1-Methylthiomethylene-2-[(1′*S*,2′*S*)-1′,2′-epoxy-1′,5′-dimethyl-4′-hexenyl]-3-methoxycyclohexane-1,2,4-triol (11). To a stirred solution of **9** (26 mg, 0.087 mmol) in 2 mL of DMF was added thiomethoxide (18 mg, 0.26 mmol) at room temperature. The reaction mixture was stirred for 1.5 h, then diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo, and the residue was chromatographed on silica gel (ethyl acetate:hexane, 1:2 used as the eluent) to give 21 mg (70.0%) of the product as colorless oil. IR (neat) cm^{-1}: 3430, 2924, 1441, 1382, 1324, 1154, 1100, 1057, 1037, 833; ^1H NMR (500 MHz, $CDCl_3$) δ 5.19 (1H, t, $J=7.3$ Hz), 4.33–4.29 (1H, m), 3.70 (1H, br, s), 3.49 (3H, s), 2.97 (1H, t, $J=6.5$ Hz), 2.90 (1H, d, $J=13.2$ Hz), 2.76 (1H, d, $J=13.2$ Hz), 2.60–2.40 (2H, m), 2.25–2.17 (1H, m), 2.14 (3H, s), 1.97–1.81 (3H, m), 1.73 (3H, s), 1.66 (3H, s), 1.62–1.54 (1H, m), 1.49 (3H, s); MS(FAB) m/z: 469.5 ($M+Na^+$, 100).

(1*R*,2*S*,3*R*,4*R*)-1-Methylthiomethylene-2-[(1′*S*,2′*S*)-1′,2′-epoxy-1′,5′-dimethyl-4′-hexenyl]-3-methoxy-4-*O*-(*N*-chloroacetyl)carbamoyl-cyclohexane-1,2,4-triol (13). To a stirred solution of **11** (30 mg, 0.087 mmol) in 2.5 mL of CH_2Cl_2 was added chloroacetyl isocyanate (11 mg, 8 mL, 0.094 mmol) at 0 °C. The reaction mixture was stirred for 1.5 h at room temperature, then diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was chromatographed on silica gel (ether:hexane, 1:2 used as the eluent) to give 35 mg (86.3%) of the product as colorless oil. IR (neat) cm^{-1}: 3485, 3263, 2964, 2925, 1753, 1719, 1501, 1376, 1202, 1158, 1105, 1072, 1028; ^1H NMR (500 MHz, $CDCl_3$) δ 8.26 (1H, br, s), 5.51 (1H, d, $J=2.9$ Hz), 5.18 (1H, t, $J=7.3$ Hz), 4.42 (2H, s), 3.81 (1H, br, s), 3.43 (3H, s), 3.05 (1H, t, $J=6.6$ Hz), 2.95 (1H, d, $J=13.7$ Hz), 2.84 (1H, m), 2.48–2.40 (1H, m), 2.23–2.16 (1H, m), 2.14 (3H, s), 2.04–1.97 (1H, dm), 1.95–1.80 (2H, series of m), 1.78–1.50 (3H, series of m), 1.72 (3H, s), 1.66 (3H, s), 1.47 (3H, s); MS(FAB) m/z 488.1 ($M+Na^+$, 100).

Cell culture. To isolate splenocytes, mice were sacrificed by asphyxiation, and their spleens were removed and pulverized between ground glass slides. Cells were filtered through nylon mesh and erythrocytes lysed in 17 mM Tris–HCl/140 mM NaCl for 5 min at 37 °C. Intact cells were washed several times in 4% fetal bovine serum (FBS) in PBS and resuspended in culture medium (RPMI with 10% FBS, 50 μM β-mercaptoethanol, 50 units/mL penicillin and 50 μg/mL streptomycin). Human PBMCs were isolated by step gradient centrifugation on Ficoll-Paque Plus (Pharmacia) and cultured in RPMI containing 10% human type AB serum, 50 μM β-mercaptoethanol, 50 units/mL penicillin and 50 μg/mL streptomycin. HUV-EC-C cells were obtained from the American Type Culture Collection and cultured in Kaign's modification of Ham's F12 media containing 10% FBS, 100 μg/mL heparin, and 30 μg/mL endothelial cell growth supplement (Sigma), 50 units/mL penicillin and 50 μg/mL streptomycin. Flasks and plates were pretreated with endothelial cell attachment factor (Sigma).

Mixed lymphocyte reaction. Splenocytes from responder (Balb/c) and stimulator (C57/Black 6) mice (5×10^5 of each per well) or human PBMCs from two donors (1×10^5 of each per well) were cultured in a volume of 200 μL in U-bottom 96-well plates in the presence of varying concentrations of drugs or carrier alone (0.5% ethanol) for either 96 h (mouse) or 154 h (human). Cells were treated with 1 μCi [methyl-^3H] thymidine (6.7 Ci/mmol) per well for the last 8 h of culture and then harvested onto glass fiber paper for scintillation counting.

Endothelial cell proliferation assay. HUV-EC-C cells (5000 per well) were seeded into 96-well plates and grown for 96 h in the presence of varying concentrations of drugs or carrier alone (0.5% ethanol). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 25 μL, 10 mg/mL in PBS) was added during the final 4 h after which cells were extracted with 100 μL of

10% SDS/0.1 M HCl per well for 16 h at 37 °C. Plates were read at 600 nm.

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