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Investigation of novel fumagillin analogues as angiogenesis inhibitors

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Abstract—Modification of fumagillin was conducted to develop MetAP-2 inhibitors with desirable pharmacological properties. Replacement of the C4 side chain by benzyloxime preserves the inhibitory activity against MetAP-2 enzyme. Fumagillin analogues containing the C4 benzyloxime moiety were found to be very sensitive to the nature of the C6 substituent on the inhibition activity of HUVEC proliferation. This lack of correlation between MetAP-2 and HUVEC activities might be due to the cellular metabolism of the compounds by epoxide hydrolase, which is present in the cell. Compound (*E*)-**3d**, containing ethylpiperazinyl carbamate at C6 position, exhibited antiangiogenic effects similar to TNP-470 on matrigel plug assay and rat corneal micropocket assay.
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Inhibitors of angiogenesis hold promise as therapeutic agents for treatment of a variety of diseases.[1,2] A great deal of effort has been devoted to the development of antiangiogenic treatments particularly for cancer[3] since Folkman reported that tumor growth and metastasis depend on blood vessel growth.[4] Antiangiogenic agents are expected to be less susceptable to mechanism of resistance, even after protracted treatment, since the actively dividing cancer cells are not the direct target of the therapeutic agent. Furthermore, because angiogenesis is down regulated in most adult processes except reproduction, an antiangiogenic agent may have minimal side effects.[5]

Fumagillin (**1**),[6] a natural product isolated from *Aspergillus fumigatus*, was found to strongly inhibit endothelial cell proliferation.[7] TNP-470 (**2**), which resulted from the subsequent search for improved fumagillin analogues and was found to have greater potency and lower toxicity than fumagillin,[7] is one of the first inhibitors of angiogenesis to reach clinical trials. Fumagillin and TNP-470 are proposed to inhibit angiogenesis by selective inhibition of methionine aminopeptidase type 2 (MetAP-2)[8] through covalently binding to the His231 residue of MetAP-2 by opening the spiro-epoxide.[9] Significant correlation has been reported between inhibition of MetAP-2 and the inhibition of bovine aortic endothelial cell (BAEC) proliferation.[10]

In the clinical trials of TNP-470, however, its poor pharmacokinetic behavior and dose-limiting toxicity remain obstacles to its use as an anticancer agent.[11] The instability and toxicity of TNP-470 are likely due, at least in part, to the presence of two epoxides and a chloroacetyl groups. We envisioned that improvement of TNP-470 would be attained by replacing one or more of these functional groups. Here, we report (*E*)-**3d**, an analogue of fumagillin without the sidechain epoxide and chloroacetylcarbamate group of TNP-470, which showed comparable in vitro and in vivo activity to TNP-470 (Fig. 1).

TNP-470 has three functional groups that are either chemically labile or metabolically unstable in its structure, namely the two epoxides (spiro-epoxide and the one on C4 side chain) and the chloroacetylcarbamate moiety at C6. Reactivity of the spiro-epoxide functional group seems crucial for the binding of fumagillin analogues. Therefore, we first explored C4 sidechain modifications, since crystallographic information indicated that there was significant space within the pocket for further optimization.[9] Modification of the C4 chain was possible by a semisynthetic approach starting from fumagillol (**4a**). Reduction of the side-chain epoxide to the diene (*E*)-**6b** was performed in a four-step sequence,

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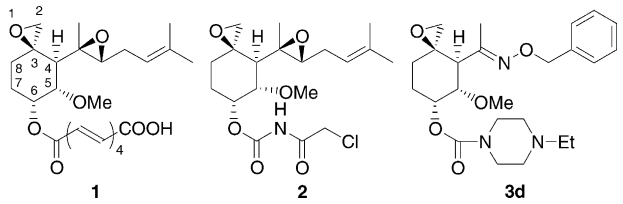
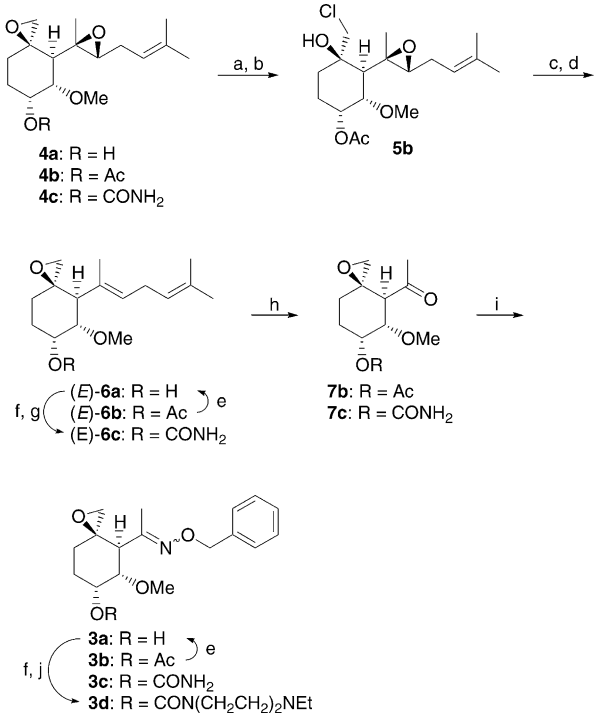


Figure 1.

with some improvement in yields over the procedure of Liu et al.[12,13] as shown in Scheme 1. Opening of the spiro-epoxide before the reduction of the side-chain epoxide to olefin[14] is primarily responsible for the improvement. The diene (*E*)-**6b** was converted by ozonolysis to the methylketone **7b**, a common precursor for the C4 modifications.

According to the data reported previously by Liu et al., compounds with a rotatable single bond between C1′ and C2′ carbons on the C4 side chain show reduced inhibitory activity against MetAP-2 and BAEC proliferation, compared to analogues bearing more rigid epoxide and diene side chains.[13] We hypothesized that planarity of the C1′ and C2′ atoms of the C4 side chain was essential for optimal activity. Compounds with the diene moiety at the C4 chain (**6b** and **6c**) exhibited significant but somewhat weaker enzyme activities toward MetAP-2 (Table 1). Among several moieties investigated for fumagillin C4 side-chain replacement, we found benzyloxime ether to be the best bioisostere.[16] The IC_{50} values of compound (*E*)-**3b** and (*E*)-**3c** toward



Scheme 1. Reagents and conditions: (a) LiCl, AcOH, THF, rt, 96%; (b) Ac_2O, DMAP, CH_2Cl_2, 0 °C, 86%; (c) WCl_6, *n*-BuLi, THF, −78 °C, 92%; (d) *t*-BuOK, THF, 0 °C, 77%; (e) K_2CO_3, MeOH, rt, 100%; (f) ClCOOPh, DMAP, CH_2Cl_2, 0 °C, 100%; (g) NH_4OH, EtOH, 0 °C, 85%; (h) O_3, CH_2Cl_2, MeOH, −78 °C, then Me_2S, 74%; (i) H_2NOBn, *p*-TsOH, 4 Å sieves, THF, 50 °C, 40% (*E*-isomer), 17% (*Z*-isomer); (j) $HN(CH_2CH_2)_2NEt$, CH_2Cl_2, rt, 78%.

Table 1. Activities of selected fumagillin analogues in enzymatic and HUVEC-based assays

Compd	MetAP-2[a] IC_{50} (nM)	HUVEC[b] EC_{50} (nM)
1	0.63[c]	0.82±0.25
2	0.43±0.13[d]	0.52±0.03[e]
4b	0.51[c]	3.0±1.1
4c	0.39[c]	0.65±0.10
(*E*)-**6b**	8.9±2.2	ND
(*E*)-**6c**	1.7±0.5	490±100
(*E*)-**3b**	1.2±0.32	450±180
(*Z*)-**3b**	25±2	ND
(*E*)-**3c**	1.2±0.17[f]	88±55
(*Z*)-**3c**	57±22	ND
(*E*)-**3d**	0.8±0.08	0.90±0.18

[a] Assays were duplicated, unless otherwise indicated.
[b] EC_{50}'s were measured using the sulforhodamine B (SRB) dye binding assay.[15] HUVEC proliferation assays were triplicated, unless otherwise indicated.
[c] $n=1$.
[d] $n=27$.
[e] $n=10$.
[f] $n=5$.

MetAP-2 were approximately 1 nM, and comparable to epoxides **4b** and **4c**, respectively. Comparing the two benzyloxime isomers, the each *E*-isomer was more active than the *Z*-isomer in the enzyme assay and was consistently the major product in the reaction.

With the benzyl oxime moiety established at the C4 position, optimization of the C6 substituent was performed. X-ray crystal structures of fumagillin-MetAP-2 complex showed that the C6 side chain is placed outside of the MetAP-2 binding pocket.[9] From this X-ray structure we expected that the C6 sidechain would not have a significant effect on enzyme inhibition. Optimization of the C6 substituent of fumagillin has been studied extensively.[7,17–19] Previous reports clearly showed though that C6 substituents significantly affect the activity of these analogues.[7,17–19] Most benzyloxime bearing analogues exhibited excellent activities ($\leq$1 nM) in the MetAP-2 assay, whereas inhibition of the human umbilical vein endothelial cell (HUVEC) proliferation varied significantly depending on the C6 substituent. Among the compounds tested, the ethylpiperazinyl carbamate, (*E*)-**3d**, was found to be as active as fumagillin, TNP-470, and AGM-1883 (**4c**) in the HUVEC-based proliferation assay.[20]

Although a reasonable correlation between MetAP-2 inhibition and the inhibition of BAEC proliferation has been reported,[10] we discovered an apparent lack of correlation between MetAP-2 activity and HUVEC proliferation. Table 2 shows that most compounds of this series exhibited similar stability in cell culture medium (67–75% intact after 20 h). In cell lysate, however, the stability of fumagillin analogues varied considerably. For example, less than 3% of compound (*E*)-**3c** and (*E*)-**6c**, compared to ~76% of **4c**, remained after 20 h in HUVEC lysate. Interestingly, compound (*E*)-**3d** was reasonably stable in the cell lysate.

We speculated that the decomposition of fumagillin analogues might be due to epoxide hydrolase (EH) present in

Table 2. Stability of fumagillin analogues in HUVEC culture medium and in HUVEC lysate[a]

Compd	% of the compound remaining[b]					
	Cell culture medium			Cell lysate		
	0 h	4 h	20 h	0 h	4 h	20 h
4d	100	95	75	100	91	76
(*E*)-**6c**	100	76	68	100	36	1
(*E*)-**3c**	100	90	69	100	20	3
(*E*)-**3d**	100	96	67	100	97	68

[a] Error of this measurements is estimated to be 10–15%.
[b] Each compound (1 μM) was incubated in HUVEC lysate at 37 °C for up to 20 h.

HUVEC since HUVEC has been reported to express microsomal epoxide hydrolase gene products.[21] Microsomal epoxide hydrolase is known to hydrolyze the spiro-epoxide of metabolite IV (**4c**) during the metabolism of TNP-470.[22] When compound **4c** was treated with EH,[23] it was readily hydrolyzed as expected (Table 3). On the other hand, hydrolysis of **4c** was inhibited completely when cyclohexene oxide, a specific EH inhibitor,[24] was added to the incubation mixture. This suggested that the hydrolysis of **4c** was mainly due to the enzyme, EH. Interestingly, compound (*E*)-**6c** seemed much less stable toward EH than **4c** since it was hydrolyzed even in the presence of cyclohexene oxide. Thus, it seems that the C4 side-chain epoxide impacts on the stability of the spiro-epoxide in fumagillin analogues. Compound (*E*)-**3c** appears more labile than **4c** but more stable than (*E*)-**6c** based on its rate of hydrolysis in the presence of the inhibitor.

The introduction of an ethylpiperazinyl carbamate group at the C6 position clearly increased the stability of the spiro-epoxide of (*E*)-**3d** against EH activity. When compound (*E*)-**3d** was treated with EH in the presence of cyclohexene epoxide, its hydrolysis was almost completely inhibited as was observed in the case of **4c**. This suggested that introduction of an ethylpiperazinyl carbamate group at the C6 position makes the compound significantly less susceptible to EH. The stability of fumagillin analogues against EH activity correlates with the HUVEC activity while the enzymatic activities of this class of compounds are all quite good.

Compound (*E*)-**3d** was found to dose-dependently (all data not included) inhibit angiogenesis induced by basic fibroblast growth factor (bFGF) in a mouse matrigel plug assay (Table 4), as measured by hemoglobin content of the subcutaneous plug. Compound (*E*)-**3d** was administered at 2.5 mg/kg/day for 7 days by an osmotic pump, and exhibited an anti-angiogenic effect similar to TNP-470 (76% vs 71% inhibition). In the bFGF-induced rat corneal micropocket assay, compound (*E*)-**3d** also showed a similar dose-dependent (all data not included) anti-angiogenic effect (86%) to TNP-470 (90%) as shown in Table 5. In both in vivo models, (*E*)-**3d** caused weight changes similar to those observed with TNP-470. Thus, compound (*E*)-**3d** was as effective as TNP-470 in inhibition of MetAP-2, HUVEC proliferation, and angiogenesis.

Modification of C4 side chain of fumagillin scaffold was accomplished by a semisynthetic approach. Fumagillol was converted to the diene (*E*)-**6b** in 58% yield by a four-step sequence, improving the reported procedure of Liu et al.[13,16,20] The *E*-benzyl oxime moiety, lacking the potentially labile epoxide group, was found to be the best substitute for the C4 side chain of fumagillin. Fumagillin analogues containing

Table 4. Antiangiogenic activity in mouse matrigel assay[a]

Compd	% Inhibition	% Body weight change
2	71	−11
(*E*)-**3d**	76	−13

[a] Dose, 2.5 mg/kg/d for 7 days, s.c. $n = 8$ mice/group.

Table 5. Antiangiogenic activity in rat corneal micropocket assay[a]

Compound	% Inhibition	% Body weight change[b]
2	90	+7
(*E*)-**3d**	86	+10

[a] Dose, 2.5 mg/kg/d for 5 days, s.c.
[b] % Body weight change of the control group was +28% regardless with or without bFGF. $n = 8$ eyes/group.

Table 3. Stability of fumagillin analogues in the presence of epoxide hydrolase (EH)[a]

		% of the compound remaining or produced[b]								
		Control			With EH			With EH and an inhibitor[c]		
		3 min	0.5 h	2 h	3 min	0.5 h	2 h	3 min	0.5 h	2 h
4c	Substrate	100	103	106	84	ND	ND	98	109	107
	Hydrolysate	ND	ND	ND	22	103	100	ND	ND	ND
(*E*)-**6c**	Substrate	100	92	90	18	ND	ND	108	75	5
	Hydrolysate	ND	ND	ND	77	79	100	ND	21	87
(*E*)-**3c**	Substrate	100	103	100	10	2	1	106	95	60
	Hydrolysate	ND	ND	ND	93	95	100	ND	15	60
(*E*)-**3d**	Substrate	100	103	102	96	ND	ND	110	101	106
	Hydrolysate	ND	ND	ND	6	96	100	ND	ND	ND

[a] Error of this measurements is estimated to be 10–15%.
[b] Each compound (2 μM) was incubated with or without epoxide hydrolase at 37 °C. The protein concentration was 1 mg/mL in the final incubation mixture.
[c] Cyclohexene oxide (50 mM) was used as an inhibitor of epoxide hydrolase.

E-benzyloxime moiety at C4 sidechain generally showed good MetAP-2 inhibition activity whereas HUVEC-based activities varied significantly depending on the C6 substituent. The poor correlation between enzyme activity and HUVEC activity might be due to the metabolic stability of the analogues in the HUVEC assay. The C6 substituents of fumagillin analogues were found to influence the susceptibility of the analogues to epoxide hydrolase activity, which might deactivate fumagillin analogues in HUVEC. Among the compounds tested, compound (*E*)-**3d** exhibited the best activity in the HUVEC-based assay, and is roughly equipotent to TNP-470. Compound (*E*)-**3d** also showed activities similar to TNP-470 in the matrigel assay and the rat corneal micropocket assay.

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