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Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2

(TNP-470/cancer/endothelial/catalysis)

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ABSTRACT **Angiogenesis inhibitors are a novel class of promising therapeutic agents for treating cancer and other human diseases. Fumagillin and ovalicin compose a class of structurally related natural products that potently inhibit angiogenesis by blocking endothelial cell proliferation. A synthetic analog of fumagillin, TNP-470, is currently undergoing clinical trials for treatment of a variety of cancers. A common target for fumagillin and ovalicin recently was identified as the type 2 methionine aminopeptidase (MetAP2). These natural products bind MetAP2 covalently, inhibiting its enzymatic activity. The specificity of this binding is underscored by the lack of inhibition of the closely related type 1 enzyme, MetAP1. The molecular basis of the high affinity and specificity of these inhibitors for MetAP2 has remained undiscovered. To determine the structural elements of these inhibitors and MetAP2 that are involved in this interaction, we synthesized fumagillin analogs in which each of the potentially reactive epoxide groups was removed either individually or in combination. We found that the ring epoxide in fumagillin is involved in the covalent modification of MetAP2, whereas the side chain epoxide group is dispensable. By using a fumagillin analog tagged with fluorescein, His-231 in MetAP2 was identified as the residue that is covalently modified by fumagillin. Site-directed mutagenesis of His-231 demonstrated its importance for the catalytic activity of MetAP2 and confirmed that the same residue is covalently modified by fumagillin. These results, in agreement with a recent structural study, suggest that fumagillin and ovalicin inhibit MetAP2 by irreversible blockage of the active site.**

Angiogenesis has been established as an important step for the pathogenesis of a number of human diseases, including diabetic retinopathy, rheumatoid arthritis, and cancer (1–3). In particular, it has been unequivocally shown that tumors cannot grow or metastasize without the formation of new blood vessels (4, 5). Inhibition of angiogenesis, therefore, is emerging as a promising new strategy for the treatment of cancer.

Fumagillin and ovalicin are among the most potent natural-product inhibitors of angiogenesis (6, 7). They act by directly inhibiting endothelial cell proliferation. In an effort to understand the molecular basis of angiogenesis inhibition by these natural products and to unravel the molecular mechanisms controlling endothelial cell proliferation, we and others identified a common molecular target for this class of inhibitors, a bifunctional protein known as methionine aminopeptidase 2 (MetAP2, EC 3.4.11.18; refs. 8 and 9). In addition to its ability to catalyze the cleavage of N-terminal methionine from nascent polypeptides (10), MetAP2 also is known to affect translational initiation through its ability to associate with eukaryotic initiation factor 2α (eIF-2α; ref. 11). We showed that binding of MetAP2 to these inhibitors specifically blocks its aminopeptidase activity without interfering with its association with eIF-2α (8). The exquisite specificity of these inhibitors for MetAP2 was underscored by the lack of effect of these drugs on the closely related type 1 methionine aminopeptidase (MetAP1) both *in vitro* and *in vivo* (8, 9). We further demonstrated that these inhibitors bind to MetAP2 covalently, which may explain their extremely high potency for inhibition of endothelial cell proliferation. The relevance of the MetAP2 interaction was supported by a strong correlation between inhibition of endothelial cell proliferation in cell culture and MetAP2 enzymatic activity *in vitro* for a number of fumagillin and ovalicin analogs (8, 12).

One of the unique structural features of fumagillin and ovalicin is that they possess two potentially reactive epoxide groups (Fig. 1). In light of our prior observation that both of these inhibitors covalently modify MetAP2, the questions became which, if any, of the two epoxide groups is involved in the covalent binding of the drugs to MetAP2 and to what extent each of the epoxide groups contributes to the high-affinity binding of these inhibitors to the enzyme. A closely related question was which amino acid residue(s) in MetAP2 is covalently modified by the drugs. To address these questions, we prepared deoxyfumagillin analogs in which one or both epoxide groups were removed and determined their activity in a MetAP2 enzymatic assay and an endothelial cell proliferation assay. We have found that the ring epoxide is crucial for the activity of fumagillin, whereas the side chain epoxide group is dispensable. In agreement with these observations, we also have found that only the ring epoxide group is involved in the covalent modification of MetAP2. By using a fluorescently tagged fumagillin analog, we have identified His-231 in MetAP2 as the residue that is covalently modified by fumagillin, which is in agreement with the recently determined single-crystal x-ray structure of the MetAP2–fumagillin complex (23). By using a H231N mutant, we have shown that His-231 is required for the covalent binding of fumagillin to MetAP2. Furthermore, we have found that the same residue is essential for the catalytic activity of MetAP2. These results provide important information on the molecular recognition of the fumagillin/ovalicin class of angiogenesis inhibitors by MetAP2 and shed light on the mechanism of catalysis by this class of hydrolytic enzymes.

MATERIALS AND METHODS

Materials. All chemical reagents used in organic synthesis were purchased from Aldrich. All molecular biology reagents and kits were from New England Biolabs, Strategene, and Bio-Rad. The

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Abbreviations: MetAP, methionine aminopeptidase; MetAP1, type 1 MetAP; MetAP2, type 2 MetAP; B–F, biotin–fumagillin conjugate; B–F1, biotin–C1–deoxyfumagillin; B–F2, biotin–C1′–deoxyfumagillin; B–F12, biotin–C1, C1′–dideoxyfumagillin; eIF-2α, eukaryotic initiation factor-2 α.

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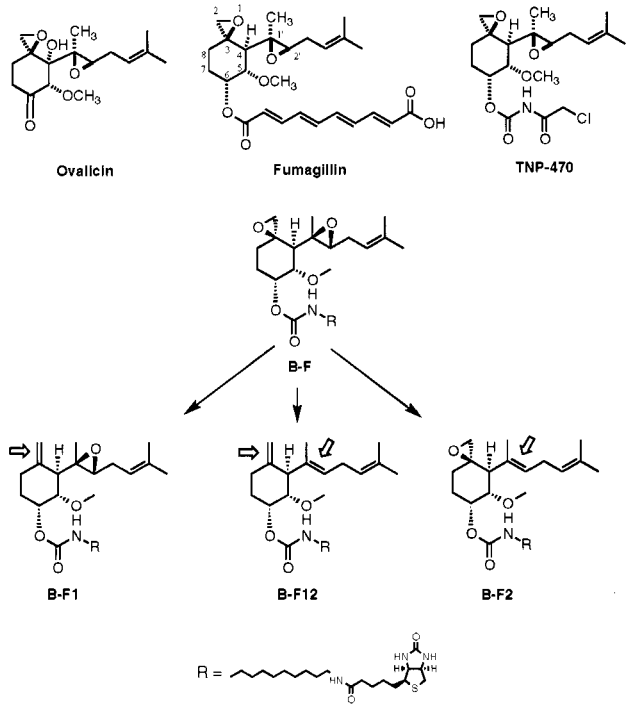

FIG. 1. Chemical structures of ovalicin, fumagillin, TNP-470, biotin–fumagillin (B–F) conjugate and the corresponding deoxyfumagillin analogs. The numbering of the relevant atoms in fumagillin is shown. Epoxide groups that are converted to olefins are indicated by open arrows.

Bac-to-Bac baculovirus expression system was purchased from GIBCO/BRL.

Syntheses of Deoxyfumagillin Analogs and Fluorescein–Fumagillin Conjugate. The detailed synthetic procedures will be published elsewhere due to space limitation. They are available upon request. Each compound was characterized by ^{1}H-NMR, MS, and IR.

Cell Culture. Sf9 (GIBCO/BRL) and High Five cells (Invitrogen) were maintained in Grace's insect cell medium as per manufacturer's instructions. Bovine aortic endothelial cells were grown in low-glucose (DMEM–LG) medium up to passage 18. Endothelial cell proliferation assays were performed as described (8).

MetAP2 Enzymatic Assay. The enzyme assay was performed in a 96-well format. Various concentrations of the inhibitors or a solvent control were incubated with 1 nM recombinant MetAP2 in buffer A (20 mM Hepes, pH 7.5/40 mM KCl/1.5 mM $CoCl_2$) for 1 hr at 4°C. To begin the enzymatic reaction, Met-Gly-Met-Met was added to a final concentration of 4 mM and incubated at 37°C. The reaction was quenched after 20 min by adding EDTA to a concentration of 10 mM. Released methionine was quantitated as reported (13).

MetAP2 Covalent Binding Assay (Near-Western Blot). Covalent binding of fumagillin–biotin to wild-type and mutant MetAP2 was determined essentially as described by Griffith *et al.* (8). To assay deoxyfumagillin analogs, 100 ng of recombinant MetAP2 was incubated with the deoxyfumagillin analogs (40 μM) after preincubation with or without 50 μM ovalicin competitor. The nonspecific carrier protein casein was added to the samples at a concentration of 20 μM. Covalent binding was then assayed as described above.

Identification of the Covalently Modified MetAP2 Residue by Using Fluorescein–Fumagillin. One milligram of recombinant human MetAP2 was incubated with 50 μM ovalicin or ethanol carrier in 1 ml of buffer B (10 mM Hepes, pH 8.0/100 mM KCl/1.5 mM $MgCl_2$/10% glycerol) at 4°C for 2 hr, followed by incubation with 10 μM fluorescein–fumagillin for another 2 hr at 4°C. Samples were dialyzed against 7 liters of 100 mM NH_4HCO_3 overnight to remove unbound drug. After dialysis, samples were concentrated by SpeedVac (Savant) to 50 μl. Ten microliters of 45 mM DTT was added, and the samples were incubated at 50°C for 15 min. To alkylate free sulfhydryls, 10 μl of 100 mM iodoacetamide was added, and the samples were incubated for 15 min at room temperature. Fifty micrograms of sequencing grade chymotrypsin (Boehringer Mannheim) was added to each sample and diluted to 150 μl in 100 mM NH_4HCO_3. Samples were allowed to digest for 24 hr at room temperature. The digested samples were subjected to HPLC separation (Hewlett–Packard model 1100) by using a 2.1 × 250 mm Vydac 214TP52 column with a gradient of 100% buffer A:0% buffer B to 25% buffer A:75% buffer B (buffer A, 0.05% trifluoroacetic acid in H_2O; buffer B, 0.043% trifluoroacetic acid/80% CH_3CN in H_2O) for 90 min and a flow rate of 0.2 ml/min. The absorbance of each eluate was monitored at 280 nm and 496 nm simultaneously, and each peak at 496 nm was collected separately. The fractions corresponding to the two peaks that were absent in the ovalicin-treated sample were subjected to Edman sequencing by using standard procedures.

Expression and Purification of Recombinant Human MetAP2. Recombinant His-tagged human MetAP2 and mutants were expressed by using the Bac-to-Bac baculovirus expression system (GIBCO/BRL). Recombinant baculovirus stocks were generated and amplified as per the manufacturer's instructions. Protein was harvested 36 hr after baculovirus infection of two 15-cm plates of High Five cells. The cell pellet was weighed and lysed (in 5 ml/g of wet pellet) in prechilled lysis buffer [buffer B + 1% Nonidet P-40/1 mM phenymethylsulfonyl fluoride (PMSF)/2 μg/ml leupeptin/2 μg/ml aprotinin/1 μg/ml pepstatin]. The lysate was incubated on ice for 10 min and centrifuged at 10,000 × *g* for 10 min. The supernatant was diluted to 6 ml in buffer B and incubated for 1 hr at 4°C with 1 ml of pre-equilibrated Talon resin (CLONTECH). The Talon resin was pelleted by centrifugation at 1,200 × *g* for 3 min and washed with 4 × 10 ml of buffer B. During the final wash, the resin was slurried into a Bio-Rad Econo column. The 6 × His-tagged MetAP2 was eluted with 6 ml of 50 mM imidazole in buffer B, and 0.5 ml fractions were collected. The amount and purity of recombinant MetAP2 were analyzed by the absorbance at 280 nm and SDS/PAGE (10% gel). The fractions containing the highest amounts of MetAP2 were pooled and dialyzed against 3 liters of buffer B overnight before storage at 4°C.

Construction of Human MetAP2 Mutants. The wild-type human MetAP2 coding region (Y.-H.C.) was PCR-amplified by using a 5′ primer (5′-GATC*GAATTC*ATGGCGGGTGTGG-AGGAG) tagged with an *Eco*RI site (italicized) and a 3′ primer (5′-CATG*CTCGAG*TTAATAGTCATCTCCTCT) tagged with an *Xho*I site (italicized). The product was digested and ligated into pFastBac HTa (GIBCO/BRL). All mutants were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) as per manufacturer's instructions.

RESULTS

Synthesis of Deoxyfumagillin Analogs. To gain insight into the molecular recognition of fumagillin and ovalicin by MetAP2, we first focused our attention on the two potentially reactive epoxide groups. The prior observation that these inhibitors bind covalently to MetAP2 suggested that one or both of these epoxide groups may be involved in covalent binding. Therefore, we sought to determine which of the two epoxide groups was involved in the covalent modification of MetAP2.

Of the potential functional groups, we chose to convert the epoxide groups to olefins, as they are of similar size and conformational flexibility as epoxide groups without additional substituents that would otherwise complicate the interpretation of the results. We also decided to tether each analog with biotin so that the conjugates could be used to evaluate covalent binding to MetAP2 and tested for inhibition of enzymatic activity and cell

proliferation. A number of reagents were examined for the deoxygenation reaction. One reagent (consisting of tungsten hexachloride and *n*-butyllithium) was found to be sufficient for the synthesis of all three deoxyfumagillin analogs (ref. 14; Fig. 2*A*). Thus, when unprotected fumagillol (**1**) was treated with this reagent, C2-deoxyfumagillol (**3**) was obtained as the exclusive product, albeit with low yield. This selectivity for the C2 epoxide is possibly caused by the presence of the C6 hydroxyl group that may direct the tungsten reagent to the ring epoxide. In contrast, when the C6 hydroxyl group was protected by the tributylsilyl group (**2**), the same reaction conditions gave rise to a 1:1 mixture of dideoxyfumagillol (**5**) and the chlorohydrin intermediate (**4**). Treatment of **4** with potassium *tert*-butoxide reformed the ring epoxide, which, on deprotection, yielded the other desired monodeoxyfumagillol (**7**). The three fumagillol intermediates, as well as fumagillol, were converted into the corresponding biotin conjugates by using similar synthetic methods (not shown).

Assessment of the Importance of the Two Epoxide Groups in MetAP2 Binding by Using Deoxyfumagillin Analogs. We next determined the activity of the biotin–deoxyfumagillin analogs along with biotin–fumagillin and TNP-470 in the methionine aminopeptidase assay *in vitro* by using recombinant MetAP2. As shown in Table 1, attachment of biotin to C6 of fumagillin led to an approximately 90% decrease in potency in comparison to TNP-470 or fumaginone, which bears a ketone at C6 (8). In comparison to B–F, the removal of the ring epoxide group biotin–C1–fumagillin (B–F1) caused a decrease in potency to 1/1000 original levels, suggesting that the ring epoxide plays a crucial role in the binding of fumagillin to MetAP2. The removal of the side chain epoxide biotin–C1′–fumagillin (B–F2), however, had little, if any, effect on MetAP2 inhibition. Interestingly, the dideoxyfumagillin analog biotin–C1,C1′–fumagillin (B–F12) was least active, with an IC_{50} value >100 μM, the solubility limit of the conjugate in the assay buffer. When tested in the endothelial cell proliferation assay, the activity of the deoxyfumagillin analogs was found to correlate with their potency in the enzymatic assay

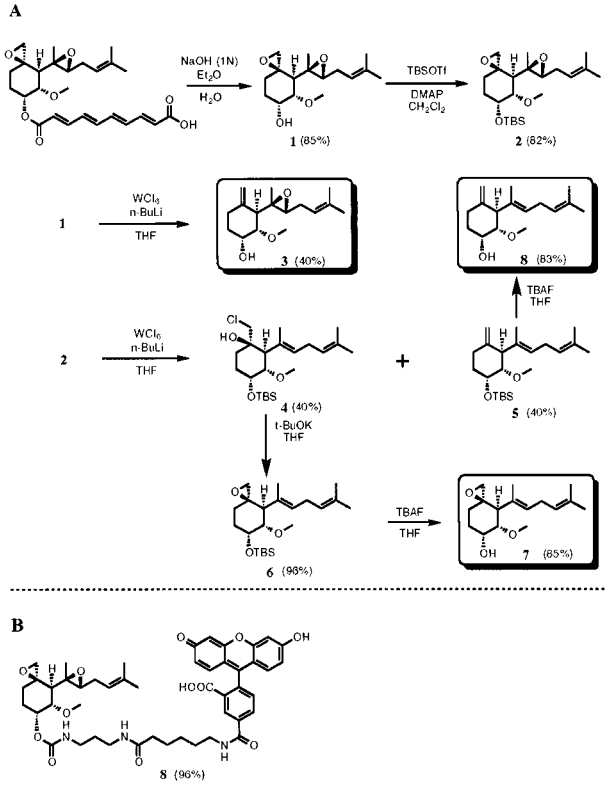

FIG. 2. Syntheses of deoxyfumagillin analogs (*A*), and the structure of the fluorescein–fumagillin conjugate (*B*). The reagents and solvents are indicated. The yields are indicated in parentheses.

Table 1. IC_{50} values for deoxyfumagillin analogs in MetAP2 assay and in endothelial proliferation

Compound	IC_{50}, M	
	MetAP2 activity	BAEC proliferation
TNP–470	1.3×10^{-9} ($\pm 1.0 \times 10^{-10}$)	1.15×10^{-11} ($\pm 4.1 \times 10^{-12}$)
B–F	1.04×10^{-8} ($\pm 1.2 \times 10^{-9}$)	1.77×10^{-10}) ($\pm 6.3 \times 10^{-11}$)
B–F1	4.00×10^{-5} ($\pm 1.3 \times 10^{-6}$)	1.59×10^{-7} ($\pm 7.1 \times 10^{-8}$)
B–F2	3.40×10^{-9} ($\pm 9.0 \times 10^{-10}$)	5.35×10^{-9} ($\pm 2.2 \times 10^{-9}$)
B–F12	$> 1.0 \times 10^{-4}$	8.25×10^{-5} ($\pm 1.9 \times 10^{-5}$)

(Table 1). The apparently higher potency of these analogs in the cell-based assay is caused by the need to use >1 nM recombinant MetAP2 in the enzyme assay—the minimum concentration of the enzyme required to detect the cleaved methionine. Together, these results strongly suggest that the ring epoxide group at C2 is crucial for the activity of fumagillin, whereas the epoxide group on the side chain is dispensable.

To determine which epoxide group is involved in the covalent modification of MetAP2, we employed near-Western blot (so named as it employs a small biotin–fumagillin conjugate instead of an antibody to visualize the MetAP2 protein) by taking advantage of the biotin moiety in the conjugates (8). As previously shown, the biotin–fumagillin conjugate remained bound to MetAP2 after SDS/PAGE and transfer to nitrocellulose membrane and can be visualized by blotting directly with streptavidin–horseradish peroxidase conjugate (Fig. 3, lane 2). This interaction is specific because preincubation of MetAP2 with excess ovalicin prevented binding of biotin–fumagillin (Fig. 3, lane 3). The deoxyfumagillin analogs were found to react nonspecifically with proteins under the reaction conditions as shown by their ability to bind the nonspecific protein casein. After normalization of the background casein band and quantification of the signal, B–F and B–F2 binding to MetAP2 was found to decrease by >50% on ovalicin treatment, whereas the other deoxyfumagillin analogs showed no reduction binding (Fig. 3, lanes 2 and 3 and lanes 6 and 7). Thus, only B–F and B–F2 (compounds that retained the ring epoxide) showed specific covalent binding to MetAP2. Although both B–F1 and B–F12 appeared to bind MetAP2 covalently, such binding was insensitive to excess ovalicin, indicating that this interaction is nonspecific. This nonspecific binding is likely the result of the olefin groups rather than any remaining epoxides, because even the dideoxyfumagillin analog gave a strong interaction (Fig. 3, lanes 8 and 9). Thus, in agreement with the MetAP2 enzymatic assay, the side chain epoxide appears dispensable for specific covalent binding to MetAP2, whereas the ring epoxide is required for this interaction.

Synthesis and Testing of the Fluorescein–Fumagillin Conjugate. Having assessed the importance of the two epoxide groups in fumagillin and ovalicin in the binding to MetAP2, we then turned to the determination of the MetAP2 residue(s) covalently

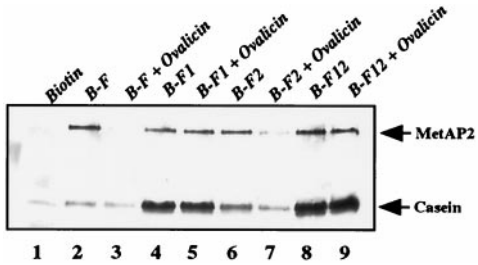

FIG. 3. Near-Western blot to determine the covalent binding of biotin–deoxyfumagillin analogs to MetAP2.

modified by the drugs. In our initial experiments identifying MetAP2 as a fumagillin-binding protein that used a biotin–fumagillin conjugate, no peptide that contained a biotin–fumagillin adduct was identified (8). Several attempts to isolate the biotin–fumagillin adduct of MetAP2 after trypsin digestion with streptavidin-affinity resin did not yield clear results. We reasoned that the inability to isolate the biotin–fumagillin-modified peptide might have been the result of a number of problems, including nonspecific binding of the putative peptide(s) to the affinity resin. To bypass the affinity purification step, we chose to replace biotin with a fluorescent tag that could be used to detect the peptide(s) covalently modified by the corresponding fumagillin conjugate. Fluorescein has a unique absorbance at 494 nm with reasonable intensity (ε = 73,000 $cm^{-1}\cdot M^{-1}$), easily distinguishable from that arising from amino acid side chains. We thus synthesized a fluorescein–fumagillin conjugate (**8**) by using a procedure similar to that for the synthesis of the biotin–fumagillin conjugate (Fig. 2*B*). We determined the activity of the fluorescein–fumagillin conjugate in the MetAP2 enzyme assay and found that it had comparable activity to biotin–fumagillin (data not shown).

Identification of the MetAP2 Residue Covalently Modified by Fumagillin. Recombinant human MetAP2 was incubated with fluorescein–fumagillin, and unbound drug was removed by extensive dialysis. The sample then was reduced and alkylated with iodoacetamide and digested with chymotrypsin. The resulting peptide mixture was subjected to preparative HPLC separation with the detection wavelength set at 496 nm to follow the fluorescein tag. As a control for nonspecific interactions, a second MetAP2 sample preincubated with ovalicin was processed in parallel. Comparison of the two chromatograms showed two unique peaks (Fig. 4*A,* peaks I and II) that were absent in the control sample (data not shown). Edman degradation of peak I gave the sequence of a peptide corresponding to residues 224–232. As expected, Cys-228 migrated to a position corresponding to an adduct with iodoacetamide. In addition, His-231 failed to give any signal, suggestive of chemical modification by fluorescein–fumagillin (Fig. 4*B*). Analysis of peak II yielded a mixture of two peptides in roughly equimolar ratio. One peptide yielded a complete sequence corresponding to residues 218–224, whereas the other peptide corresponded to residues 226–232, overlapping the sequence obtained from peak I. Both Cys-228 and His-231 produced blank cycles in the second peptide (Fig. 4*B*). Together, these results suggested that His-231 in MetAP2 was the residue covalently modified by the fluorescein–fumagillin conjugate, but the role of Cys-228 remained ambiguous. Cys-228 was not covalently modified by fumagillin in the peptide from peak I, but the fact that it did not give a signal corresponding to an iodoacetamide adduct in the second peptide from peak II could be the result of either incomplete alkylation by iodoacetamide or covalent modification by fumagillin.

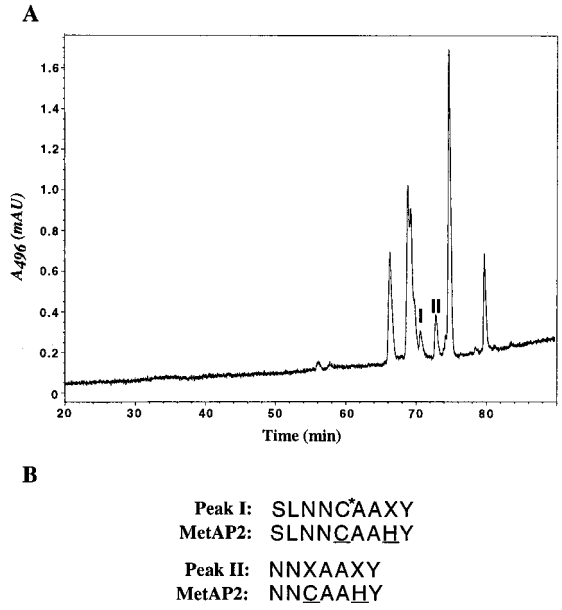

FIG. 4. Identification of MetAP2 residue that is covalently modified by fumagillin. (*A*) HPLC chromatogram of the chymotryptic peptides derived from MetAP2 bound by fluorescein–fumagillin conjugate in the presence (data not shown) and absence (*A*) of ovalicin. Peaks I and II, absent in the control samples, are indicated in *A*. (*B*) Results of Edman degradation of peak I and peak II in *A*. The single-letter amino acid code was used. X denotes a blank signal for the corresponding sequencing cycle. C* denotes a cysteine-*S*-acetamide derivative. The corresponding sequences in MetAP2 are shown below the peptide sequences. Residues that gave either no signal or modified signal in Edman sequencing are underlined.

Site-Directed Mutagenesis of Cys-228, His-231, and His-339 to Assess Their Roles in Drug Binding and Catalysis. To resolve the role of His-231 and Cys-228 in the covalent binding of fumagillin and ovalicin to MetAP2, the corresponding H231N and C228A mutants were created by site-directed mutagenesis. As a control, we mutated His-339, predicted to be at the active site based on the *Escherichia coli* MetAP crystal structure (15). We note that at this time we were informed that His-231 is covalently modified in the crystal structure of MetAP2–fumagillin complex (J. Clardy, personal communication). To confirm that His-231 is indeed the modified residue (as shown by the biochemical and structural studies), we proceeded to produce all three mutants in baculovirus-driven High Five insect cells and purified them to near homogeneity by Talon affinity chromatography. The enzymatic activity of the mutants was then determined, along with that of the wild-type enzyme. The C228A mutant was found to retain almost full catalytic activity, whereas mutation of His-339 resulted in a dramatic decrease in activity (Fig. 5*A*). Both C228A and H339N mutants remained sensitive to ovalicin, with IC_{50} values of 1.1 and 1.5 nM, respectively, in comparison to the wild-type enzyme (IC_{50} = 1.5 nM). In contrast, mutation of His-231

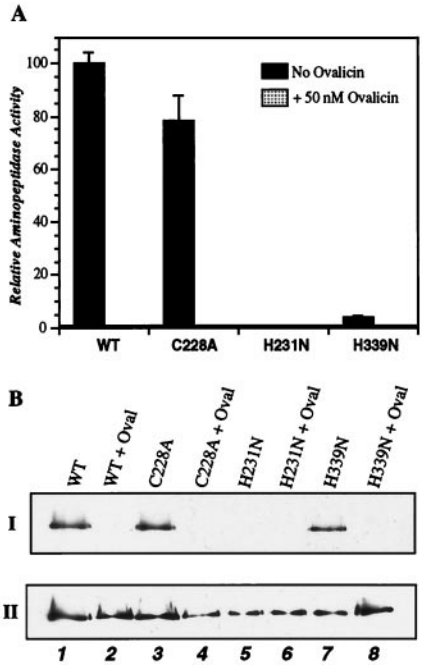

FIG. 5. Determination of catalytic activity, covalent binding to biotin–fumagillin, and tryptic digestion pattern of MetAP2 mutants. (*A*) Methionine aminopeptidase activity of mutant and wild type (WT) MetAP2 and their sensitivity to ovalicin. (*B*) Near-Western blot of WT, H231A, C227A, and H339A MetAP2 mutants by using biotin–fumagillin.

resulted in a complete loss of catalytic activity. The structural integrity of the H231N mutant as assessed by circular dichroism spectrometry and partial proteolysis by using trypsin was found to be indistinguishable from wild-type MetAP2 (data not shown), suggesting the lack of activity of His-231 was not caused by misfolding of the mutant protein. We then examined whether any of the MetAP2 mutants retained the ability to bind biotin–fumagillin covalently by near-Western blot. As shown in Fig. 5*B*, both the C228A and H339A mutants remained capable of covalent binding to biotin–fumagillin, similar to the wild-type enzyme. The H231N mutant, however, was unable to bind to biotin–fumagillin covalently, confirming that His-231 was the site of covalent modification of MetAP2 by this class of angiogenesis inhibitors.

DISCUSSION

The natural products fumagillin and ovalicin have been shown to inhibit endothelial cell proliferation and MetAP2 enzymatic activity with high potency (6–9, 12). How such high-affinity molecular recognition is achieved at the molecular level has remained inscrutable. The presence of two epoxide groups in these inhibitors and the covalent binding of the inhibitors to MetAP2 raised the possibility that one or both of the epoxide groups could be involved in the covalent modification of the enzyme. Reciprocally, it also was of great interest to identify the MetAP2 residue covalently modified by the drugs and to delineate the role of that residue in both catalysis and drug binding.

We took a chemical approach to assess the importance of each of the two epoxide groups in MetAP2 binding and to determine which of the epoxide groups is involved in the covalent modification of the enzyme by converting each of the two epoxide groups to olefins. The reactivity of the two epoxide groups was found to be influenced by the presence of a hydroxyl group at C6 when reacted with tungsten hexachloride and *n*-butyllithium. The ring epoxide group was preferentially deoxygenated in the presence of the C6 hydroxyl group, whereas the side chain epoxide appeared to be more reactive when the C6 hydroxyl was protected. This complete reversal of reactivity was likely caused by a combination of neighboring group participation from the C6 hydroxyl group when it was unprotected and some steric hindrance for the ring epoxide when the C6 hydroxyl group was protected by the tributylsilyl group. The elaboration of a biotin group tethered to the deoxyfumagillin analogs paved the way for the subsequent determination of covalent binding between MetAP2 and these compounds by using near-Western blotting.

The importance of the two epoxide groups for MetAP2 binding was assessed by measuring their IC_{50} values for MetAP2 enzymatic activity *in vitro*. B–F1, lacking the ring epoxide, was found to be about 1/1000 as potent as B–F, whereas B–F2, lacking the side chain epoxide, retained full activity. These results clearly indicate the importance of the ring epoxide in MetAP2 binding. It was somewhat surprising that the dideoxyfumagillin analog B–F12 had a further decrease in activity compared with B–F1, whereas B–F2 was as active as B–F. Thus, the elimination of the side chain epoxide group affects MetAP2 binding affinity differently depending on the presence of the ring epoxide. This result is likely caused by the fact that B–F and B–F2 bind irreversibly in the active site, whereas B–F1 and B–F12 bind reversibly. The covalent binding of B–F2 to MetAP2 masks the decrease in affinity for MetAP2 caused by removal of the side chain epoxide. The further decrease in activity of B–F12 in comparison to B–F1 reflects more closely the true effect of removal of the side chain epoxide group and suggests that it does play a significant role in binding of fumagillin to MetAP2. In addition to the enzymatic assay, the IC_{50} values for these compounds also were determined in endothelial proliferation assay. Although the IC_{50} values for endothelial cell proliferation were about two orders of magnitude lower, as more MetAP2 was required for the enzymatic assay *in vitro*, the changes in potency for different deoxyfumagillin analogs mirrored those seen in the enzymatic assay. This result extends the correlation found between MetAP2 inhibition and inhibition of endothelial cell proliferation for fumagillin analogs (8, 12), further supporting the hypothesis that MetAP2 inhibition mediates the antiproliferative effects of the drugs.

By using the biotin–fumagillin analogs in a near-Western blot, we found that of the three deoxyfumagillin analogs, only B–F2 retained drug-sensitive covalent binding to MetAP2, strongly suggesting that the ring epoxide is involved in the specific covalent modification of MetAP2 (Fig. 3). This result is consistent with the finding that the ring epoxide was crucial for MetAP2 binding and indicates that covalent modification of MetAP2 by fumagillin is largely responsible for the potent inactivation of the enzyme (Table 1). We note that the deoxyfumagillin analogs also were capable of increased nonspecific interaction with proteins. This property was not caused by the remaining epoxide group because the dideoxyfumagillin analog B–F12 also exhibited strong nonspecific interactions. Although the precise mechanism of the increased chemical reactivity of the deoxyfumagillin analogs remains unknown, the relative specificity of fumagillin, which contains chemically more reactive epoxide groups, underscores the exceptional design of natural products, which have undergone extensive selection over time.

TNP-470, a synthetic analog of fumagillin, is currently undergoing clinical trials for treatment of several types of cancer. One of the major limitations of TNP-470 has been its short serum half

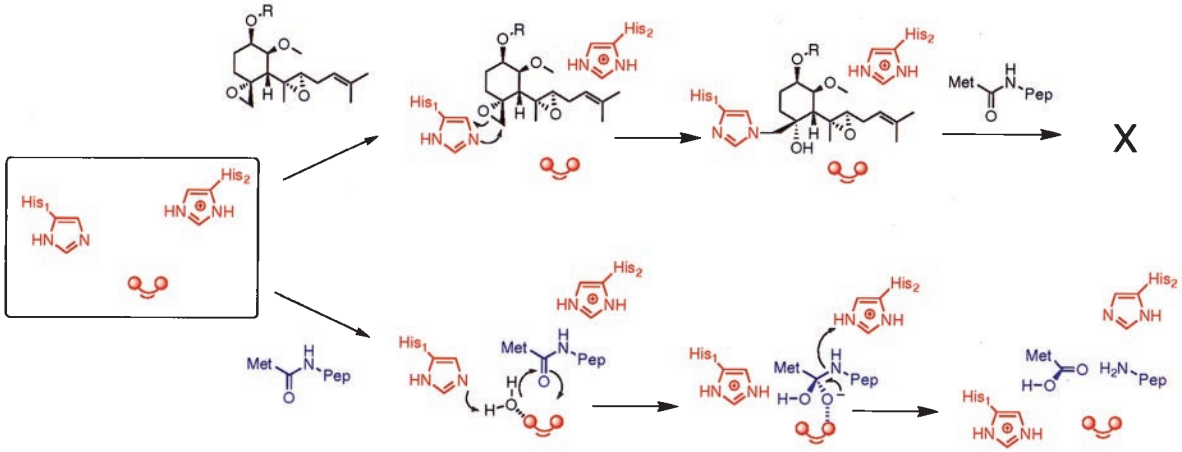

FIG. 6. Scheme of the proposed mechanism of catalysis of MetAP2 and its inhibition by fumagillin highlighting the roles of the binuclear metal center and two His residues. R denotes the side chain in fumagillin and ovalicin (see Fig. 1). Pep represents polypeptide as part of an oligopeptide or a protein substrate.

life, likely because of the presence of the chloroacetyl and the two epoxide groups (16, 17). The high potency of ovalicin and fumaginone clearly demonstrates that the chloroacetyl group is dispensable for activity, and the design of new MetAP2 inhibitors devoid of epoxide groups may help to further alleviate this problem. Our study demonstrates that the side chain epoxide group can be eliminated from irreversible inhibitors without causing a decrease in activity. We also showed that the ring epoxide group and its covalent modification of MetAP2 were crucial for potent inhibition of the enzyme and activity in endothelial cells. Earlier work showed that the ring epoxide could be converted into a sulfonium salt without significantly decreasing its activity (18), but it is possible that the hydroxyl group at C3 cyclizes to reform the ring epoxide. It remains to be seen whether the ring epoxide group can be transformed into other functional groups to give high-affinity, reversible inhibitors of MetAP2 that may have improved pharmacokinetic properties.

To identify the residue in MetAP2 covalently modified by fumagillin and ovalicin, we synthesized a fluorescein–fumagillin conjugate that was used to follow the modified MetAP2 peptides after digestion with chymotrypsin. Comparison of HPLC chromatograms of MetAP2 samples bound to fluorescein–fumagillin in the presence and absence of ovalicin yielded two unique peaks associated with fluorescein. We expected that the residue modified by fluorescein–fumagillin would give either no signal or a unique signal distinct from the expected amino acid during Edman sequencing of the modified peptides. This analysis was somewhat complicated by the presence of cysteine, itself a likely candidate for modification by epoxides. Sequencing of peak I suggested that His-231 was modified by the conjugate, whereas Cys-228 was not, as the latter was alkylated by iodoacetamide. Sequencing of peak II, however, gave a somewhat ambiguous result. In addition to the missing signal for His-231, Cys-228 also gave no signal, suggesting that Cys-228 was either not alkylated by iodoacetamide or was modified by fumagillin. This ambiguity was resolved by site-directed mutagenesis of the two residues.

Both Cys-228 and His-231 were mutated to assess their role in fumagillin-binding to MetAP2. As an additional control, a second active-site histidine mutant, H339N, was generated. Neither mutation of Cys-228 to alanine nor mutation of His-339 to asparagine had an effect on inhibition of MetAP2 by TNP-470 or covalent drug binding. In contrast, mutation of His-231 completely eliminated covalent binding to fumagillin. Because this mutant also was found to lack detectable catalytic activity, its structural integrity was assayed by using circular dichroism and partial proteolysis and found to be indistinguishable from the wild-type enzyme. These results confirm that His-231 is the site of covalent modification of MetAP2, likely because of the nucleophilic attack of the ring epoxide group by the imidazol side chain. Importantly, our results are also in accordance with the recent crystal structure of the MetAP2–fumagillin complex.

The finding that His-231 was the MetAP2 residue covalently modified by fumagillin was unexpected. Whereas certainly capable of acting as a nucleophile, the imidazole ring of histidine is relatively unreactive compared with other nucleophilic side chains. However, some precedent can be found in which an active-site histidine is specifically alkylated by inhibitors such as the inhibition of chymotrypsin by tos-L-phenylchloromethylketone (19). In addition, as one of the hallmarks of fumagillin and ovalicin is their exquisite specificity for MetAP2 over MetAP1 (8, 9), one might have expected that the inhibitors would covalently modify a residue that is unique to MetAP2. Interestingly, His-231 is conserved between both the type 1 and type 2 enzymes, indicating that the specificity of the inhibitors does not come from the presence of His-231 *per se*, but from other drug-recognition elements specific to MetAP2. Further structural and biochemical analysis will be required to understand the basis for the extraordinary specificity of these inhibitors.

The fact that mutation of both active-site histidines results in a dramatic loss in activity suggests that these residues may play important roles in catalysis by MetAP2. Although neither residue is predicted to be involved in the coordination to the putative cobalt ions at the active site based on the crystal structure of the *E. coli* enzyme (15), both residues are positioned in close proximity to the binuclear metal center and are conserved in all known MetAPs. This requirement of at least two His residues in addition to a binuclear metal center for efficient catalysis by MetAP2 makes it tempting to speculate that the mechanism of peptide-bond hydrolysis by this class of enzyme may resemble that of the ribonucleases (20–22). Thus, one of the His residues may serve as a general base that acts along with the coordinated metal ions to activate a water molecule for attack on the scissile amide bond, whereas a second His residue serves as a general acid to protonate the amide nitrogen leaving group, facilitating the peptide bond cleavage (Fig. 6). The fact that His-231 serves as a nucleophile to open the ring epoxide of fumagillin suggests that this residue may serve as a general base in catalysis, whereas His-339 may serve as a general acid. Although the precise roles of His-231 and His-339 in catalysis will have to await further biochemical and structural analysis of MetAP2, it is clear that the covalent modification of His-231 by fumagillin, ovalicin, and TNP-470 serves to irreversibly block the active site of MetAP2, preventing substrate binding and catalysis (Fig. 6). It is this inhibition of MetAP2 enzymatic activity that appears to serve as the molecular basis of inhibition of endothelial cell proliferation by this class of angiogenesis inhibitors.

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