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Development of Synthetic Aminopeptidase N/CD13 Inhibitors to Overcome Cancer Metastasis and Angiogenesis

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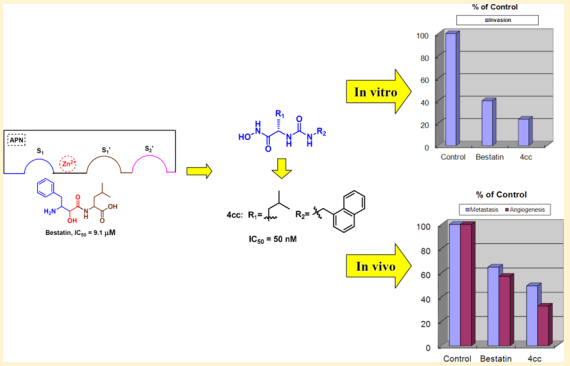
Supporting Information

ABSTRACT: Cancer metastasis is a major barrier to its treatment and an important cause of patient death. Antimetastatic agents hold promise for patients with advanced metastatic tumors. Aminopeptidase N/CD13 (APN) is being pursued by many as an important target against cancer metastasis and angiogenesis, but there are few reports on the *in vivo* evaluation of synthetic APN inhibitors. Herein, a series of compounds targeting APN were synthesized and evaluated for their antimetastasis and antiangiogenesis potency both *in vitro* and *in vivo*. Excitingly, compounds **4m**, **4t**, and **4cc**, with the most potent APN inhibitory activities, displayed significant antimetastasis and antiangiogenesis effects *in vitro* and *in vivo*, suggesting that those synthetic APN inhibitors have the potential to overcome cancer metastasis and angiogenesis.



KEYWORDS: *APN/CD13, inhibitors, anticancer, metastasis, angiogenesis*

An enduring problem in cancer treatment is the high failure rates in patients with advanced metastatic cancers.[1] Cancer metastasis is considered as a key cause of operation failure, postoperative relapse, and ultimately death.[2] Metastasis is a complex, multistep process, in which cancer cells spread from primary sites to new places.[3] Cancer cell invasion is the key stage, and angiogenesis is the prerequisite.[4] The extracellular matrix (ECM) is the main barrier of malignant cell dissemination. The ECM is a substrate of aminopeptidase N (APN/CD13, EC 3.4.11.2) and matrix metalloproteinases (MMPs). Targeting MMPs in the treatment of cancer metastasis has yielded unsatisfactory results.[5] Consequently, in recent years APN has become one of the most studied cancer therapeutic targets.[6]

APN is a zinc-dependent integral peptidase,[7] belonging to the M1 family of the MA clan of peptidases.[8] As an exopeptidase, APN preferentially cleaves neutral amino acids from the *N*-terminal of various oligopeptides, including enkephalins, neurokinins,[9] angiotensins, and some cytokines.[10] Human APN exists in many tissues, organs, and cell types, including kidney proximal tubule cells, stem cells, epithelial cells, endothelial cells, fibroblast cells, leukemia cells, and immune cells, etc.[7,11] APN degrades ECM to promote malignant cell invasion into the bloodstream.[11] In the angiogenesis process, neo-endothelial cells invading through the ECM also need APN.[12] Therefore, APN would be a key target in cancer metastasis and angiogenesis.[13] Meanwhile, the dysregulated expression and high-level exopeptidase activity of APN are detected in various mammalian cancer cell lines[14] such as melanoma, prostate, ovarian, colon, renal and pancreas carcinomas, etc., as well as neo-endothelial cells. The statistical survival rates for patients with high APN expression solid tumors were reported to be significantly lower than those of patients with APN-negative solid tumors.[15] So far, reported APN inhibitors can be divided into natural products and synthetic compounds. Among them, bestatin (Ubenimex[16]) may be the most studied one. It is isolated from a culture filtrate of *Streptomyces olivoreticuli* and is a cancer chemotherapeutic drug in some countries (Figure 1A).[17]

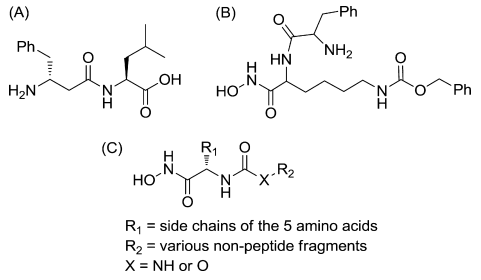


Figure 1. (A) Chemical structure of bestatin; (B) chemical structure of D24; (C) target compounds.

Bestatin is a potent APN inhibitor with a K_i value of 3.03 μM.[18] Saiki et al. found that bestatin significantly inhibited cancer cell invasion in a dose-dependent manner.[19] However, research on synthetic compounds only aimed at evaluating their APN and cell proliferation inhibition. There are few reports on their *in vivo* antimetastatic and antiangiogenic effects.

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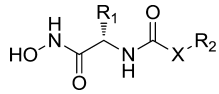
Therefore, in this paper, potent synthetic small-molecule APN inhibitors were designed and synthesized, and their antimetastasis and antiangiogenesis effects were evaluated *in vitro* and *in vivo*.

In 2006, Yoshimoto et al. reported the crystal structure of APN from *Escherichia coli* with the active site occupied by bestatin.[20] Recently, Gu et al. engineered *Thermoplasma acidophilum* factor F3 (a homologous protein of human APN) with two-point directed mutation, which obtained the protein with an identical active site to human APN. Structural biology studies were then performed on the cocrystal structure of this protein and one active APN inhibitor (D24, Figure 1B) developed by our group. The result suggested that engineered factor F3 mutant has a larger binding pocket than *E. coli* APN.[21] According to the chemical structures of bestatin and D24, both of them have a zinc-binding group (ZBG) and a neutral amino acid residue. In our ongoing studies, hydroxamic acid as strong ZBG in D24 was kept and five neutral amino acids (L-leucine, L-phenylglycine, L-isoleucine, L-phenylalanine, and L-methione) were introduced to the new target structures. The nonpeptide structure in D24 was replaced by various aromatic rings or an aromatic fragment with an alkyl linker so as to increase interaction with the larger pocket of human APN (Figure 1C).

The target compounds, **4a-4cc**, **7**, and **9** were first examined for their inhibitory activities toward APN from porcine kidney. Most compounds exhibited more potent inhibitory activities than bestatin, some of which had 10-fold or more improvement (Table 1). The substituents greatly influenced the compounds potency. Replacement of the ureido linker provides a serious potency decrease, such as for **7** and **9**. With the same

Table 1. Structures and IC_{50} Values of the Target Compounds[a]



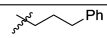
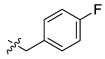
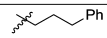
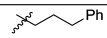
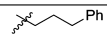
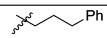
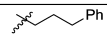
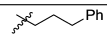
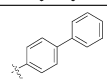
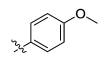
Compd	R_1	R_2	X	IC_{50} (μM) towards APN from porcine kidney
4a	$-CH_2CH(CH_3)_2$		NH	3.4 ± 0.2
4b	-Ph		NH	5.0 ± 0.2
4c	$-CH_2CH_2SCH_3$		NH	7.0 ± 0.3
4d	-Ph		NH	2.2 ± 0.2
4e	$-CH_2CH(CH_3)_2$		NH	2.3 ± 0.2
4f	$-CH(CH_3)CH_2CH_3$		NH	> 10
4g	$-CH_2Ph$		NH	2.8 ± 0.2
4h	-Ph		NH	5.5 ± 0.3
4i	-Ph	$-CH_2Ph$	NH	0.98 ± 0.08
4j	-Ph	$-CH_2CH_2Ph$	NH	1.9 ± 0.2
4k	$-CH_2CH(CH_3)_2$	-Ph	NH	2.7 ± 0.2
4l	$-CH_2CH(CH_3)_2$		NH	2.3 ± 0.2
4m	$-CH_2CH(CH_3)_2$		NH	0.099 ± 0.009
4n	$-CH_2CH(CH_3)_2$		NH	2.1 ± 0.2
4o	$-CH_2CH(CH_3)_2$		NH	0.57 ± 0.03
4p	$-CH_2CH(CH_3)_2$		NH	2.5 ± 0.1
4q	$-CH_2CH(CH_3)_2$		NH	0.33 ± 0.02
4r	$-CH_2CH(CH_3)_2$		NH	0.66 ± 0.04
4s	$-CH_2CH(CH_3)_2$		NH	0.57 ± 0.04
4t	$-CH_2CH(CH_3)_2$		NH	0.29 ± 0.03
4u	$-CH_2CH(CH_3)_2$		NH	0.69 ± 0.06

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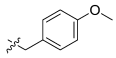
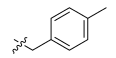
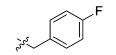
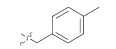
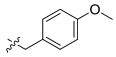
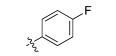
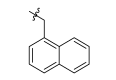
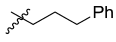
Table 1. continued

Compd	R_1	R_2	X	IC_{50} (μM) towards APN from porcine kidney
4v	$-CH_2CH(CH_3)_2$		NH	0.83± 0.08
4w	$-CH_2CH(CH_3)_2$		NH	0.41 ± 0.04
4x	$-CH_2CH(CH_3)_2$		NH	0.73 ± 0.05
4y	-Ph		NH	1.64 ± 0.09
4z	-Ph		NH	1.03 ± 0.08
4aa	-Ph		NH	9.9 ± 0.7
4bb	-Ph	-Ph	NH	5.9 ± 0.2
4cc	$-CH_2CH(CH_3)_2$		NH	0.050 ± 0.002
7	$-CH_2CH(CH_3)_2$		O	> 10
9	$-CH_2CH(CH_3)_2$	-Ph	CH2	> 10
Bestatin	-	-	-	9.1 ± 0.6

[a]All compounds were assayed three times, and the results are expressed with standard deviations.

R_2 substituent, L-leucine and L-phenylglycine residues contributed more to the potency than the other residues. As for R_2 substituents, a long side chain between the aromatic ring and the ureido group decreased the potency, such as for **4i**, **4j**, **4d**, and **4h**. Their potencies increased along with the chain length decline, while not so regularly in **4k**, **4a**, and **4e**. Most of the L-leucine based compounds with substituted phenyl or benzyl on the R_2 position were much more potent. **4zb** with phenyl was less potent than **4i** with benzyl, which could also be seen in **4b** versus **4za** with fluorine, and in **4p** versus **4x** with an L-leucine residue. But the opposite phenomenon was seen in **4q** versus **4w** and in **4o** versus **4v**, with methyl or methoxy. As heterocycle R_2 substituents, thiophene (**4r**) contributed more to the potency than furan (**4l**). For the L-leucine-based compounds with substituted benzyl at R_2, electron donating groups seemed better than electron withdrawing groups. For instance, **4t** was more potent than **4u**, as well as **4w** versus **4x**, **4o** and **4q** versus **4p**, also seen in **4y** and **4z** versus **4b**, except **4v**. This might also demonstrate that methyl or methoxy fit into the pocket better than fluorine. The substituted position on benzyl also influenced the potency. With methoxy substituted benzyl, **4t** (meta-) seemed better than **4s** (ortho-) and **4v** (para-), while this is not so obvious in the fluorine substituted ones **4u** and **4x**. The most potent compounds were **4m** (IC_{50} = 99 nM) with 1-naphthyl and **4cc** (IC_{50} = 50 nM) with 1-naphthylmethyl, suggesting that a larger group was better here, though **4n** with a biphenyl group was much less potent.

ES-2 cells with high APN expression were used as human APN, which makes more sense to the following *in vitro* and *in vivo* results. We only assessed the ability of the nanomolar range compounds to inhibit human APN on cultured ES-2 human ovarian clear cell carcinoma cells (Table 2). Those compounds were still better than bestatin, some even with more than 10-fold improvement. The effects of the more potent ones (**4m**, **4q**, **4t**, **4x**, and **4cc**) on ES-2 cell survival were evaluated, and

Table 2. IC_{50} Values and Inhibition Rates of the Compounds[a]

		growth inhibition rates toward ES-2 cells			
compd	IC_{50} (μM) toward APN on the surface of ES-2 cells	5 μg/mL	50 μg/mL	IC_{50} (μM) toward MMP-2	IC_{50} (MMP-2)/ IC_{50} (APN from procine kidney)
4m	0.48 ± 0.05	<1.0%	3.63%	>1000	$>10^6$
4o	6.4 ± 0.6	<1.0%	6.85%	>1000	$>10^6$
4q	0.94 ± 0.08	<1.0%	1.78%	573 ± 35	1740
4s	1.1 ± 0.1	<1.0%	3.29%	>1000	$>10^6$
4t	0.79 ± 0.07	<1.0%	<1.0%	>1000	$>10^6$
4u	3.0 ± 0.3	<1.0%	2.81%	>1000	$>10^6$
4v	6.9 ± 0.6	<1.0%	<1.0%	>1000	$>10^6$
4w	1.5 ± 0.1	<1.0%	1.83%	>1000	$>10^6$
4x	0.91 ± 0.09	<1.0%	11.9%	>1000	$>10^6$
4cc	0.42 ± 0.03	<1.0%	6.67%	545 ± 30	10900
bestatin	16.0 ± 1.6	<1.0%	2.89%	276 ± 19	30.2

[a]The compounds were assayed three times, and the results are expressed with standard deviations.

Table 2 showed that at low concentration all compounds had almost no influence on ES-2 cell survival. At high concentration, there was only a slight antiproliferative effect for most of the compounds, except **4x**. Matrix metalloproteinase-2 (MMP-2) is also a zinc-dependent metalloproteinase responsible for cancer invasion, so MMP-2 inhibitory activity was also tested. **4m**, **4q**, **4t**, and **4cc** exhibited very high selectivity for APN over MMP-2 (Table 2).

Cell migration is an important step during the invasion process. Agents blocking cell motility would also exhibit anti-invasion

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effects. A cell migration assay was performed on transwell chambers without Matrigel coating. Figure 2A showed that all

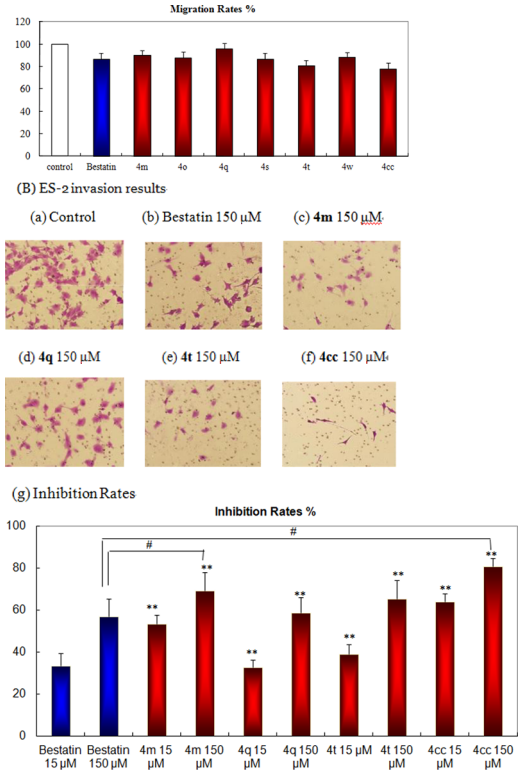

Figure 2. (A) ES-2 cell migration. (B) ES-2 cell invasion inhibition: (a) control; (b) bestatin 150 μM; (c) **4m**, 150 μM; (d) **4q**, 150 μM; (e) **4t**, 150 μM; (f) **4cc**, 150 μM; (g) statistical chart. Each column represents the mean values with SD for three independent experiments.***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, versus the control. ###, $P < 0.001$; ##, $P < 0.01$; #, $P < 0.05$, versus Bestatin treated groups.

the tested compounds (150 μM) had only a slight inhibitory tendency to ES-2 cell migration, without statistical significance. An anti-invasion assay was performed on transwell chambers coated with Matrigel. According to the result, ES-2 cells could freely invade and pass through Matrigel, but this was significantly blocked by bestatin, **4m**, **4q**, **4t**, and **4cc**. In 1989 Azuma et al. reported the tumor cell invasion block effects of bestatin in various conditions to mouse B16BL6 and 3LL cells. The result indicated that bestatin had approached 50−80% inhibitory rates at the 100 mg/mL level, with a dose-dependent tendency.[22] According to our results on human ES-2 cell invasion inhibition, **4m** and **4cc** were more potent than bestatin at the concentrations of 15 μM and 150 μM (Figure 2B(g)). Figure 2 shows that **4m**, **4q**, **4t**, and **4cc** were able to significantly inhibit ES-2 cell invasion in a dose-dependent tendency without obvious influence on ES-2 cell survival and migration, which indicated their potential therapeutic application in overcoming cancer metastasis. The ECM gel (Matrigel)-induced human umbilical vascular endothelial cell (HUVEC) capillary tubular structure formation assay was used as an *in vitro* measurement of angiogenesis.[23] Bestatin, **4m**, **4t**, and **4cc** decreased the number of branch points formed by HUVECs (data not shown), which demonstrated the potential antiangiogenesis effects of the APN inhibitors.

B16BL6 cells, which represent an advanced malignant murine tumor more metastatic than B16,[24] were selected to examine **4m**, **4t**, and **4cc** on tumor-induced angiogenesis and experimental metastasis *in vivo*. **4m**, **4t**, and **4cc** effectively inhibited murine APN activity and had quite low antiproliferative effects on B16BL6 (Figure 3A). In 2004, Saiki et al. demonstrated a significant

(A) The IC_{50} and GI_{50} values of **4m**, **4t** and **4cc** [a]

Compd	IC_{50} (μM) towards APN on the surface of B16BL6	GI_{50} (μM) towards B16BL6
4m	2.26	> 500
4t	5.61	> 500
4cc	1.82	> 500
Bestatin	23.2	> 500

[a]The compounds were assayed three times, and the standard deviations are < 10% of the means.

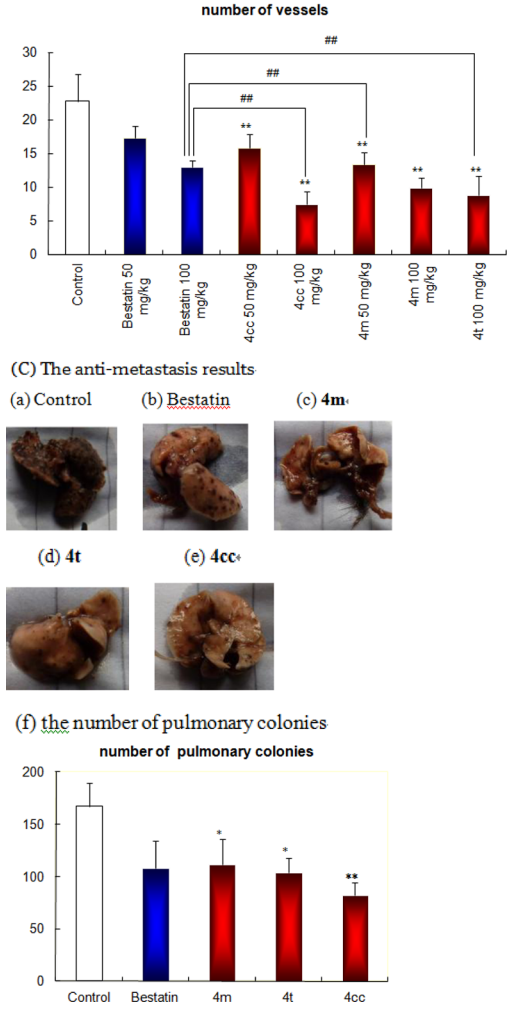

Figure 3. (A) The IC_{50} and GI_{50} values of **4m**, **4t**, and **4cc**. (B) **4m**, **4t**, and **4cc** inhibit B16BL6 induced angiogenesis *in vivo*. **, $P < 0.01$, versus the control. ##, $P < 0.01$, versus bestatin treated groups. (C) B16BL6 pulmonary colonies. (a) Control, (b) bestatin, (c) **4m**, (d) **4t**, (e) **4cc**, (f) statistical chart. Each column represents the mean values with SD *, $P < 0.05$, versus the control, **, $P < 0.01$, versus the control.

antiangiogenesis effect of bestatin (100 or 200 mg/kg, p.o.) on the C57BL6 mouse dorsal air sac model with the B16BL6 cell line.[25] In our assay, angiogenesis was induced by the implantation of B16BL6 cells into the intradermal site of C57BL/6 mouse backs,[26] and it quantified by counting the number of vessels oriented toward the tumor mass[27] under a dissecting microscope.

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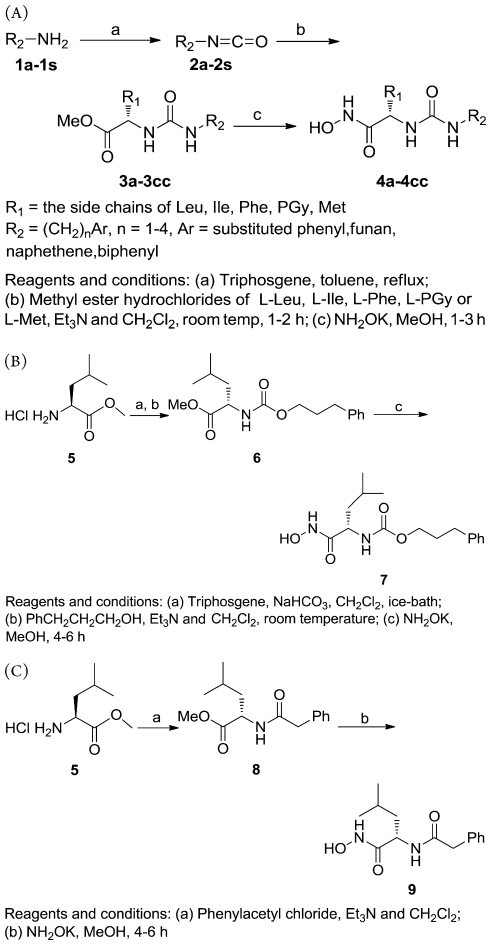
Figure 3B showed that the intraperitoneal administration of **4m**, **4t**, and **4cc** at the 100 mg/kg level led to significant anti-angiogenesis effects, which were significantly better than those for bestatin ($P < 0.01$). In 2006, our group reported a murine H22 liver cancer experimental metastasis on Kunming mice, and bestatin showed potency (60−70% inhibitory rate) at 50 mg/kg orally.[28] At this time, further study on APN inhibitor *in vivo* antimetastasis effects was performed through choosing a much higher advanced metastatic cancer cell line, B16BL6. The result showed that intravenous inoculation of B16BL6 resulted in the establishment of lung metastatic sites in C57BL/6 mice (as high as 170 nodes in the control group), much more serious than H22 (only 33 nodes). A significant decrease of the B16BL6 pulmonary colonies was detected in **4m**, **4t**, and **4cc** treated groups (Figure 3C).

The synthetic methods for compounds **4a**−**4cc** are shown in Scheme 1A. The amines listed were transformed into

Scheme 1



isocyanates through triphosgene. The ureido linker was obtained from the isocyanates coupled with the amino acid methyl esters. Without further purification, they were directly transformed into hydroxamic acids as the target compounds. **7** was synthesized according to Scheme 1B. L-Leucine methyl ester was converted into isocyanate[29] and coupled with 1-phenyl-1-propanol to obtain carbamate linker, which was finally transformed into hydroxamic acid. And the synthetic method of **9** was shown in Scheme 1C. The amide bond was formed through the coupling between L-leucine methyl ester and phenacetyl chloride, and the hydroxamic acid was obtained by the same method as above.

We have designed and synthesized a series of small-molecule synthetic APN inhibitors. The APN inhibitory activity demonstrated that most compounds were more potent than bestatin. The three most potent candidates, **4m**, **4t**, and **4cc**, with a nanomolar range IC_{50}, exhibited significant anti-invasion effects to ES-2 cells *in vitro* without obvious cytotoxicity, which is better than bestatin. In the *in vivo* evaluation including murine B16BL6 induced angiogenesis and experimental lung metastasis assays, **4m**, **4t**, and **4cc** exhibited significant antiangiogenesis and antimetastasis effects without obvious cytotoxicity toward B16BL6 cells. Such results suggest that those three synthetic APN inhibitors are potential candidates which can be developed to overcome cancer metastasis and angiogenesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of compounds, experimental procedures, characterization of new compounds, ^{1}H NMR, ^{13}C NMR, and biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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