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Evaluation of chelating agents as anti-angiogenic therapy through copper chelation

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Abstract—The evaluation of several sets of polyamine donor chelating agents including a selection of novel hexadentate 1,3,5-*cis*,*cis*-triaminocyclohexane (tach) based derivatives were performed in an in vitro endothelial cell proliferation assay to assess their cytotoxicity and selectivity as novel anti-angiogenic agents. The selective nature of the anti-angiogenic agents for human umbilical vein endothelial cells (HUVEC) was compared to a normal fibroblast cell line and a human Glioma cell line to evaluate these compounds. Linear tri- and tetra-polyamines were superior to both macrocyclic and the tach based polyamine chelating agents in terms of selectivity of its inhibitory activity toward the proliferation of HUVEC cells compared to the fibroblast and human Glioma cells. The linear polyamine, triethylenetetramine (**22**), previously reported to possess anti-angiogenic properties failed to demonstrate any selectivity for inhibiting the proliferation of HUVEC cells compared to the fibroblast and human Glioma cells.
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1. Introduction

In the normal adult, the process of angiogenesis is tightly regulated that occurs during wound healing and in the proliferating endometrium during menstruation.[1] However, in several pathologic conditions including tumor formation, diabetic retinopathy, and rheumatoid arthritis angiogenesis plays a key role.[1] The proliferating endothelium can be targeted through anti-angiogenic drug therapy in an effort to arrest aberrant growth of vessels.[2] Though there are several known targets for anti-angiogenic therapy, one of the most intriguing is the proliferating endothelial cell's requirement for copper as a co-factor in its cellular process.[3] Therefore, potential anti-angiogenic agents might be determined by their ability to decrease the amount of available copper at the endothelial level.[4,5]

The body of evidence supporting the importance of copper in tumor formation is sizable, but also somewhat inferential. Biochemical studies have demonstrated higher levels of copper in tumor tissue as compared to nontumor tissue.[6,7] Also, multiple stimulators of angiogenesis that bind copper with high affinity include fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).[8,9] Furthermore, in the rabbit corneal assay frequently employed to assess angiogenic activity, both $CuSO_4$ and ceruloplasmin (a copper transport containing protein) can stimulate vessel growth while the noncopper binding form of ceruloplasmin lacked activity.[10,11] The first studies in animal tumor models, which linked impeded angiogenesis and therefore tumor growth with reduced copper levels were conducted using penicillamine, an early and routinely employed chelation therapy agent, and/or a low copper diet.[12,13] The efficacy of copper binding agents in the treatment of human disease has been well demonstrated in the treatment of Wilson's disease an inherited metabolic disorder resulting in improper copper sequestration and toxicity in the body. Thus, the precedent for copper sequestration therapy has significant standing and clinical experience of patient tolerance undergoing such therapy is well established.

Current therapies for Wilson's disease includes the use of copper binding molecules such as penicillamine and more recently tetrathiomolybdate (TM) along with also the use of zinc salts to alter the biological distribution of +2 metal ions and eliminate Cu(II).[14,15] Combining the anti-tumor observations in animals and the clinical

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utility of TM in patients with Wilson's disease provided impetus for clinical trials using TM in patients with metastatic cancer. Early results have demonstrated reduced copper levels and that the drug is well tolerated during the treatment.[16]

In our previous studies we had focused on the screening of a small select series of *cis*,*cis*-*N*,*N*′,*N*″-1,3,5,-triaminocyclohexane (tach) compounds for anti-angiogenic activity in an in vitro endothelial cell proliferation assay to assess their cytotoxicity and selectivity.[17] These previously evaluated compounds were primarily polyaminopyridyl compounds such as *N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis*,*cis*-1,3,5,-triaminocyclohexane (tachpyr) that had shown significant cytotoxicity against cancer cell lines when evaluated as iron depletion therapy drugs.[18] Due to their metal binding cavity sizes and that these compounds had also demonstrated significant stability as Cu(II) complexing agents,[19] they and some related compounds were studied for anti-angiogenic activity. The results of the previous studies in fact eliminated these specific compounds, not on the basis of low cytotoxicity, but rather on a lack of selective cytotoxicity and thus a lack of anti-angiogenic activity.[17] Recent efforts to determine intracellular metal ion selectivity of tachpyr compounds has demonstrated that their activity stems primarily from iron and zinc sequestration, and not from significant copper complex formation.[20] Surprisingly, the compounds that emerged from our previous study as potential leads were acyclic analogs of tachpyr that had demonstrated low or no biological activity as iron sequestration agents.[17] Additionally, *N*,*N*′,*N*″-tri(2-mercaptoethyl)-*cis*,*cis*-1,3,5-triaminocyclohexane (tachENSH), the sole tach based thiol donor compound, emerged as the overall lead compound.[17] Continued evaluation of this compound and its direct analogs are an ongoing study.

Due to the limited number of compounds previously examined and therefore limited conclusive data, the current study focuses principally on polyamine chelating agents wherein the donor geometry and number are the variables (Figs. 1 and 2). Donor geometry includes: (1) a selection of tach based hexadentate polyamine ligands to assess this pre-organized donor geometry in conjunction with this donor set; (2) a set macrocyclic ligands wherein both the donor number and binding cavity is varied to isolate optimal copper chelation chemistry for this specific application; and (3) a selection of acyclic polyamines with varying donor number and spatial arrangement. The first set was prepared in part for this study (Fig. 1) to provide a unique group of hexamine donor ligands wherein an octahedral geometry within a pseudo-adamantyl carbon cage about the metal is formed that has been well characterized.[19,21] To this end, seven compounds of this category are included with varying alkyl and aryl substituents. The second set of compounds were chosen based on their association with Cu(II) complexation use in radiopharmaceutical development as well as in Cu(II) metal extraction applications.[22–26] The third set of compounds was included because there had been previous reports of simple linear polyamines having anti-angiogenic activity and on that basis an examination of permutation of donor number and arrangement was deemed important to include in this study.[5]

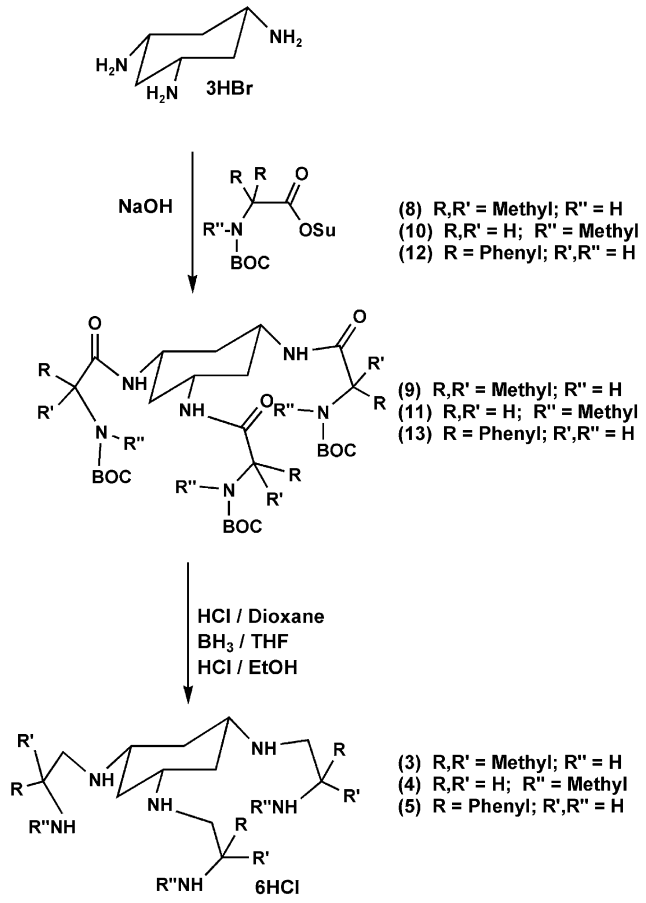

Figure 1. Synthesis of novel hexadentate 1,3,5-*cis*,*cis*-triaminocyclohexane (tach) based chelating agents for evaluation of their selective inhibition of the proliferation of HUVEC cells as compared to fibroblast and human Glioma cells.

The determining characteristic of a compound that identifies it as an anti-angiogenic agent is selective cytostasis of endothelial cells excluding all other cell types.[27] To evaluate the ability of all the chelating agents in this report to function as anti-angiogenic agents, their ability to selectively inhibit the proliferation of human umbilical vein endothelial cells (HUVEC) in vitro while not inhibiting the proliferation of nonendothelial cells was determined. A normal fibroblast cell line (NIH3T3) and a human glioma tumor cell line (U251) were utilized as control nonendothelial cell types. Therefore, a compound that demonstrates growth inhibition of the HUVEC cell line at a concentration significantly lower then the concentration needed to inhibit the normal and tumor cell lines may be a promising lead compound for further study as an anti-angiogenic agent.

Herein we report the design and synthesis of novel tach based hexadentate hexamine chelating agents, their biological evaluation as potential anti-angiogenic agents, and the biological evaluation of a select set of previously reported or commercially available acyclic and cyclic polyamines.

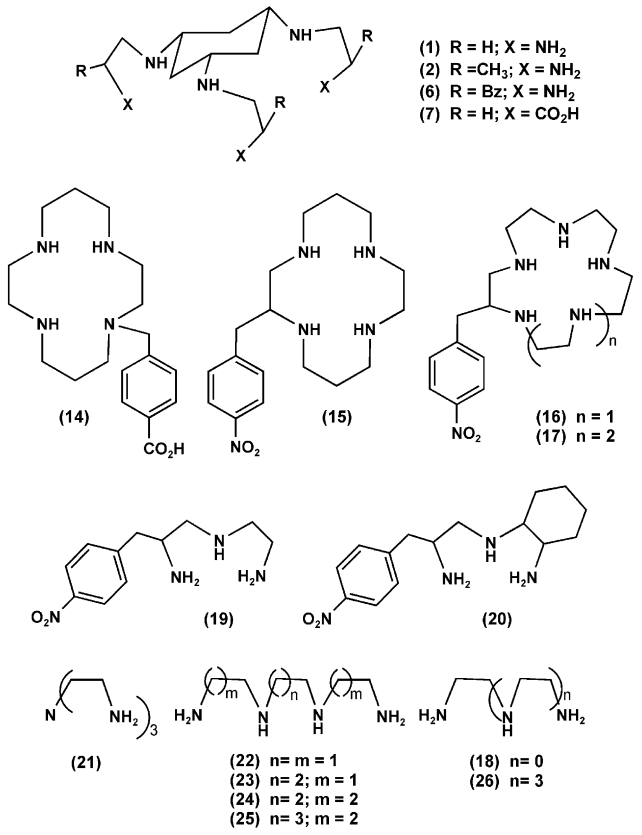

Figure 2. Linear and macrocyclic tri-, tetra-, penta-, and hexa-amine chelating agents evaluated for their selectivity of in inhibitory activity of the proliferation of HUVEC cells as compared to fibroblast and human Glioma cells.

2. Results and discussion

The synthesis of the novel tach based ligands **3**–**5** (Fig. 1) was performed analogously to that of the previously prepared compounds **1**, **2**, and **6**, with some small variations to adjust for solubility of intermediates during isolation. Thus, reaction of the free base of tach generated in situ with the selected carbamate protected amino acid active ester cleanly provided the tris-amide adducts that could be isolated either by precipitation of the product or by an extractive process. The carbamate protection groups were removed by treatment with acid and the amides reduced with diborane that after acidic work up provided the desired tris-substituted ethylenediamine arm derivates of tach. No attempts were made to optimize yields of methods and all of the tach based ligands were carried forward to biological evaluation as their respective hexa-hydrochloride salts.

The tach based ligands therefore provided an assessment of various donor patterns within the geometric constrainment of the hexadentate octahedral coordination sphere conferred by these compounds. Thus, as a direct consequence of being able to take advantage of the range of available amino acid, a range of mono- and di-substitution on the ethylene coordinating arm was available and included H, methyl, phenyl, and benzyl along with dimethyl. The derivatives that possess mono-substitution on the ethylene coordinating arm were also prepared as their single enantiomers to eliminate issues pertaining to steric hindrance in metal ion complex formation as the structures of these complexes tend to form with varying degrees of spiral character and that an inappropriately arranged substituent would be expected to disrupt complexation of metal ions.[21] A single polyaminocarboxylate class ligand, **7**, was included as well as we had previously reported preparation of its respective Cu(II) complex.[28]

The macrocyclic and linear polyamines chosen for assessment were either commercial in origin or had been previously prepared as reported.[21–25,28–31] The macrocyclic chelating agents were chosen based upon ring cavity size and because cyclam derivatives **14** and **15** and numerous related derivatives had also been investigated for chelating Cu(II) radionuclides of interest in nuclear medicine applications wherein exceptionally strong complexation of the metal ions is a requirement. The two larger polyamine macrocycles **16** and **17** were chosen to examine what impact size of the ring and number of metal binding donors would have on biological activity.

A selection of linear amines was included in this study since **22** had been previously reported to exert an anti-angiogenic effect on tumor.[5] To examine some of the variables surrounding this reported activity, this selection of analogs varied the number donor amines, the carbon framework spacing between the amines, and in some respect geometric organization of the amines.

All the chelating agents were evaluated in a cell proliferation assay whereby normal, tumor, and endothelial cells were treated with various concentrations of each respective agent.[17] The efficacy of each compound as a potential anti-angiogenic agent was determined by the relative level of cytotoxicity exhibited specific to endothelial cells versus normal and/or tumor cells. While the sequestration of Cu(II) has been hypothesized as the potential mode of action being evaluated, actual determination of the metal(s) being complexed has not been performed. Thus, the possibility that chelation of other metal ions such as Zn(II) cannot be ruled out as being involved. However, as application of the experimental design to exploit differential sensitivity between HUVEC and other cell lines specifically suggests Cu(II) complexation as the mode of action. In support of this interpretation, the determination of intracellular metal ion selectivity of tachpyr compounds demonstrated that their activity originates primarily from iron and zinc chelation, and not from significant copper complex formation.[20] This directly correlates with the results of non-specific toxicity against all of the cell lines in the assay including HUVEC.

Somewhat surprisingly, none of the tach based agents exhibited any differential or selective cytoxicity toward the HUVEC cells (Table 1). Compound **6** with benzyl groups was found to be the most active though thoroughly unselective confirming previous results with the analogous methyl substituted compound exhibiting intermediate activity.[21] Interestingly, the

Table 1. Hexadentate 1,3,5-*cis*,*cis*-triaminocyclohexane (tach) based chelating agents evaluated for selective activity (comparative activity values are mM concentration IC_{50} measurement to determine differential cytotoxicity for HUVEC vs control cell lines)[a]

Compound	HUVEC	NIH3T3	U251
1	1.0	1.0	1.0
2	0.008	0.02	0.009
3	1.0	1.0	1.0
4	1.0	1.0	1.0
5	0.02	0.02	0.02
6	0.003	0.004	0.001
7	0.01	0.04	0.01

[a] Error in IC_{50} measurements does not exceed ±3.0%.

Table 2. Macrocyclic polyamines evaluated for selective activity (comparative activity values are mM concentration IC_{50} measurement to determine differential cytotoxicity for HUVEC vs control cell lines)[a]

Compound	HUVEC	NIH3T3	U251
14	0.5	0.5	1.0
15	**0.09**	0.25	0.76
16	0.004	0.008	0.004
17	0.19	0.02	0.02

[a] Error in IC_{50} measurements does not exceed ±3.0%.

polyaminocarboxylate ligand **7** was also found to possess considerable, albeit unselective, cytotoxicity in this assay.

The macrocyclic ligands **14** and **15** were expected to possess some selectivity for chelating Cu(II) while the larger ring compounds **16** and **17** were unknown in their selectivity toward forming Cu(II) complexes (Table 2). As partially expected, **15** was found to possess significant selective cytotoxicity toward the HUVEC cell line, however, **14** failed to exhibit this property. There are some significant differences between these two cyclam derivatives that may explain this result. Cyclam **14** has a tertiary amine functionalized with an aryl carboxylate group that may in fact hinder formation of the Cu(II) complex. This group also then provides a zwitterion that may limit traversal of the cell membrane and thus charge may play a role in the lack of its activity. Cyclam **15** is also functionalized, however, this compound is C-functionalized, the array of secondary amines is maintained, and this status may exert influence on both selectivity and complex stability. Upon examination of the results obtained for the larger ring polyamines, **16** and **17**, both were found to exhibit considerable cytotoxicity with **16** having considerable activity. Yet, in both cases neither **16** nor **17** have any selectivity against HUVEC cells and thus their metal binding activity in this arena appears to not be adequately selective for intracellular Cu(II).

The linear polyamines (Table 3) were evaluated with some expectation that these reagents would exhibit some level of activity in this assay as **22** had been previously reported as an effective anti-angiogenic agent.[5] The amines were selected to address variation in the number of amines as well as the carbon framework spacing between them as this could strongly affect Cu(II) binding by altering the bite angle in complex formation to either favorable or disfavorable geometries.[32] Three diethylenetriamine derivatives were evaluated. No significant activity was expected from any of these as the number of donors was deemed inadequate for this application and that at least four amines would be preferable. This hypothesis held true initially with diethylenetriamine itself, **18**, and with **19**, a simple C-functionalized analog. However, the more geometrically pre-organized diethylenetriamine **20** was found to exhibit considerable selective cytotoxicity against the HUVEC cell line. Differences that may account for this activity include (1) the stereospecific pre-organizational geometry of the ligand being capable of forming a far more stable complex with Cu(II) than with either **19** or **18** and (2) that **20** might also be somewhat more lipophilic in nature with inclusion of the cyclohexane ring into the structure. The polyaminocarboxylate formed from this triamine has been shown to possess considerably enhanced stability for several medically useful radionuclides as compared to the analogous compound formed from **19**.[33] The uniquely arranged polyamine **21** also was found to possess considerable selectivity against the HUVEC cell line and again this might be attributable to a combination of coordination number and geometry. The selection of tetraamines includes the simplest polyethylene bridged compound, **22**, and three related compounds that vary the lengths of the three carbon bridges with either a propylene or butyl central bridge. Surprisingly, in our hand **22** exhibited no selective activity at all. All three compounds with a 3 carbon central bridge, **23**, **24**, and **25**, were found to possess activity in the assay, although **24** with 3 propylene bridges appeared to be selective toward the U251 and not the HUVEC cell line. Compounds **23** and **25** were both found to be selective toward the HUVEC versus the NIH3T3 and U251 cell lines. However, **23** also exhibited moderate relative activity toward U251 while **25** exhibited moderate relative activity toward both the NIH3T3 and U251 cell lines. The higher activity found for **25** was somewhat unexpected in that the butane spacer would result in seven-membered chelate ring for a 1:1 Cu(II) complex, which was not predicted as favorable for forming a stable complex.

Table 3. Macrocyclic polyamines evaluated for selective activity (comparative activity values are mM concentration IC_{50} measurement to determine differential cytotoxicity for HUVEC vs control cell lines)[a]

Compound	HUVEC	NIH3T3	U251
18	1.0	1.0	1.0
19	0.44	0.37	0.27
20	**0.05**	0.14	0.11
21	**0.32**	1.0	0.69
22	1.0	1.0	1.0
23	**0.25**	1.0	0.66
24	0.39	0.45	0.05
25	**0.05**	0.25	0.27
26	1.0	1.0	1.0

[a] Error in IC_{50} measurements does not exceed ±3.0%.

One overall criteria that seems evident from the data acquired from evaluating these polyamines in this assay is

the consistent requirement for optimal arrangement of the amines to form Cu(II) complexes. Specifically, inclusion of propylene bridges between the amine donors appears to be a key component since formation of six-membered chelate rings tends to be favored for the smaller ionic radius transition metals.[32] A second criteria that also appears influential is a combination of charge and ability to actually access intracellular Cu(II). This is apparent from the results from the functionalized acyclic and 14-membered ring cyclam ligands where functionalization alters either charge or provides altered and enhanced relative lipophilicy. Lastly, the results acquired for the two functionalized acyclic ligands **19** and **20** demonstrate the importance of pre-organization and perhaps specific stereochemistry that should be included in the design of future Cu(II) chelating agents for anti-angiogenic agents.

3. Experimental

3.1. Ligand syntheses

3.1.1. Materials and methods. All the reagents and solvents listed below were obtained from Aldrich, Fluka, Kodak, or Sigma and were used without further purification. Et_2O was distilled from Na and used immediately. The *cis*,*cis*-1,3,5-triaminocyclohexane trihydrobromide (tach $(HBr)_3$) was prepared as reported in the literature.[34] Compounds **1**, **2**, **6**, **7**, **14**–**17**, **19**–**20** were prepared as previously reported.[21–25,28–31]

^1H and ^{13}C NMR were obtained using a Varian Gemini 300 instrument and chemical shifts are reported in ppm on the δ scale relative to TMS or TSP. Proton chemical shifts are annotated as follows: ppm (multiplicity, integral, coupling constant (Hz)). Chemical ionization mass spectra (CI-MS) were obtained on a Finnnegan 3000 instrument. Fast atom bombardment (FAB-MS) mass spectra were taken on an Extrel 400 instrument. Optical rotations were measured using a Perkin–Elmer 341 instrument with a 100 mm cell at 20 °C. Elemental analyses were performed by Atlantic Microlabs (Atlanta, Georgia).

3.1.2. General procedure for preparation of tach amino acid triamides (9, 11, 13). The tach $(HBr)_3$ (1 equiv) was treated with NaOH (3 equiv) in H_2O (10 mL) in a two-necked flask at room temperature fitted with a Dean–Stark trap and condenser. After formation of a clear homogeneous solution, benzene (200 mL) was added, and the H_2O was removed via azeotropic distillation. The benzene solution was then reduced to ca. 25 mL and cooled to room temperature. After dilution with DMF (150 mL), the appropriate amino acid active ester (3 equiv) in DMF (30 mL) was added. The temperature was raised to ca. 45 °C and the solution was stirred for 18 h. The DMF solution was concentrated to near dryness (vacuum rotary evaporation), the solid residue was suspended in EtOAc (200 mL) and vigorously stirred. Those derivatives that precipitated directly at this point were collected on a Buchner funnel, washed with a small portion of hexanes, and dried. Those that cleanly dissolved in EtOAc were transferred to a separatory funnel, extracted with 5% NaHCO3 (3×100 mL), brine (100 mL), 1 N HCl (3×100 mL), and brine (100 mL), dried over $MgSO_4$, filtered, and either rotary evaporated to dryness or to a generate a precipitate, which after cooling was collected, washed with hexanes, and vacuum dried.

3.1.3. General procedure for preparation of hexamines 3–5.

3.1.3.1. Deprotection—triamides 9, 11. The triamide was added to anhydrous 1,4-dioxane saturated with $HCl_{(g)}$ (200 mL) and stirred for 18 h. The precipitate was collected, washed with Et_2O, and dried under vacuum.

3.1.3.2. Deprotection—triamide 13. The phenylalanine triamide was treated with trifluoroacetic acid (50 mL) for 6 h, which, after solvent removal by rotary evaporation and vacuum drying, left a white foam.

3.1.3.3. Borane reduction. The deprotected material was washed into a three-necked round bottom flask with THF (150 mL) and 1 M BH_3. THF was added while cooling the suspension with an ice bath. The reaction mixture was then vigorously refluxed for 6 d, after which any excess borane was decomposed with MeOH. The solution was rotary evaporated to a gummy solid and taken up in 100% EtOH (150 mL). The EtOH solution was saturated with $HCl_{(g)}$ while cooling with an ice bath and then the acidic solution was refluxed for 6 h during which a white precipitate formed. The flask was held at 4 °C for 8 h and the precipitate was collected on a Buchner funnel, washed with Et_2, and dried in vacuo.

3.1.4. Boc-*N*-methyl-glycine hydroxysuccinimidyl ester (10). The amino acid (10.00 g, 112 mmol), Et_3N (24 mL, 173 mmol), and Boc-ON (27.7 g, 112 mmol) were suspended in 50% aqueous dioxane (300 mL) and stirred for 18 h. The solution was transferred to a separatory funnel, H_2O (200 mL) was added, and the aqueous layer extracted with EtOAc (3×150 mL). The aqueous layer was returned to the flask, cooled in an ice bath, and acidified to pH 2.0 with 3 N HCl. The solution was rapidly extracted then with EtOAc (3×150 mL), the combined extracts dried over $MgSO_4$, filtered, and rotary evaporation to leave the product (18.0 g, 85%).

^1H NMR ($CDCL_3$) δ 9.00 (br s, 1H), 3.989 (d, 2H, $J = 23.1$), 2.941 (s, 3H), 1.457 (d, 9H, $J = 11.1$); Mass Spect. (CI/NH_3) 190, 207 (M^++1, M^++18).

The Boc protected acid (11.00 g, 58.2 mmol), *N*-hydroxysuccinimide (6.70 g, 58.3 mmol), and EDC (11.16 g, 58.2 mmol) were suspended in EtOAc (200 mL) to which DMF (100 mL) was added to form a clear solution. After stirring for 18 h, the reaction solution was diluted with EtOAc (100 mL) and sequentially extracted with H_2O (100 mL), 5% $NaHCO_3$ (100 mL), and salt solution (100 mL). After drying over Na_2SO_4, filtration, and rotary evaporation, a white solid (13.8 g) was obtained, which was further dried under high vacuum.

^{1}H NMR (DMSO-d_6) δ 4.394 (s, 2H), 2.819 (s, 4H), 1.409 (s, 3H,), 1.377 (s, 9H); Mass Spect. (CI/NH_3) 304 (M^++18). The active ester was used without further purification.

3.1.5. *cis*,*cis*-1,3,5-Triaminocyclohexane-tris(*N*-*tert*-butyloxycarbonyl-*N*-methylaminoacetamide) (11). The tach $(HBr)_3$ (3.90 g, 10.5 mmol) was treated as described above with NaOH (1.26 g, 31.5 mmol) and **10** (9.00 g, 31.5 mmol) to produce a white solid (4.18 g, 62%).

^{1}H NMR (DMSO-d_6) δ 7.851 (d, 1H, J = 7.8), 3.717, 3.672 (2s, 3H), 3.330 (m, 1H), 2.782 (s, 2H), 1.821 (br d, 1H, J = 8.7), 1.390 (s, 3H), 1.328 (s, 6H), 1.117 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 167.67, 155.07, 78.44, 51.63, 44.75, 37.82, 35.59, 27.99; Mass. Spect. (FAB/glycerol) 643 (M^++1). Anal. Calcd for $C_{30}H_{54}N_6O_9$: C, 56.04; H, 8.48; N, 13.04. Found: C, 56.25; H, 8.44; N, 12.81.

3.1.6. *cis*,*cis*-1,3,5-Triamino-tris(*N*-methyl-2-aminoethylene)cyclohexane (4). Boc-triamide **11** (3.25 g, 5.06 mmol) was deprotected with $HCl_{(g)}$ as described above and after isolation and drying, reduced with 1 M BH_3·THF (50 mL). After the acidic work-up, the product was obtained as a white solid (1.78 g, 68%).

^{1}H NMR (D_2O) δ 3.330 (s, 3H), 3.12 (m, 1H), 2.783 (s, 2H), 2.494 (d, 1H, J = 11.7), 1.390 (q, 1H, J = 11.7); ^{13}C NMR (D_2O) δ 55.49, 47.60, 43.72, 36.23, 33.40; Mass Spect. (CI/NH_3) 301 (M^++1). Anal. Calcd for $C_{15}H_{36}N_6(HCl)_6$: C, 34.69; H, 8.17; N, 16.18. Found: C, 34.18; H, 8.38; N, 16.06.

3.1.7. Boc-[*R*]-phenylglycine hydroxysuccinimidyl ester (12). The amino acid (15.00 g, 99.0 mmol), Et_3N (20.6 mL, 148 mmol), and Boc-ON (24.4 g, 99.0 mmol) were suspended in 50% aqueous dioxane (400 mL) and stirred for 18 h. The solution was transferred to a separatory funnel, H_2O (200 mL) was added, and the aqueous layer extracted with EtOAc (3 × 150 mL). The aqueous layer was returned to the flask, cooled in an ice bath, and acidified to pH 2.0 with 3 N HCl. The solution was rapidly extracted then with EtOAc (3 × 150 mL), the combined extracts dried over $MgSO_4$, filtered, and rotary evaporation to leave the product (24.3 g, 94%).

^{1}H NMR (DMSO-d_6) δ 8.032 (d, 1H, J = 4.2), 7.48–7.27 (m, 5H), 5.129 (d, 1H, 5.1), 1.434 (s, 3H), 1.211 (s, 6H); Mass Spect. (CI/NH_3) 269 (M^++18).

The Boc protected acid (22.00 g, 87.5 mmol), *N*-hydroxysuccinimide (10.1 g, 87.8 mmol), and EDC (16.6 g, 87.8 mmol) were dissolved in DMF (200 mL) was added to form a clear solution. After stirring for 18 h, the reaction solution was diluted with EtOAc (500 mL) and sequentially extracted with H_2O (100 mL), 5% $NaHCO_3$ (100 mL), and salt solution (100 mL). After drying over Na_2SO_4, filtration, and rotary evaporation, a white solid (25.5 g) was obtained, which was further dried under high vacuum.

^{1}H NMR (DMSO-d_6) δ 8.158 (d, 1H, J = 8.1), 7.50–7.34 (m, 5H), 5.639 (d, 1H, J = 8.4), 2.774 (s, 4H), 1.409 (s, 9H); Mass Spect. (FAB/glycerol) 371 (M^++23). The active ester was used without further purification.

3.1.8. *cis*,*cis*-1,3,5-Triaminocyclohexane-tris(*N*-*tert*-butyloxycarbonyl-2-[*R*]-phenylacetamide) (13). The tach $(HBr)_3$ (5.00 g, 13.4 mmol) was treated as described above with NaOH (1.613 g, 40.0 mmol) and **12** (14.03 g, 40.4 mmol) to produce a white solid (8.9 g, 77%).

^{1}H NMR (DMSO-d_6) δ 8.225–8.075 (m, 1H), 7.42–7.11 (m, 5H), 5.073 (q, 1H, J = 9.6), 3.576 (m, 1H), 1.90–1.60 (m, 1H), 1.362 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 172.90, 154.82, 139.02, 128.23, 127.48, 126.91, 78.44, 57.67, 44.78, 37.25, 28.17, 25.27; Mass Spect. (FAB/glycerol) 864 (M^++1). $[\alpha]_D^{23}$ −8.0 (DMSO, c = 0.001); Anal. Calcd for $C_{48}H_{60}N_6O_9$: C, 66.63; H, 7.01; N, 9.72. Found: C, 66.52; H, 6.85; N, 9.54.

3.1.9. *cis*,*cis*-1,3,5-Triamino-tris(2-phenyl-2-aminoethylene)cyclohexane (5). Boc-triamide **13** (10.9 g, 1.32 mmol) was deprotected with trifluoroacetic acid as described above and after isolation and drying, reduced with 1 M BH_3·THF (160 mL). After the acid work-up, the product was obtained as a white solid (7.8 g, 88%).

^{1}H NMR (D_2O) δ 7.533 (s, 5H), 4.554 (t, 1H, J = 7.2), 3.436 (br s, 2H), 2.915 (m, 1H), 2.242 (br d, 1H, J = 10.2), 1.148 (q, 1H, J = 11.1); ^{13}C NMR (D_2O) δ 136.26, 133.14, 132.61, 130.42, 56.26, 55.93, 50.44, 36.58; Mass Spect. (FAB/glycerol) 487 (M^++1). $[\alpha]_D^{23}$ −8.0 (DMSO, c = 0.18); Anal. Calcd for $C_{30}H_{42}N_6(HCl)_6$: C, 51.06; H, 6.87; N, 11.91. Found: C, 50.85; H, 6.58; N, 12.02.

3.1.10. Boc-2,2-dimethyl-glycine hydroxysuccinimidyl ester (8). The amino acid (10.30 g, 100 mmol), Et_3N (20.8 mL, 150 mmol), and Boc-ON (24.6 g, 100 mmol) were suspended in 50% aqueous dioxane (400 mL) and stirred for 18 h. The solution was transferred to a separatory funnel, H_2O (150 mL) was added, and the aqueous layer extracted with EtOAc (3 × 150 mL). The aqueous layer was returned to the flask, cooled in an ice bath, and acidified to pH 2.0 with 3 N HCl. The solution was rapidly extracted then with EtOAc (3 × 150 mL), the combined extracts dried over MgSO4, filtered, and rotary evaporation to leave the product (17.5 g, 86%).

^{1}H NMR ($CDCl_3$) δ 1.530 (s, 6H), 1.442 (s, 9H); Mass Spect. (CI/NH_3) 221 (M^++18).

The Boc protected acid (11.00 g, 54.2 mmol), *N*-hydroxysuccinimide (6.23 g, 54.2 mmol), and EDC (10.4 g, 54.2 mmol) were suspended in EtOAc (250 mL) to which DMF (100 mL) was added to form a clear solution. After stirring for 18 h, the reaction solution was diluted with EtOAc (100 mL) and sequentially extracted with H_2O (100 mL), 5% $NaHCO_3$ (100 mL), and salt solution (100 mL). After drying over Na_2SO_4, filtration, and rotary evaporation, a white solid (13.0 g) was obtained, which was further dried under high vacuum.

^{1}H NMR (DMSO-d_6) δ 2.777 (s, 4H), 1.481 (s, 6H), 1.393 (9H); Mass Spect. (CI/NH_3) 318 (M^++18). The active ester was used without further purification.

3.1.11. *cis*,*cis*-1,3,5-Triaminocyclohexane-tris(2,2-dimethylaminoacetamide) (9). The tach $(HBr)_3$ (4.96 g, 13.3 mmol) was treated as described above with NaOH (1.60 g, 40 mmol) and **8** (12.00 g, 40 mmol) to produce a white solid (7.0 g, 77%). A small portion (~50 mg) was treated with trifluoroacetic acid to generate a more interpretable set of NMR data.

^{1}H NMR (DMSO-d_6) δ 3.909 (tt, 1H, J = 12.9, 3.9), 2.067 (d, 1H, J = 12.0), 1.597 (s, 6H), 1.353 (q, 1H, J = 12.9); ^{13}C NMR (DMSO-d_6) δ 174.18, 58.94, 47.89, 37.69, 25.00; Mass Spect. (CI/NH_3) 685 (M^++1). Anal. Calcd for $C_{36}H_{60}N_6O_9$: C, 57.85; H, 8.85; N, 12.27. Found: C, 57.45; H, 8.66; N, 12.14.

3.1.12. *cis*,*cis*-1,3,5-Triamino-tris(2,2-dimethyl-2-aminoethylene)cyclohexane (3). Boc-triamide **9** (2.00 g, 2.92 mmol) was deprotected as described above and after isolation and drying, reduced with 1 M BH_3·THF (50 mL). After the acid work-up, the product was obtained as a white solid (1.34 g, 82%).

^{1}H NMR (D_2O) δ 3.31–3.20 (m, 1H), 3.252 (s, 2H), 2.586 (d, 1H, J = 10.8), 1.553 (q, 1H, J = 11.7), 1.468 (s, 6H); ^{13}C NMR (D_2O) δ 57.25, 56.22, 55.55, 35.46, 26.05; Mass Spect. (CI/NH_3) 343 (M^++1). Anal. Calcd for $C_{18}H_{42}N_6(HCl)_6$: C, 38.51; H, 8.64; N, 14.97. Found: C, 38.21; H, 8.58; N, 14.50.

3.2. Biological methods

3.2.1. In vitro cellular proliferation assay. HUVEC (Clonetics), U251, and NIH-3T3 (ATCC) were grown in T75 flasks in EBM-2 media supplemented with the EGM-2 bullet (Clonetics). At confluence the cells were washed once with sterile PBS and released with 1.5 mL of trypsin. The cells were then placed in a 50 mL conical flask containing 50 mL of supplemented EBM-2 media. Cells were then evenly seeded into four 24 well plates and allowed to adhere for 24 h at 37 °C in a 5% CO_2 atmosphere. The media was then removed and the study drug was added to the wells, four wells per drug dilution, in 400 μL of un-supplemented media and allowed to incubate for 30 min. At which time 400 μL of media containing twice the supplementation was added and incubated for 72 h. Positive controls had stimulated media alone while negative controls had un-stimulated media added. Positive and negative controls were added to every plate to generate an IC_{50} for each drug. After 72 h the media was removed, the cells were trypsinized with 400 μL volume, and the total cell number counted in a Coulter Counter. IC_{50} values were generated by comparison of the proliferation of the cells, done in quadruplicate, with various concentrations of drug versus the stimulated positive controls and the un-stimulated negative controls. An IC_{50} value was that number where the cellular proliferation was halfway between the stimulated positive control and the unstimulated negative control.

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