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Evaluation of Copper Chelation Agents as Anti-Angiogenic Therapy

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Abstract—The design, synthesis and evaluation of *N,N′,N″*-tris(2-pyridylmethyl)-*cis,cis*-1,3,5,-triaminocyclohexane (tachpyr, **1**) derivatives as novel anti-angiogenic agents were performed in an in vitro endothelial cell proliferation assay to assess their cytotoxicity and selectivity. 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. *N,N′,N″*-tris(2-mercaptoethyl)-*cis,cis*-1,3,5-triaminocyclohexane trihydrochloride (**3b**) was superior to tachpyr in terms of selectivity of its inhibitory activity toward the proliferation of Huvec compared to the fibroblast and human Glioma cell lines.

Introduction

Angiogenesis is a tightly regulated process that in a normal adult is only found in the proliferating endometrium during menstruation and during wound healing. However, angiogenesis is common in many pathologic conditions including tumor formation, diabetic retinopathy and rheumatoid arthritis.[1] Anti-angiogenic drug therapy targets the proliferating endothelium in an effort to arrest aberrant growth of vessels.[2] Multiple targets for anti-angiogenic drug therapy exist. An intriguing target is the proliferating endothelial cell's requirement for copper as a co-factor in its cellular process.[3] Therefore, drugs that could decrease the amount of available copper at the level of the endothelium could become anti-angiogenic agents.[4,5]

The evidence that copper is important in tumor formation is inferential. First, biochemical studies have demonstrated a higher copper concentration in tumor tissue as compared to non-tumor tissue.[6,7] Second, multiple stimulators of angiogenesis including fibroblast growth factor and vascular endothelial growth factor bind copper with a high affinity.[8,9] Third, both $CuSO_4$ and ceruloplasmin (a copper containing protein) can stimulate angiogenesis while the non-copper binding form of ceruloplasmin cannot stimulate vessel growth in the rabbit corneal assay.[10,11] The first examination of the effects of copper deprivation in animal tumor models utilized penicillamine, an early and routinely employed chelation therapy agent, and/or a low copper diet, and demonstrated a decreased level of angiogenesis and hence, controlled tumor growth.[12,13]

Certain chelating agents have been shown to bind copper with a high affinity.[12] Previous work on copper chelation agents have focused on Wilson's disease, which is an inherited metabolic disease of copper toxicity that is fatal if left untreated. Early agents employed to combat copper overload included penicillamine and trientine (triethylenetetramine).[14,15] However, some patients developed severe toxicity to these agents. New therapy for Wilson's disease includes the use of zinc acetate and tetrathiomolybdate (TM).[16,17] Combining the anti-tumor observations in animals and the clinical utility of TM in patients with Wilson's disease provided impetus for an ongoing Phase I trial of TM in patients with metastatic cancer. Early results have demonstrated the therapy to be well tolerated and that copper levels appeared to be reduced.[18]

One family of chelating agents that have yet to be investigated for this application are those based upon *cis,cis*-1,3,5,-triaminocyclohexane (TACH),[19] specifically those

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agents prepared that employ this triamine as a platform to append additional metal ion binding sites. Of this class of compounds, the tris-pyridyl derivative known as tachpyr (**1**) (Fig. 1) has been extensively investigated. Recent publications have reported on the formation and stability of radioactive Cu(II) complexes as well as the synthesis of several closely related compounds.[20] Based upon the high stability and affinity for copper as expressed in these radiopharmaceutical investigations, the potential to exploit the properties of these compounds as Cu(II) sequestration and/or deprivation agents appeared attractive. The previously reported compounds included permutations of methylation wherein a methyl group had been added to the pyridyl ring in all positions, *N*-methylation of the secondary amines, and *C*-methylation of the ligand framework between the secondary amine and pyridyl groups. Additional analogues have also been prepared to initiate investigation into donor character, exchanging imidazole, hydroxyl, and mercapto donors for pyridyl, as well as inclusion of some less configurationally restrained tachpyr analogues.

One of the essential characteristics of anti-angiogenic agents, that is used to screen prospective agents, is a compound's ability to inhibit endothelial cell proliferation while not inhibiting the proliferation of other cell types, that is selective cytostasis.[21] To evaluate the ability of all of the chelating agents in this report to function as anti-angiogenic agents, their ability to inhibit the proliferation of human umbilical vein endothelial cells (Huvec) in vitro while not inhibiting the proliferation of non-endothelial cells was determined. A normal fibroblast cell line (NIH3T3) and a human glioma tumor cell line (U251) were utilized as control non-endothelial cell types. A compound that inhibits the proliferation of the Huvec cell line at a concentration lower than the concentration needed to inhibit the other cell lines may be an attractive anti-angiogenic agent. Herein, we report the design, synthesis and biological evaluation of these compounds.

Results and Discussion

The tachpyr (**1**) family of compounds had previously been investigated for its formation of Cu(II) complexes in regards to use as radiopharmaceuticals and also for their application as therapeutics via iron depletion. For this study these chelators were chosen as they had demonstrated significant stability in forming Cu(II) complexes and were thought then as viable Cu(II) depletion agents as well.[20,22]

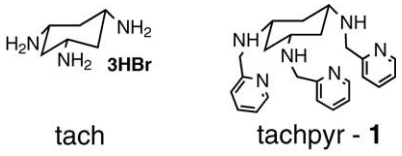
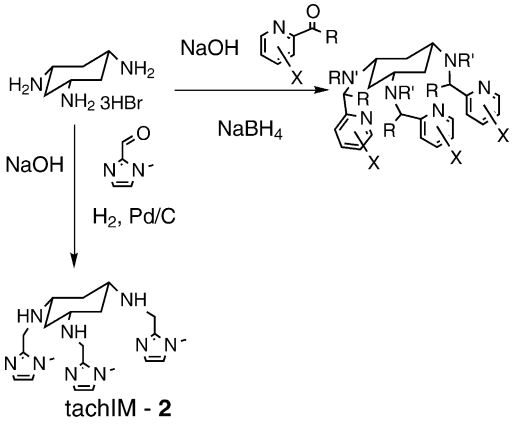


Figure 1.

Compound **1** and the direct analogues **1a–f** thereof with methyl groups (Fig. 2) on the various positions of the pyridyl ring or elsewhere were prepared from TACH and the appropriately substituted carboxaldehydes (**1–1d**) or ketone (**1e**) as previously reported.[19,20,22] In brief, the triamine was reacted with minimal excess of the ketone with elimination of water (Dean–Stark distillation) and the resultant tris-imine then reduced with excess $NaBH_4$. The synthesis of **1f** was also performed from the *N*,*N*′,*N*″-tris-methylated parent amine directly alkylated with 2-(chloromethyl)pyridine.

Tris-imidazole **2** was also formed from the respective tris-imine, however, reduction of the tris-imine was performed by direct hydrogenation to yield the triamine as hydride mediated reduction was either unsuccessful or complicated by numerous side products.[19]

Novel chelators **3a** and **3b** (Fig. 3) were prepared from *cis,cis*-1,3,5,-triaminocyclohexane by first tris-acylation employing an active ester derivative to introduce the two carbons bearing a protected hydroxyl or thiol. Attempts at direct acylation by either acid chlorides where possible or mediated by diimide coupling reactions generated mixtures of products in poor yields. Several protecting groups were investigated for this step including benzyl and benzoyl, however, while some provided a measure of desired product, all were hampered by complications due to incomplete acylation or requirements of chromatographic isolation. Additionally, use of an acid labile protecting group in both cases was desirable since this would then be removed during the acidic cleavage of the polyamine boron adducts that frequently complicate polyamine synthesis. Use of the *tert*-butyl group for the synthesis of **3a** was found to be very convenient and was efficiently removed to provide the final product in the last step. The EOE protected



R,R'= H, X= 3-Me; tach(3-Me)pyr - **1a**
R,R'= H, X= 4-Me; tach(4-Me)pyr - **1b**
R,R'= H, X= 5-Me; tach(5-Me)pyr - **1c**
R,R'= H, X= 6-Me; tach(6-Me)pyr - **1d**
R= CH_3, R',X= H; tachpyr(C-Me) - **1e**
R,X= H, R'= CH_3; tach(*N*-Me)pyr - **1f**

Figure 2.

thiol active ester had been prepared for the preparation of bifunctional chelating agents for either ^{99m}Tc or ^{186,188}Re and served to introduce the ethylene thiol functionality after amide reduction and acid cleavage of the protecting group.[23]

Synthesis of **4a** and **4b** (Fig. 4) from the commercial triamine, tris(2-aminoethyl)amine (tren), were both performed in a very straightforward manner by modification of previously reported synthesis of the tris-imine to directly form the two respective tris-imines with subsequent reduction with excess $NaBH_4$.

There were numerous compounds within the **1**–**1f** family of chelators that inhibited Huvec proliferation at a micromolar concentration (Table 1). There were also many derivatives that also inhibited the proliferation of either the NIH3T3 or U251 cell lines. Thus, while cytotoxic, these derivatives exhibited little differential between any of the cells lines used for this purpose. Two compounds, **1e** and **1f,** were also evaluated in part as a control for **1** in that the previous investigations indicated their unfavorable stability with radio-copper. Surprisingly, **1f** demonstrated at least a 2-fold greater inhibition in Huvec proliferation as compared to either

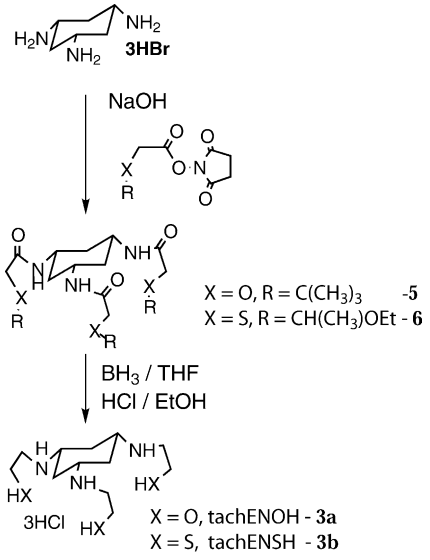


Figure 3.

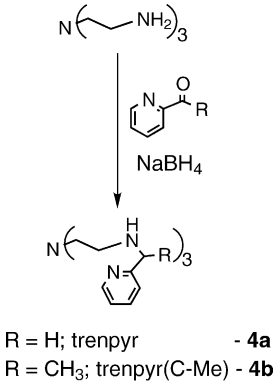


Figure 4.

the NIH3T3 or U251 cell lines. The rest of the compounds demonstrated either no activity or very little differential in the inhibition of the cell lines.

Compound **4a** was included to provide a much less geometrically constrained coordination sphere as compared to **1** yet maintain the same donor character and number permitting some evaluation of this specific variable. Similar activity and selectivity results were obtained using **4a** as compared to **1** which tend to indicate that there may be no advantage gained by the preorganization of the donor components as achieved by use of **1** in comparison to the much more unrestricted **4a**.

In parallel, to that reported above, the analogous **4b** was also prepared and evaluated to determine whether again the more open geometry could be more efficient and whether the additional *C*-methylation would have any effect. Similarly to **1e**,**4b** was active, but both **1e** and **4b** demonstrated no greater selectivity towards the Huvec cell line than their parental ligands. There are several possible simple explanations for this result that involve either modified coordination geometry, either 4-coordination of metal ion or some form of multimeric coordination, or in conjunction with this explanation, *C*-methylation may be a source of significant change in lipophilicity thereby permitting greater cell membrane translation. The impact of increasing lipophilicity with metal chelators to provide a significant enhancement of activity has been reported wherein cyclam was found to lack cytotoxicity while tetra-alkylated analogues were very active while the intermediate amides were also inactive.[24] Additionally, there was no control of the stereochemistry of these methyl groups through these syntheses. The crystal structure of the Cu(II) complex of **1** clearly indicates that from the spiral nature that this complex would only be stable if the methyl groups could be aligned in a consistent configuration.[20] This current result supports a coordination geometry then that would be either 4-, 5-, or some multimeric arrangement for the **1e** while in the case of the **4b** the decreased geometric constraints of the ligand may permit this chelator to function well to sequester Cu(II).

One of other goals of this preliminary investigation was to determine the proper donor character set of the ligand. With this in mind, the pyridyl groups were exchanged for imidazole giving **2** which was predicted in part to have some selectivity for Cu(II) over iron and therefore exhibit differential toxicity towards the Huvec cell line. This indeed was the result, and **2** demonstrated

Table 1. IC_{50} values (μM) of the compounds **1a**–**f** for inhibition of proliferation of Huvec, NIH3T3 and U251 cells

Compd	R	R′	X	Huvec	NIH3T3	U251
Tachpyr (**1**)	H	H	H	1	2.1	0.35
1a	H	H	3-Me	1.24	4.0	1.7
1b	H	H	4-Me	1.4	1.6	1.5
1c	H	H	5-Me	1.3	1.5	2.0
1d	H	H	6-Me	100	100	100
1e	CH_3	H	H	20	50	20
1f	H	CH_3	H	50	500	100

at least a 2-fold greater inhibition in Huvec proliferation as compared to either the NIH3T3 or U251 cell lines. The magnitude of this result was not large, and again may stem from the donor atoms of **2** being excessively constrained to less than optimal coordination geometry for this select purpose. With that being stated, efforts to prepare the tren-based analogue of **2** are ongoing to relieve possible steric inhibitions to efficient complexation.

Lastly, recognizing that heterocyclic amines might not be an optimal choice for chelation of intracellular Cu(II), the pyridyl groups were replaced with either an alkyl hydroxyl or thiol generating **3a** and **3b**, respectively, with the latter compound being of primary interest and the former acting as a control for coordination number and geometry. Gratifyingly, **3b** (Fig. 5) did indeed demonstrate significant activity as well as the best differentiation in activity towards the Huvec cell line as compared to either NIH3T3 or U251 cells, respectively, while **3a** was completely inactive. Inhibition of the Huvec cell line was 10-fold greater than the inhibition of the NIH3T3 cell line and 18-fold greater than the U251 cell line. This differential should lead to effective anti-angiogenic therapy with minimal toxicity.

Clearly, the result observed for **3b** is related to the donor character of this chelating agent. Thiols are known to be good donors for binding Cu(II) being of a 'soft' nature and thiol donors have been extensively investigated for myriad applications.[25] As observed in the results, that is zero activity, the contrary condition of the 'hard' hydroxy being a poor donor for Cu(II) was equally demonstrated with **3a**. There were some concerns that thiols would be too reactive in a biological milieu and be subject to significant oxidation deleting this component from being available for binding Cu(II) and that perhaps thioethers might be a somewhat better choice despite some loss of binding strength. In spite of this concern, this compound appears not to suffer from a compromising reactivity and displays not only reactivity, but also promising selectivity.

Taken in combination with all the information garnered through this study, pre-organization on the order of requiring the TACH scaffolding seems not a major prerequisite for activity as evidenced by the tren based compounds. However, issues of selectivity appear to be related to both some measure of lipophilicity and hence cellular availability and then again donor character. Further studies of novel compounds to optimize these conditions of hexacoordination, soft donor character, and moderate pre-organization are ongoing.

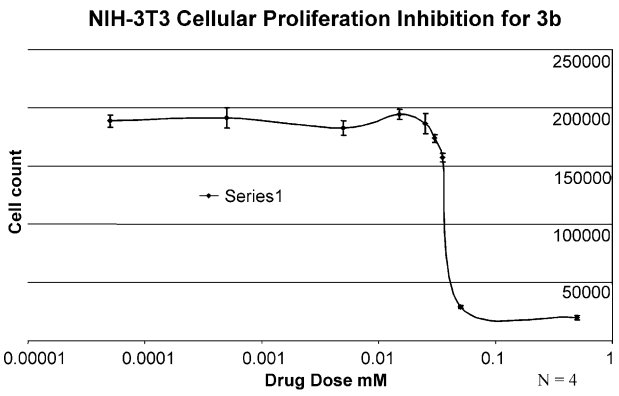


Figure 5.

Conclusion

Copper chelation is an attractive anti-angiogenic strategy to prevent tumor endothelial cell proliferation and tumor vascularization. Previous work with copper chelators has focused on Wilson's disease and the neurological manifestations of that disease.[14,15,17] Recent studies have examined the role of TM in the treatment of cancer patients in a Phase I study.[18] The goal of our study was to use Huvec proliferation assays to evaluate **1**, **4**, and their derivatives for their ability to differentially inhibit the proliferation of Huvec versus a fibroblast and tumor cell line. Compounds **1f**, **2** and **3b** demonstrated a differential inhibition of Huvec with **3b** having the greatest effect. This lead compound and **3a**, a closely related compound, but of different donor character, that is hydroxyl versus thiol, that did not show activity, will now be tested in vivo for their ability to inhibit angiogenesis and prevent tumor formation. From this preliminary evaluation compound **3b** emerged as a promising lead compound that will serve as a starting point for novel variants to be derived to obtain an agent with the greatest anti-angiogenic effect. To help validate that the anti-proliferative effect observed against Huvec was due to copper chelation, studies to determine relative affinity constants of several of the compounds reported in this work for Cu, Zn and Fe have been initiated and will be reported in due course. Anti-angiogenic compounds have also demonstrated value added when combined with radiotherapy and this strategy will also be employed with these compounds.

Experimental

Materials and methods

All chemicals and solvents were purchased from Fluka, Sigma, or Aldrich and were used as received. The *cis,cis*-1,3,5-triaminocyclohexane trihydrobromide (TACH) was prepared as reported in the literature.[19] Compounds **1**, **1a–f**, and **2** were prepared as previously reported.[19,20,22] The *N*-hydroxysuccinimidyl *S*-(1-ethoxyethyl)mercaptoacetate was prepared as reported by Kasina et al.[23] The precursor tris-imines for **4a**,**b** were prepared by modification of a reported procedure.[26]

^{1}H 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, TSP, or solvent. Proton chemical shifts are annotated as follows: ppm [multiplicity, integral, coupling constant (Hz)]. Chemical ionization mass spectra (CI-MS) were obtained on a Finnegan 3000 instrument. Fast atom bombardment mass spectra (FAB-MS) were taken on a Extrel 400.

Elemental analyses were obtained from Atlantic Microlabs (Norcross, GA, USA).

Analytical HPLC was performed using a Beckman gradient system equipped with Model 114M pumps controlled by System Gold software and a Model 165 dual wavelength detector set at 254 and 280 nm. An Altex C-18 reverse-phase column (5-μm particles, 4.6×250 mm) and a binary gradient of 0–100% B/25 min (Solvent A = 0.05 M Et_3N/HOAc, pH 5.5, Solvent B = MeOH) at 1.0 mL/min was used for all analyses.

Chemistry—ligand synthesis

***N*-Hydroxysuccinimidyl glycolate *tert*-butyl ether. Methyl glycolate *tert*-butyl ether.** Methyl glycolate (20 g, 222 mmol) was dissolved in dry cyclohexane (500 mL) and *tert*-butyltrichloroacetimidate (50 g, 229 mmol) was added. $BF_3{\cdot}Et_2O$ (1.75 mL) was added by syringe and a white precipitate gradually formed. After 18 h, solid Na_2CO_3 was added, and after stirring for 1 h, the suspension was filtered through a silica pad on a coarse glass frit. The filtrate was carefully rotary evaporated to a light-yellow oil which was vacuum distilled (11 mm, 70 °C) to isolate the product (21.4 g, 66%). ^{1}H NMR ($CDCL_3$) δ 4.046 (s, 2H), 3.753 (s, 2H), 1.240 (s, 9H); MS (CI/NH_3) 164 ($M^+ + 18$).

Glycolic acid *tert*-butyl ether. The above ester methyl glycolate *tert*-butyl ether (12.0 g, 82.3 mmol) was added to EtOH (200 mL) in which KOH (82.3 mmol) was been previously dissolved and the solution was stirred for 18 h. The EtOH was removed by rotary evaporation and the residue taken up in H_2O. The aqueous solution was acidified with 3 N HCl and immediately extracted with $CHCl_3$ (3×100 mL). The combined extracts were dried over Na_2SO_4, filtered, and rotary evaporated to leave a clear oil (10.5 g, 97%). ^{1}H NMR ($CDCL_3$) δ 4.049 (s, 2H), 1.27 (s, 9H); Mass Spect. (CI/NH_3) 150 ($M^+ + 18$). The crude acid (8.4 g, 63.6 mmol), *N*-hydroxysuccinimide (7.32 g, 6.37 mmol), and EDC (12.2 g, 63.7 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 (11.8 g) was obtained which was further dried under high vacuum. ^{1}H NMR (DMSO-d_6) δ 4.46 (s, 4H), 2.822 (s, 2H), 1.185 (s, 9H); MS (CI/NH_3) 247 ($M^+ + 18$). The active ester was used without further purification.

***cis,cis*-1,3,5-triaminocyclohexane-tris(*O*-*tert*-butylglycolamide) (5).** TACH $(HBr)_3$ (2.5 g, 6.75 mmol) and NaOH (0.81 g, 20.3 mmol) were dissolved in H_2O (10 mL). After a clear solution had formed, benzene (180 mL) was added and the H_2O eliminated by a Dean-Stark trap. The benzene solution was then concentrated to ca. 30 mL by distillation. After cooling to room temperature, DMF (150 mL) was added followed by addition of the above prepared active ester (5.0 g, 20.3 mmol) in DMF (30 mL). The reaction solution was stirred for 18 h. The solvent was removed by vacuum rotary evaporation and the residue was taken up in EtOAc (300 mL). The solution was sequentially extracted with salt solution (100 mL), 5% $NaHCO_3$ (2×100 mL), salt solution (100 mL), dried over Na_2SO_4, filtered, and rotary evaporated to a white solid (2.18 g, 69%).

^{1}H NMR (DMSO-d_6) δ 7.314 (d, 1H, J = 7.8), 3.743 (s, 2H), 3.620 (m, 1H), 1.791 (br.d, 1H, J = 11.7), 1.377 (q, 1H, J = 11.7), 1.159 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 169.489, 73.986, 62.096, 44.539, 37.193, 27.054; MS (CI/NH_3) 472,489 ($M^+ + 1$,18). Anal. calcd for $C_{24}H_{45}N_3O_6$; C, 61.10; H, 9.63; N, 8.91. Found: C, 61.05; H, 9.59; N, 8.79.

***N,N′,N″*-Tri(2-hydroxyethyl)-*cis,cis*-1,3,5-triaminocyclohexane trihydrochloride.** Amide **5** (1.00 g, 2.12 mmol) was dissolved in THF (75 mL) in a two-necked flask, cooled in an ice bath, and 1 M BH_3THF (13 mL) was injected via syringe. The reaction was allowed to come to room temperature over 1 h, after which the reaction was vigorously refluxed for 24 h. After cooling to room temperature, the excess hydride was decomposed with MeOH, the solution concentrated to a gum by rotary evaporation. The residue was taken up in 100% EtOH (50 mL) and while cooling with an ice bath, saturated with $HCl_{(g)}$. The acidic solution was then vigorously refluxed for 18 h during which a fine white precipitate formed. The suspension was cooled at 4 C for 8 h, collected on a Buchner funnel, washed with Et_2O, and dried in vacuo (0.50 g, 63.4%). ^{1}H NMR (D_2O) δ 3.874 (t, 2H, J = 4.8), 3.503 (tt, 1H, J = 11.5,3.0), 3.288 (t, 2H, J = 4.8), 2.681 (br.d, 1H, J = 10.8), 1.709 (q, 1H, J = 11.7); ^{13}C NMR (D_2O) δ 59.803, 54.946, 49.846, 33.089; MS (FAB/glycerol) 262 ($M^+ + 1$). Anal. calcd for $C_{12}H_{27}N_3O_3(HCl)_3$; C, 38.87; H, 8.17; N, 11.33. Found: C, 38.93; H, 8.10; N, 10.94.

***cis,cis*-1,3,5-Tris(1-ethoxyethylmercaptoacetamido) cyclohexane (6).** TACH $(HBr)_3$ (5.00 g, 13.44 mmol) and NaOH (1.61 g, 40.25 mmol) were dissolved in H_2O (10 mL). After a clear solution had formed, benzene (120 mL) was added and the H_2O was distilled off by use of a Dean–Stark trap. The benzene solution was then concentrated to ~30 mL. After cooling to room temperature, DMF (120 mL) was added followed by addition of *N*-hydroxysuccinimidyl *S*-(1-ethoxyethyl) mercaptoacetate[23] (10.53 g, 40.3 mmol) in DMF (20 mL). The reaction solution was warmed mildly and stirred for 18 h. The solvent was removed by vacuum rotary evaporation and the residue suspended in EtOAc (300 mL). The precipitate was collected on a Buchner funnel and washed with EtOAc to leave the product as a white powder (5.64 g, 74%). ^{1}H NMR (DMSO-d_6) δ 7.967 (d, 1H, J = 7.8), 4.766 (q, 1H, J = 6.3), 3.70–3.55 (m, 2H), 3.44–3.32 (m, 1H), 3.106 (s, 2H), 1.829 (br. d, 1H, J = 11.1), 1.425 (d, 3H, J = 6.0), 1.099 (t, 4H, J = 6.6, signal for the ring proton obscured by the signal for the methyl group); ^{13}C NMR (DMSO-d_6) δ 168.214, 80.826, 62.212, 44.964, 37.474, 31.547, 22.684, 14.891; MS (CI/NH_3) 568 ($M^+ + 1$). Anal. calcd for $C_{24}H_{45}N_3O_6S_3$; C, 50.74; H, 8.00; N, 7.40. Found: C, 50.48; H, 7.90; N, 7.35.

***N,N′,N″*-Tri(2-mercaptoethyl)-*cis,cis*-1.3.5-triaminocyclohexane trihydrochloride (3b).** Amide **6** (4.087 g, 7.199 mmol) was suspended in THF (100 mL) in a two-necked flask, cooled in an ice bath, and 1 M BH_3THF (86 mL) was injected via syringe. The reaction was allowed to come to room temperature over 1 h, after which the reaction was vigorously refluxed for 120 h. After cooling to room temperature, the excess hydride was decomposed with MeOH. The solution was concentrated to a gum by rotary evaporation and held under vacuum for 72 h. The residue was taken up in 100% EtOH (200 mL) and while cooling with an ice bath, saturated with $HCl_{(g)}$. The acidic solution was then vigorously refluxed for 18 h during which a fine white precipitate formed. The suspension was cooled at 4 °C for 8 h, collected on a Buchner funnel, washed with Et_2O, and dried in vacuo (1.93 g, 64%). ^{1}H NMR (D_2O) δ 3.485 (tt, 1H, J = 11.7, 3.6), 3.346 (t, 2H, J = 6.9), 2.866 (t, 2H, J = 6.9), 2.644 (br.d, 1H, J = 8.7), 1.703 (q, 1H, J = 12.0); ^{13}C NMR (D_2O) δ 54.940, 50.562, 33.268, 23.076; MS (FAB/glycerol) 310 ($M^+ + 1$). Anal. calcd for $C_{12}H_{27}N_3S_3(HCl)_3$; C, 34.39; H, 7.23; N, 10.03. Found: C, 34.26; H, 7.41; N, 10.07.

Tris[*N*-(2-pyridylmethylene)-2-aminoethyl]amine (4a). Tris(2-aminoethyl)amine (3.00 g, 20.5 mmol) and 2-pyridine-2-carboxaldehyde (6.585 g, 61.55 mmol) were dissolved in benzene (150 mL) and the resultant H_2O was removed by Dean–Stark distillation for 18 h. After cooling to room temperature, the solution was decanted and rotary evaporated to isolate the crude tris-imine that was then taken up in MeOH (150 mL). The imine was then treated with $NaBH_4$ (2.34 g, 61.6 mmol) and stirred for 18 h. The reaction solution was then rotary evaporated to a solid and $CHCl_3$ (125 mL), saturated salt solution (50 mL), and 5% $NaHCO_3$ (50%) were added and vigorously stirred for 2 h. The mixture was then poured into a separatory funnel, the organic layer retained, dried over $MgSO_4$, filtered, and then rotary evaporated to leave the product as an oil (7.39 g, 86%). ^{1}H NMR ($CDCl_3$) δ 8.480 (d, 1H, J = 4.8, 0.09), 7.577 (dt, 1H, J = 7.5, 2.1), 7.306 (d, 1H, J = 7.8), 7.110 (dd, 1H, J = 6.9, 4.5), 3.918 (s, 2H), 3.10 (br.s, 1H), 2.769 (t, 2H, J = 5.7), 2.669 (t, 2H, J = 5.7); ^{13}C NMR ($CDCl_3$) δ 159.24, 149.08, 136.28, 122.14, 121.77, 54.70, 54.03, 47.05; MS (CI/NH_3) 420 ($M^+ + 1$). Anal. calcd for $C_{24}H_{33}N_7$; C, 68.69; H, 7.94; N, 23.37. Found: C, 68.45; H, 7.48; N, 23.69.

Tris[*N*-(2-pyridyl(2-ethylene))-2-aminoethyl]amine (4b). Tris(2-aminoethyl)amine (3.00 g, 20.5 mmol) and 2-acetylpyridine (7.46 g, 61.65 mmol) were dissolved in benzene (150 mL) and the resultant H_2O was distilled off by use of a Dean–Stark trap. After cooling to room temperature, the solution was decanted and rotary evaporated to isolate the crude tris-imine that was taken up in MeOH (200 mL). The imine was then treated with $NaBH_4$ (2.34 g, 61.6 mmol) and stirred for 18 h. The reaction solution was then rotary evaporated to a solid and $CHCl_3$ (125 mL), saturated salt solution (50 mL), and 5% $NaHCO_3$ (50%) were added and vigorously stirred for 2 h. The mixture was then poured into a separatory funnel, the organic layer retained, dried over $MgSO_4$, filtered, and then rotary evaporated to leave the product as an oil (7.75 g, 82%). ^{1}H NMR ($CDCl_3$) δ 8.55 (m, 1H), (td, 1H, J = 6.6, 1.8), (dt, 1H, J = 8.1, 0.9), (ddd, 1H, J = 7.2, 4.8, 1.2), 3.870 (q, 1H, J = 6.6), 2.62–2.43 (m, 4H), 1.368 (dd, 3H, J = 6.6, 0.9); ^{13}C NMR ($CDCl_3$) δ 164.57, 149.04, 136.34, 121.68, 120.82, 59.44, 53.99, 45.23, 22.66; MS (CI/NH_3) 462 ($M^+ + 1$). Anal. calcd for $C_{27}H_{39}N_7$; C, 70.23; H, 8.53; N, 21.24. Found: C, 70.12; H, 8.23; N, 21.45.

Biological methods

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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