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Preliminary evaluation of the cytotoxicity of a series of tris-2-aminoethylamine (Tren) based hexadentate heterocyclic donor agents

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Abstract—Tachpyridine is a cytotoxic metal chelator with potential anti-tumor activity. The synthesis and evaluation of a set of derivatives of the related hexadentate heterocyclic donor agents tris-2-aminoethylamine (tren) and tris[*N*-(2-pyridylmethylene)-2-aminoethyl]amine (trenpyr) was performed to compare their cytotoxic activity to tachpyridine in HeLa tumor cells. Methyl groups were added to the pyridyl ring of trenpyr, and the effects of alkyl group substitution on cell survival were assessed. Profound cytotoxicity was observed and IC_{50} data were obtained in ascending order from those compounds substituted with a methyl group at the 3-, 4-, or 5-position and lastly by the 6-methyl derivative. These results suggest that analogous derivatives with substitution at the 3-position of the pyridyl ring deserve further exploration.
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1. Introduction

Iron is a critically important metal for cell function, survival, and replication. It is a cofactor in hemoglobin and myoglobin respiratory proteins, hydroxylating enzymes, lipoxygenases, and cyclooxygenases, and ribonucleotide reductase, the enzyme catalyzing the rate-limiting step in DNA synthesis. Tumor cells have been shown to have an increased uptake and trafficking of iron with concomittant membrane expression of transferrin receptors.[1,2] Thus, depletion of intracellular iron has been hypothesized as a viable target for cancer therapy.[3] Iron chelation agents including deferrioxamine (DFO),[4,5] pyridoxal isonicotinoyl hydrazone (PIH) and related compounds,[6,7] and *N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis*,*cis*-1,3,5,-triaminocyclohexane (tachpyr) have been studied for their efficacy in this arena (Fig. 1).[8–10]

Our group has previously synthesized tachpyr and several analogs of this compound.[10–12] Tachpyr is a hexadentate metal chelator with three secondary amines and three pyridyl groups assembled in a pre-organized framework base on the *cis*,*cis*-1,3,5,-triaminocyclohexane (tach). We have previously shown that tachpyr is exceptionally cytotoxic through the binding of cellular iron and/or zinc, but not other metals, and that disruption of the ability to bind metal ions by alkylation of the secondary amines effectively deactivates these compounds.[8] Tachpyr also has been reported to induce a p53-independent apoptotic mode of death.[9,10] Tachpyr is a lipophilic molecule, which impacts on its ability to cross cell membranes.[13]

While investigations continue into a more detailed understanding of the mode of action of tachpyr and

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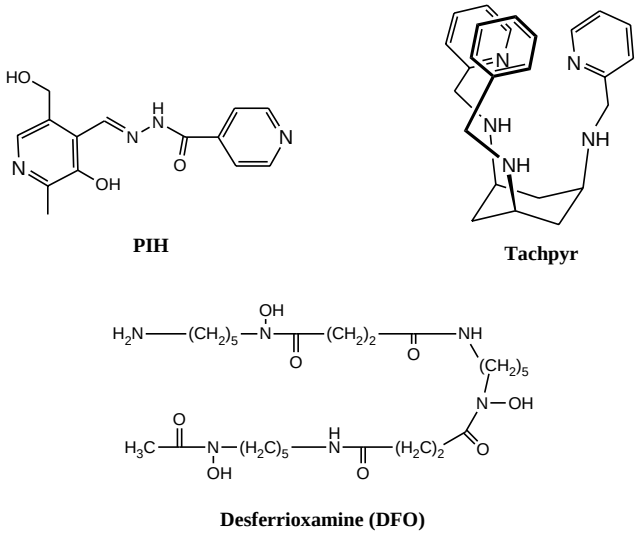

Figure 1. Chelating agents that have been evaluated as iron depletion cancer therapeutics agent.

its respective derivatives,[10,12,14] the nature of the chelating agent itself also continues to be an active area of study. While tachpyr may benefit from the tach framework conferring considerable pre-organization of the six donor amines, there is also potentially some energy cost in metal complex formation as this chelator has to alter conformation from all equatorial to an all axial conformation in which the metal ion assumes a pseudo-adamantyl position within the complex structure.[10,15] One may hypothesize that selection of the three aminomethylenepyridyl metal-binding arms assembled in a geometry that would eliminate or lower this complex formation energy barrier might also provide a more biologically active agent for depleting intracellular iron. Previous results addressing the location of alkyl groups have demonstrated significant impact on both complex formation,[10] and stability,[15] and that this in part correlates with cytotoxicity.[8]

Thus, to further investigate this possibility, we have hypothesized that ligands analogous to tachpyr based on the well-known triamine framework provided by tris-2-aminoethylamine (tren) should exhibit a similar biological activity pattern. We would also predict that while the tren-based compounds would have an analogous cytotoxicity pattern, they might also be less active as a whole due to having to counter entropy costs in complex formation. Thus, we would validate some of the physical constraints of complex formation as they impact biological activity. To this end, tren, tris[*N*-methyl-2-(aminoethyl)]amine (NMe-tren), tris[*N*-methyl-*N*-2-pyridylmethylene-2-(aminoethyl)]amine (NMe-trenpyr), tris[*N*-(2-pyridylmethylene)-2-aminoethyl]amine (trenpyr), tris[*N*-(2-pyridyl(2-ethylene))-2-2-aminoethyl]amine (tren(C-Me)pyr), and four trenpyr analogs with methyl groups at the 3-, 4-, 5-, and 6-position were prepared and evaluated versus tachpyr for cytotoxic activity in HeLa cells, a cancer cell line, as well as in normal epithelial cells.

2. Results and discussion

The tris-methylated tren compounds were prepared via a modification of a previously reported route, reduction of an ethyl carbamate, wherein substitution with benzyl carbamate was successfully employed (Fig. 2).[16] The initially planned synthesis of the tris-methylated tren compounds involved methylation of the tris-tosylated tren, which would then be deprotected to yield the desired product analogous to the preparation of tris-methylated tach. However, difficulties were encountered in completely deprotecting the isolated intermediate methylated tosylated with formation of a tosyl salt observed by NMR that was present prior to and retained even after ion-exchange chromatography. Other protecting groups were then investigated, including benzylchloroformate, which was found to conveniently serve as protecting group and be amenable to $LiAlH_4$ reduction to generate methyl groups. Alkylation of this triamine with 2-chloromethylpyridine was accomplished by a modification of a previously reported route that had been used to generate a series of analogous *N*-alkylated tachpyr derivatives.[12]

The trenpyr (**1**) family of compounds (Fig. 3) was prepared as previously described for the synthesis of trenpyr and tren(C-Me)pyr.[17] This is a modification of previously reported syntheses of these chelators that had been prepared for complexation with Fe and Cu as superoxide dismutase mimics.[18] In brief, the requisite pyridine carboxaldehydes were first prepared as

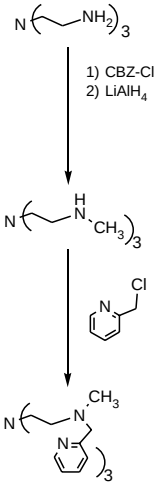

Figure 2. Synthesis and structures of tren based ligands studied.

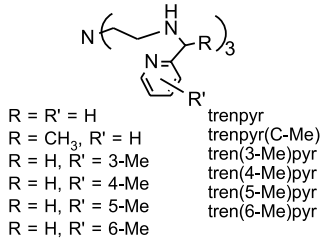

Figure 3. Structures of trenpyr based ligands studied.

previously reported by lithiation of the appropriate methyl-substituted 2-bromo-pyridine, that was quenched with dimethylformamide and isolated by fractional distillation. The aldehydes were reacted with tren to form their respective tris-imines; the reaction was driven to completion by Dean–Stark distillation. The tris-imines were then directly reduced with excess borohydride to provide, after an extractive workup, the targeted set of chelating agents.

Comparing the cytotoxicity of tachpyr to trenpyr directly by an MTT assay indicated that tachpyr possessed significantly greater activity at a lower dose of the reagent to effectively achieve 100% kill of the HeLa cells (Figs. 4 and 5). This result was somewhat of a disappointment as the more flexible chelator trenpyr was proposed to have faster complexation kinetics with

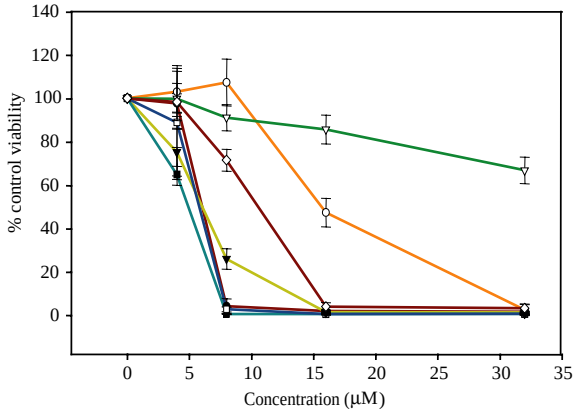

Figure 4. Effect of test compounds on cell viability. Hela cells were treated for 72 h with varying concentrations of each test compound and viability assessed using an MTT assay. Shown are means and standard errors of three independent experiments, each performed using six replicate cultures for each point. Tachpyr (●); trenpyr (○); tren (▼); tren(N-Me)pyr (■); tren(N-Me) (▽).

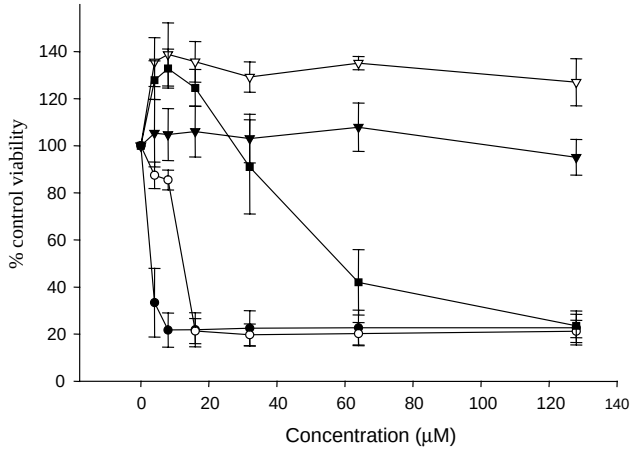

Figure 5. Effect of test compounds on cell viability. Hela cells were treated for 72 h with varying concentrations of each test compound and viability assessed using an MTT assay. Shown are means and standard errors of three independent experiments, each performed using six replicate cultures for each point. Tachpyr (●); trenpyr (○); tren(C-Me)pyr (▼); tren(3-Me)pyr (■); tren(4-Me)pyr (□); tren(5-Me)pyr (◊); tren(6-Me)pyr (▽).

intracellular iron resulting in a more toxic agent. However, the results indicate that tachpyr possesses a significant advantage originating from the more restricted geometry of this ligand. This advantage probably provides a real influence on complex formation in regard to overall lowered entropy, outweighing the energy cost of conformational change from equatorial to axial.[19]

Despite the somewhat disappointing result obtained from direct comparison of the tachpyr to trenpyr, the results obtained from examining the effects of methyl substitution, either on *N*–Me or on the pyridyl ring of trenpyr were quite interesting. From the data presented in Figures 4, and 5 and Table 1, one can clearly observe a ranking of activity wherein the tren(3-Me)pyr exhibits slightly greater activity than tachpyr, and tren(4-Me)pyr and tren(C-Me)pyr are roughly equivalent to tachpyr. The tren(5-Me)pyr appeared to be somewhat lower in activity. Most importantly, all of these compounds were found to be more active that the parent trenpyr with the exceptions being the tren(6-Me)pyr and NMe-trenpyr. Clearly, alkyl substitution of the pyridine ring as well as on the carbon framework of trenpyr has a profound effect on cytotoxicity in the HeLa cell line. The tren(3-Me)pyr, tren(4-Me)pyr, and tachpyr all clearly achieve complete cell kill at the low concentration of ~7 μM with a very steep response curve.

We also assessed the sensitivity of normal, non-cancer, epithelial cells to these compounds. As seen in Table 1, alkyl substitution had parallel effects on cytotoxic efficacy in these cells as in the malignant HeLa cells: for example, methylation at the 3-position enhanced cytotoxicity, whereas methylation at the 6-position dramatically reduced toxicity. Importantly, however, in all cases normal cells were less sensitive to the cytotoxic effect of the chelators than the cancer cells, with IC_{50} increases from 2 to 4-fold in normal cells relative to HeLa cells (Table 1). Such preferential activity on tumor cells is clearly an advantageous feature in the use of such chelators as anti-tumor drugs.

The IC_{50} for the 3-methyl-substituted compound was slightly superior to tachpyr, whereas the IC_{50} for the 4-methyl- and C-methyl-substituted compounds were comparable to tachpyr (Table 1). While the IC_{50} for the tren(5-Me)pyr was slightly higher than that of tachpyr, and the concentration required for complete cell kill

Table 1.

Compounds	Average IC_{50}[a]	
	HeLa	HMEC
Tachpyr	5.3[b]	11.5
Trenpyr	13.5	30.8
Tren-C-pyr	5.3	13.5
Tren(3-Me)pyr	3.8	14.5
Tren(4-Me)pyr	5.5	15.4
Tren(5-Me)pyr	10.1	17.2
Tren(6-Me)pyr	55.9	263.0

[a] Average of 2–4 independent experiments.
[b] Average of 12 independent experiments.

(~15 μM) was more than twice that of tachpyr, tren(5-Me)pyr was far more active than trenpyr itself. The final derivative on the group, tren(6-Me)pyr, appears to have drastically compromised activity, and this result clearly sets this substitution position off as being a negative site for modification versus all of the other ring positions. The impact of methylation of the secondary amines is also a negative indication and while the biological activity reported for analogous tachpyr compounds indicated N-alkylation essentially abrogated the cytotoxicity of tachpyr, significant activity was noted for NMe-trenpyr (Fig. 4). This result does in part substantiate our hypothesis that a more flexible geometry may be able to obviate some obstacle to complex formation and thereby retain some measure of biological activity, unlike the *N*-alkylated tachpyr compounds.[8]

The explanation of the varying degrees of activity probably lies within the structure of the Fe complex that is formed, the relative impediments to that formation, and the mode of action thereafter that appears to be an effective component of the cytotoxicity of tachpyr and thus these analogous ligands. If one examines the known structures of the analogous tachpyr complexes and thus potentially those of trenpyr, these generally appear to be octahedral in geometry with the three pyridyl donors arranged in a spiral twisted arrangement such that a pair of enantiomeric complexes are formed.[10,15] The methyl groups added to trenpyr to form tren(3-Me)pyr and tren(4-Me)pyr do not appear to provide any significant interactions or impediments in achieving this conformation about the metal; however, the addition of the methyl groups clearly provides a significant enhancement on the biological activity of these compounds. The cytotoxicity of these two compounds exceeds that of the parent trenpyr and is comparable to tachpyr itself (Fig. 5 and Table 1). The tren(5-Me)pyr, while somewhat less active than tachpyr, is also more active than trenpyr. The enhanced activity of some of these compounds may relate in part to their ability to assume the geometry required for complex formation with ease or additional driving forces; however, this enhancement is not easily explained by structural aspects of the metal complex alone for all of the studied compounds.

An explanation for the lack of activity for the tren(6-Me)pyr seems readily available from the previous literature reports. An attempt to form the iron Fe complex for the evaluation of superoxide dismutase mimic activity failed and was attributed to steric hindrance from the methyl groups in the 6-position inhibiting complex formation.[18] Complementary to this hypothesis were the crystal structures of the Cu(II) complexes of both tachpyr and analogous tach(6-Me)pyr derivative.[15] The Cu(II) complex of tachpyr itself was found to be a near regular six coordinate octahedron with minimal distortion while the complex with tach(6-Me)pyr was five coordinate with one of the pyridyl rings rotated away from metal eliminating the sixth nitrogen donor. Evaluation of the in vitro stability of a radio–copper complex of this latter ligand indicated poor complex stability thereby providing a clear explanation for the lack of activity from tren(6-Me)pyr.

The source of the increased activity of tren(3-Me)pyr versus trenpyr may be a result of a steric interaction. The hypothesized mode of biological cytotoxicity of tachpyr and its derivatives has been reported to be more than just simple sequestration of intracellular iron. These related trenpyr ligands could be expected to exert an analogous reactivity. Complex formation has been reported to involve a reduction–oxidation process wherein the initial complexed Fe(III) is reduced to Fe(II) while the ligand undergoes an oxidative dehydrogenation process that can repeatedly cycle to ultimately form the tri-imine analog of tachpyr as its Fe(II) complex.[10,19] Thus, the mode of action of these ligands in part includes formation of the tris-imine and thus a potential change in the fundamental structure of the complex. In the case of either tachpyr (6-coordinate), or trenpyr (7-coordinate) this progression to the respective tris-imine involves a 'flattening' of the spiral nature of the pyridyl rings and a rotation away from the octahedral or mono-capped anti-trigonal prism, respectively, to a more pure trigonal prism type of structures.[10,20] A careful examination of the immediate environment about the methylene protons that undergo elimination in this mechanism reveals that a potential interaction between these protons and an alkyl substituent at the 3-position on the pyridine ring exists. This steric interaction might be partially relieved through the oxidative dehydrogenation elimination reactions. The resulting sp2 centers that form then direct the remaining proton away from the alkyl substituents at the 3-position on the pyridine ring. Thus, relief of this interaction potentially provides a driving force for the elimination reaction and thus an enhanced degree of cytotoxicity and lowered IC_{50} values.

While these arguments provide some measure of explanation for the extremes in biological activity for this group of compounds, tren(3-Me)pyr versus tren(6-Me)pyr, likely explanations for the increased biological activity of the tren(4-Me)pyr, tren(5-Me)pyr, and tren(C-Me)pyr are unresolved. Differences in lipophilicity and the ability to cross cell membranes may be influenced by the addition of alkyl groups. Electronic properties of the Fe complexes may also be altered in some capacity that enhanced the elimination reaction and the redox chemistry associated with the Fe(III)/Fe(II) chemistry within the cellular environment. Thus, it seems clear that other modes of action are in play in these tren derivatives or at least are amplified in some capacity by the use of a less pre-organized triamine platform compared to tach.

In conclusion, while trenpyr itself demonstrated a somewhat decreased activity as a cytotoxic intracellular iron depletion/sequestration agent versus the analogous tachpyr, profound effects were evident from the addition of methyl groups on the pyridine rings. The 3- and 4-positions clearly appear to greatly increase biological activity to be comparable to tachpyr, along with the 5-position to a lesser extent. While tachpyr is possibly of more fundamental interest, the triamine base employed therein is considerably less available than tren. Thus, tren, being a commercially available polyamine

starting material, might serve as a more accessible entry point for investigating novel intracellular iron depletion/sequestration agents. The results reported here clearly eliminate substitution at the 6-position of the pyridyl ring in future compounds. Lastly, the results from the 3-Me derivative appear to indicate that this position is well suited for further investigation to expand understanding what limitations on substitution at this position might exist. The methyl group may well prove to be the limit in size and any larger group might provide a negative influence on biological activity. However, to address this hypothesis fully will require development of a reasonable entry into 3-alkyl-pyridine-2 carboxaldehydes. All of these variables are the topic of ongoing studies and will be reported in due course.

3. Experimental

3.1. Materials and methods

All chemicals and solvents were purchased from Fluka, Sigma, or Aldrich and were used as received. Trenpyr, tren(C-Me)pyr, 3-methyl-pyridine-2-carboxaldehyde, 4-methyl-pyridine-2-carboxaldehyde, 5-methyl-pyridine-2-carboxaldehyde, and 6-methoxy-pyridine-2-carboxaldehyde were prepared as previously reported.[12,17]

^{1}H and ^{13}C NMR were obtained using a Varian Gemini 300 instrument and chemical shifts are reported in parts per million on the δ scale relative to TMS, TSP, or solvent. Proton chemical shifts are annotated as follows: parts per million [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 system with Model 114M pumps controlled by System Gold software and a Model 165 dual wavelength detector set at 254 and 280 nm. Chromatography was performed using 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.

4. Chemistry—ligand synthesis

4.1. Tris[*N*-carbobenzyloxy-(2-aminoethyl)]amine (tren-cbz)

A solution of tren (29.20 g, 0.200 mol) in benzene (225 mL) and H_2O (100 mL) was stirred at 5 °C for 1 h. Benzylchloroformate (52.96 g, 0.312 mol) was then added drop wise to the solution. After complete addition, a second portion of benzylchloroformate (53.14 g, 0.313 mol) was added simultaneously with an 18.61 M aqueous solution of KOH (35 mL). The reaction mixture was stirred for 2 h at 5 °C and then for 18 h at room temperature. The layers were separated and the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL). The organic layers were combined, dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give the tris-carbamate (89.95 g, 82%).

^{1}H NMR (DMSO) δ 2.56 (tbd, 6H), 3.21 (tbd, 6H), 5.02 (s, 6H), 5.37 (tbd, 1H), 7.24–7.27 (m, 15H); ^{13}C NMR (DMSO) δ 38.93, 53.83, 66.64, 127.95, 128.40, 136.65, 156.93. Mass Spect. (FAB) 549 (M^++1); Anal. Calcd for $C_{30}H_{36}N_4O_6$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.70; H, 6.61; N, 10.15.

4.2. Tris-[*N*-methyl-2-(aminoethyl)]amine trihydrochloride (NMe-tren.3HCl) (2)

Tren-cbz (89.95 g, 0.164 mol) in THF (250 mL) was added drop wise to a suspension of $LiAlH_4$ (30.03 g, 0.791 mol) in THF (700 mL). After complete addition, the solution was refluxed for 18 h. The reaction mixture was then quenched (carefully with H_2O (50 mL) and 17.80 M aq KOH (50 mL) water. After filtering, the precipitate was rinsed with H_2O, the filtrate was adjusted to pH 8.0 with HCl (aq), and extracted with CH_2Cl_2 (2 × 200 mL). The organic layers were combined, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to yield an oily brown residue. This oil was then purified by vacuum distillation collecting the product at 130 °C, 5.5 mmHg. The pure oil was then converted to the Tris–HCl salt by bubbling HCl (g) through a solution in dioxane. The product was obtained as a precipitated yellow powder, 21.45 g (44%).

^{1}H NMR (D_2O) δ 2.38 (s, 9H), 2.77 (t, J = 6.90, 6H), 2.95 (t, J = 6.90, 6H); ^{13}C NMR (D_2O) δ 35.13, 46.74, 50.90; Mass Spect. (FAB) 189 (M^++1); Anal. Calcd for $C_9H_{24}N_4(H_2O)(HCl)_{2.5}$: C, 36.34; H, 9.66; N, 18.83. Found: C, 36.77; H, 9.69; N, 18.51.

4.3. Tris[*N*-methyl-*N*-2-pyridylmethylene-2-(aminoethyl)]amine (NMe-trenpyr) (5)

A suspension of NMe-tren·3HCl (0.50 g, 1.69 mmol) and Na_2CO_3 (3.04 g, mmol) in DMF (30 mL) was stirred at 80 °C as 2-chloromethylpyridine HCl (0.89 g, 5.43 mmol) in DMF (10 mL) was added. Stirring was continued for 18 h after which the reaction mixture was evaporated in vacuo. The residue was taken up in $CHCl_3$ (40 mL) and extracted with H_2O (2 × 30 mL), saturated brine solution (30 mL). The organic layer was then dried over Na_2SO_4 and filtered. The filtrate was evaporated to give leave the product as a red oil (0.54 g, 69%).

^{1}H NMR ($CDCl_3$) δ 2.24 (s, 9H), 2.53 (br t, 6H), 2.66 (br t, 6H), 3.65 (s, 6H), 7.13 (t, J = 6.75, 3H), 7.34 (d, J = 7.80, 3H), 7.62 (t, J = 9.15, 3H), 8.53 (d, J = 5.10, 3H); ^{13}C NMR ($CDCl_3$) δ 42.58, 52.56, 55.21, 63.83, 121.42, 122.53, 135.83, 148.60, 159.01; Mass Spect. (FAB) 462 (M^++1); Anal. Calcd for $C_{27}H_{39}N_7(H_2O)_{1.5}$: C, 66.36; H, 8.66; N, 20.06. Found: C, 65.82; H, 8.44; N, 20.15.

4.4. Tris[*N*-(2-(3-methyl-pyridylmethylene))-2-aminoethyl]amine (tren(3-Me)pyr)

Tren (1.00 g, 6.845 mmol) was reacted with 3-methylpyridine-2-carboxaldehyde (2.486 g, 20.55 mmol) in benzene (150 mL) at reflux and driven to completion by use of a Dean–Stark trap for 18 h. The reaction solution was decanted and concentrated to an oil by rotary evaporation. The isolated crude imine was taken up in methanol (125 mL) and reduced with sodium borohydride (0.80 g, 21 mmol) for 18 h. The solution was taken to dryness by rotary evaporation and the residue partitioned between $CHCl_3$ (125 mL) and 5% aqueous $NaHCO_3$ (125 mL) with vigorous mixing for 2 h. Thereafter, the mixture was poured into a separatory funnel, and the $CHCl_3$ solution retained. The aqueous was extracted with $CHCl_3$ (100 mL), the $CHCl_3$ portion combined, dried over $MgSO_4$, filtered, and rotary evaporated to leave the product as a dark oil, that was dried in vacuo and stored under refrigeration (2.78 g, 88%).

^{1}H NMR (DMSO-d_6) δ 8.284 (d, 1H, J = 5.1), 7.356 (d, 1H, J = 7.2), 7.012 (dd, 1H, J = 7.5, 4.5), 4.56 (br s, 1H), 3.924 (s, 2H), 2.901 (t, 2H, J = 5.7), 2.779 (t, 2H, J = 5.7), 2.251 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 153.16, 146.24, 137.43, 130.89, 121.79, 54.00, 51.37, 47.43, 18.02; Mass Spect. (FAB) 462 (M^++1); Anal. Calcd for $C_{27}H_{39}N_7$: C, 70.23; H, 8.53; N, 21.24. Found: C, 70.37; H, 8.56; N, 21.12. HPLC t_R = 17.89 min.

4.5. Tris[*N*-(2-(4-methyl-pyridylmethylene))-2-aminoethyl]amine (tren(4-Me)pyr)

Tren (1.00 g, 6.845 mmol) was reacted with 4-methylpyridine-2-carboxaldehyde (2.486 g, 20.55 mmol) in benzene (150 mL) and the crude imine was reduced with sodium borohydride (0.80 g, 21 mmol) in methanol (125 mL) to provide the final product as a dark oil (2.62 g, 83%).

^{1}H NMR (DMSO-d_6) δ 8.336 (d, 1H, J = 4.8), 7.139 (s, 1H), 6.932 (d, 1H, J = 7.2), 3.910 (s, 2H), 2.801 (t, 2H, J = 5.4), 2.695 (t, 2H, J = 5.7), 2.292 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 158.72, 149.03, 147.63, 123.33, 123.05, 54.52, 53.98, 47.14, 21.02; Mass Spect. (FAB) 462 (M^++1); Anal. Calcd for $C_{27}H_{39}N_7$: C, 70.23; H, 8.53; N, 21.24. Found: C,70.39; H, 8.53; N, 21.12. HPLC t_R = 20.12 min.

4.6. Tris[*N*-(2-(5-methyl-pyridylmethylene))-2-aminoethyl]amine (tren(5-Me)pyr)

Tren (1.00 g, 6.845 mmol) was reacted with 5-methyl-pyridine-2-carboxaldehyde (2.486 g, 20.55 mmol) in benzene (150 mL) and the crude imine was reduced with sodium borohydride (0.80 g, 21 mmol) in methanol (125 mL) to provide the final product as a dark oil (2.74 g, 87%).

^{1}H NMR (DMSO-d_6) δ 8.4–8.28 (m, 1H), 7.45–7.35 (m, 1H), 7.22–7.15 (m,1H), 3.868 (s, 2H), 3.235 (br s, 1H), 2.741 (q, 2H, J = 5.4), 2.647 (t, 2H, J = 5.4), 2.274 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 156.16, 149.60, 136.99, 131.25, 121.89, 54.37, 54.01, 47.09, 18.13; Mass Spect. (FAB) 462 (M^++1); Anal. Calcd for $C_{27}H_{39}N_7$: C, 70.23; H, 8.53; N, 21.24. Found: C, 70.60; H, 8.89; N, 21.16. HPLC t_R = 19.22 min.

4.7. Tris[*N*-(2-(6-methyl-pyridylmethylene))-2-aminoethyl]amine (tren(6-Me)pyr)

Tren (3.00 g, 6.845 mmol) was reacted with 6-methylpyridine-2-carboxaldehyde (7.46 g, 20.55 mmol) in benzene (150 mL) and the crude imine was reduced with sodium borohydride (0.80 g, 21 mmol) in methanol (125 mL) to provide the the final product as a dark oil (2.90 g, 92%).

^{1}H NMR (DMSO-d_6) δ 7.46 (t, 1H, J = 7.8), 7.099 (d, 1H, J = 7.8), 6.968 (d, 1H, J = 7.5), 3.863 (s, 2H), 3.14 (br s, 1H), 2.766 (t, 2H, J = 5.1), 2.677 (t, 2H, J = 5.1), 2.485 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 158.56, 157.90, 136.71, 121.51, 119.19, 54.92, 54.16, 47.28, 24.50; Mass Spect. (FAB) 462 (M^++1); Anal. Calcd for $C_{27}H_{39}N_7$: C, 70.23; H, 8.53; N, 21.24. Found: C, 70.50; H, 8.71; N, 21.56. HPLC t_R = 19.03 min.

5. Biological methods

5.1. In vitro cellular proliferation assay

Hela cells were obtained from the American Type Culture Collection and grown in a humidified 5% CO_2 atmosphere at 37 °C in DME medium (Gibco BRL) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Human mammary epithelial cells (HMEC) were obtained from Cambrex and propagated in a humidified 5% CO_2 atmosphere at 37 °C in mammary epithelial basal medium (Cambrex). $2–5 \times 10^3$ cells were plated in 96-well tissue culture dishes and allowed to attach overnight before test compounds were added. Six replicate cultures were used for each point. After 72 h, viability was assessed using an MTT assay in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide is added to the medium and the formation of a reduced product is assayed by measuring the optical density at 560/650 nm after 3 h. Color formation is proportional to viable cell number.[21] In some cases, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; Promega), a water soluble substrate, was used in place of MTT.

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