Synthesis and Cytotoxicity of Potential Tumor-Inhibitory Analogues of Trimelamol (2,4,6-Tris[(hydroxymethyl)methylamino]-1,3,5-triazine) Having Electron-Withdrawing Groups in Place of Methyl

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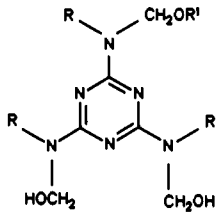
In exploring the structural features which determine the antitumor activity of 2,4,6-tris-[(hydroxymethyl)methylamino]-1,3,5-triazine (trimelamol, 1), we have synthesized analogues in which the methyl groups have been replaced by the electron-withdrawing substituents 2,2,2-trifluoroethyl (5), propargyl (13), and cyanomethyl (15) via the respective tris(alkylamino)triazines 3, 12, and 14. Three mono[(hydroxymethyl)amino]triazines (4, 7, and 10) were also prepared. All the new tris(hydroxymethyl) derivatives showed cytotoxicities toward a variety of experimental rodent and human ovarian tumor cell lines similar to those shown by 1, the cyanomethyl analogue (15) having the most favorable profile. Mono(hydroxymethyl) derivatives (4 and 7) were *ca.* one-third as toxic. The new tris(hydroxymethyl) analogues were more stable to aqueous hydrolysis than was 1. Half-life (pH 7.5) values were, for 1, 120 min, for 5, 690 min, for 13, 450 min, and for 15, 275 min, but at pH 2.0, 15 ($t_{1/2}$ 350 min) was the most stable. This cyanomethyl analogue was also the most water-soluble, being comparable to 1 whereas 5 and 13 were poorly soluble.

2,4,6-Tris[(hydroxymethyl)methylamino]-1,3,5-triazine (trimelamol, 1) is a third-generation analogue of hexamethylmelamine having potential advantages over the parent drug. Hexamethylmelamine is clinically active,



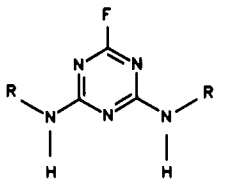
1, R = Me, R' = H

5, R = CH_2CF_3, R' = H

6, R = CH_2CF_3, R' = CH_2OH

13, R = $CH_2C{\equiv}CH$, R' = H

15, R = $CH_2C{\equiv}N$, R' = H



2, R = CH_2CF_3

11, R = $CH_2C{\equiv}CH$

particularly against ovarian carcinoma but causes severe nausea and vomiting.[1] It was hoped that the greater aqueous solubility of an analogue pentamethylmelamine would permit systemic administration, avoiding the side effects associated with oral hexamethylmelamine. However, pentamethylmelamine showed no significant antitumor activity in patients in a number of phase I trials (ref 2 and references cited therein), despite having activity in a number of preclinical models including human lung tumor xenografts.[3,4] Metabolism studies demonstrated a requirement for metabolic activation to cytotoxic *N*-(hydroxymethyl) derivatives.[5] However, whereas this occurs readily in mice, cytotoxic levels of these metabolites are never achieved in patients.[6] These findings prompted the development of 1, in which the requirement for metabolic activation is circumvented. In the phase I clinical trial, 1 proved less emetic and less neurotoxic than pentamethylmelamine and showed significant activity in patients with ovarian tumors resistant to cisplatin.[7]

These exciting findings prompted further studies designed to enhance the clinical utility of 1. However, pharmacokinetic analysis has shown 1 to have a very short plasma half-life in humans (6.3 ± 0.6 min).[7] An approach to improved therapy with this class of compound would be the design of analogues in which the hydroxymethyl functions are stabilized. Although improved stability in aqueous solution might not have a profound effect on the half-life *in vivo*, it would allow the drug to be administered by prolonged infusion. Preclinical data using the ADJ/PC6 tumor[8] and data from the phase I clinical trial of 1[7] suggest that prolonged exposure would result in an enhanced therapeutic index. A more stable analogue might also be easier to formulate, difficulty with formulation being a major obstacle to the further clinical development of 1. Stabilization could, in principle, be achieved by the use of electron-withdrawing functions in place of the methyl groups in 1. We report here the preparation of hydroxymethyl derivatives and intermediates to these containing the trifluoroethyl (2–10), propargyl (11–13), and cyanomethyl (14 and 15) functions, the aqueous stabilities and solubilities of the hydroxymethyl derivatives, and their cytotoxicities toward a variety of tumor cell lines *in vitro*.

Whereas trifluoroethyl is a more lipophilic substituent than methyl, the respective calculated π values[9] being +1.34 and +0.56, the value for cyanomethyl (−0.57) indicates it to be less lipophilic than methyl. This could be important, because it is possible that the greater CNS effects observed clinically for pentamethylmelamine compared with 1 could be related to their relative lipophilicities.[7] Low lipophilicity could therefore be a desirable feature in an analogue of 1. It is also important to establish the requirement for polyfunctionality in this class of compound. So far, the only compounds with less than three hydroxymethyl groups for which biological data have

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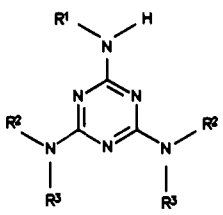

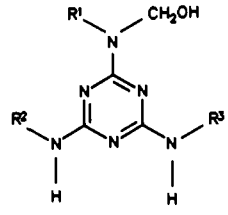

3. $R^1 = R^2 = CH_2CF_3$. $R^3 = H$

4. $R^1 = R^2 = R^3 = CH_2CF_3$

8. $R^1 = Me$; $R^2 = CH_2CF_3$. $R^3 = H$

7. $R^1 = R^2 = CH_2CF_3$. $R^3 = Me$

9. $R^1 = CH_2CF_3$, $R^2 = R^3 = Me$

10. $R^1 = CH_2CF_3$. $R^2 = R^3 = Me$

12. $R^1 = R^2 = CH_2C{\equiv}CH$. $R^3 = H$

14. $R^1 = R^2 = CH_2C{\equiv}N$. $R^3 = H$

been obtained also contain methyl groups,[3] which could be metabolized to hydroxymethyl functions, leading to ambiguity in interpreting the data. The synthesis of analogues which enable this question to be addressed also forms part of the present study.

Results and Discussion

Synthesis of Analogues. The starting material for the synthesis of 1, namely 2,4,6-tris(methylamino)-1,3,5-triazine, is made by reaction of cyanuric chloride with aqueous methylamine.[3] Even with this relatively basic amine (pK_a 10.66) displacement of all three chlorine substituents required elevated temperature. The use of both cyanuric chloride and cyanuric fluoride for the synthesis of monochloro[10] and monofluoro[11] derivatives containing one trifluoroethylamino group only has been reported, but no derivative with more than one such substituent has been reported. We anticipated that the use of the weakly basic trifluoroethylamine (pK_a 5.59) in their synthesis would certainly require the more reactive cyanuric fluoride as coreactant.[12] The bis(2,2,2-trifluoroethyl)amino derivative (2) was conveniently prepared by mixing the reagents in the absence of solvent. Displacement of the third fluorine substituent proceeded smoothly in DMF when cesium fluoride was added to give the required intermediate tris((2,2,2-trifluoroethyl)amino)-triazine (3). Alternatively, 3 could be prepared in more modest yield by conducting the reaction (without CsF) in refluxing toluene.

The reaction between 3 and 40% aqueous formaldehyde adjusted to pH 8.9, conditions we have used to prepare 1 from trimethylmelamine, gave only a mono(hydroxymethyl) derivative (4). The introduction of further hydroxymethyl substituents required the addition of the stronger base K_2CO_3. However, the product which separated from the reaction mixture under these conditions was not the desired tris(hydroxymethyl) analogue (5) but was compound 6, having one of the hydroxymethyl groups modified by further reaction with formaldehyde. This (hydroxymethoxy)methyl derivative was, however, readily and virtually quantitatively converted into the desired compound 5 by treatment with aqueous acetone. The formation of an *N*-(hydroxymethoxy)methyl derivative has a precedent in the literature. 2′,3′,5′-Tris(acetylarabinosyl)adenine afforded its 6-[[(hydroxymethoxy)-methyl]amino] derivative on reaction with aqueous formaldehyde.[13]

The availability of 2 made possible the preparation of an analogue (7) of mixed functionality. First 2 reacted with methylamine, the stronger base here catalyzing its own displacement of the remaining fluorine substituent, and the intermediate (8) then reacted with formaldehyde to form a mono(hydroxymethyl) derivative (7) analogous to 4. The ^{1}H-NMR spectrum of 7, when compared with those of 3–5, together with the presence of two fluorine signals in the ^{19}F-NMR spectrum clearly showed that the hydroxymethyl substituent was contiguous with trifluoroethyl and not with methyl. Finally, the known[11] reaction of cyanuric chloride with 2,2,2-trifluoroethylamine introduces a single trifluoroethylamino substituent, and subsequent reaction with dimethylamine gave an intermediate (9) from which a further mono(hydroxymethyl) derivative (10) was prepared.

Propargylamine (pK_a 8.15) is also a much weaker base than methylamine, and cyanuric fluoride was again the preferred coreagent, analogously yielding the monofluoro bis(propargylamino) derivative (11) and the tris(propargylamino) derivative (12). The latter could also be prepared, albeit in modest yield (32% *vs* 93%), from cyanuric chloride. The reaction of 12 with formaldehyde to form a tris(hydroxymethyl) derivative (13) again required addition of K_2CO_3. However, its omission did not yield a mono(hydroxymethyl) derivative instead: it simply prevented the separation of any product from solution.

The tris((cyanomethyl)amino)triazine 14, a potential precursor to the corresponding analogue (15) of 1, has been prepared in an impure form in two steps starting from aminoacetonitrile and cyanuric chloride.[14] In our hands the reaction with cyanuric chloride was unsatisfactory, but the reaction with cyanuric fluoride in DMF gave pure 14. Although the pK_a values of aminoacetonitrile (5.34) and trifluoroethylamine (5.59) are similar, addition of CsF was not necessary in the formation of 14. The formation of the analogue 15 in contrast to that of 5 or 13 proceeded without addition of base and without precipitation of the product from the reaction mixture.

***In Vitro* Cytotoxicities.** The data shown in Table I indicate the activity of (hydroxymethyl)melamine analogues containing electron-withdrawing groups compared with 1. In the trimelamol-sensitive cell lines Walker rat carcinoma 256 and the human small cell lung cancer H69, the analogues 5, 6, 13, and 15 show activity similar to that for 1, as seen from the IC_{50} values in Table I. Compound 15 appears the most promising in this respect. Again, in the trimelamol-sensitive ADJ/PC6 murine plasmacytoma cell line, compound 15 appears the most cytotoxic of the analogues tested, with somewhat higher IC_{50} values being obtained for compounds 5, 6, and 13. In the L1210 murine lymphocytic leukemia cell line, again 15 shows the closest activity to that of 1.

The use of GCT/GCTCisR cell lines[15] with intermediate sensitivity to 1 provides evidence for the effectiveness of

Table I. Cytotoxicities of 1 and Analogues against Various Cell Lines *in Vitro*

compound	IC_{50} (μM) Walker 256	PC6	H69	L1210	GCT	GCT/CisR
1	9.4(0.5)[a]	12.9(2.7)	8.5(2.3)	33.4(7.3)	24.3(0.9)	30.9(3.0)
4	22.5(1.5)	77.6(5.2)	42.3(3.5)	83.2(7.4)	ND[b]	ND
5	12.2(1.0)	29.5(4.5)	12.8(3.2)	55.1(12.5)	ND	ND
6	ND	29.8(5.6)	13.4(2.0)	ND	25.8(1.8)	23.7(0.3)
7	27.5(1.0)	71.4(17.7)	32.2(3.0)	112.5(7.8)	ND	ND
13	14.1(5.0)	35.2(5.5)	13.1(1.5)	47.8(7.1)	53.9(9.8)	47.3(4.0)
15	7.5(0.4)	19.4(2.0)	11.8(2.0)	32.4(0.8)	ND	ND

[a] Figures in parentheses denote the standard deviations obtained for three to six replicate analyses. [b] ND, not done.

Table II. Half-Life ($t_{1/2}$) in min for Tris(hydroxymethyl) Derivatives in Water at Varying pH at 37 °C

compound	pH 7.5	pH 5.0	pH 2.0
1	120	200	ND
5	690	ND	60
13	450	ND	40
15	275	960	350

1 and analogues against cis-platinum resistant human ovarian carcinoma cell lines. The IC_{50} values obtained in both cell lines indicate resistance factors of 1.3 and less for the three compounds tested.

Clearly those compounds containing three hydroxymethyl groups, *i.e.* **5**, **13**, and **15** (and **6**, which is a pro-drug for **5**), have superior cytotoxic activity over those compounds containing only one such group, i.e. **4** and **7**, for all the cell lines tested. The cytotoxic activity of either **4** or **7** varied from 3- to 6-fold less than that of **1**, as indicated by IC_{50} values.

Stability Studies. The influence of the trifluoromethyl substituent on the stability of the hydroxymethyl function under simulated physiological conditions was determined first, by comparing compound **10**, containing contiguous hydroxymethyl and trifluoroethyl functions, with *N*-(hydroxymethyl)pentamethylmelamine.[3] The $t_{1/2}$ values for the release of the respective products **9** and pentamethylmelamine in human plasma at 37 °C were respectively 45 and 30 min. Hence, replacement of methyl by trifluoroethyl does give a useful degree of stabilization which should be enhanced by multiple substitution.

The stabilities of the tris(hydroxymethyl) analogues and **1** were compared in aqueous solution at 37 °C (Table II). At approximately physiological pH, all analogues show superior stability over that for **1**. The trifluoroethyl analogue **5**, showing a 4-fold increase in stability, appears the most stable compound at this pH. The rankings of **5** and **13** at this pH were as expected from their relative electron-withdrawing effects reflected in the pK_a values of the appropriate amines. The reason for the surprisingly lower stability of **15** at this pH requires further explanation. On the basis of mechanisms previously proposed[16] for the loss of formaldehyde from **1**, an acid-catalyzed mechanism in which exocyclic nitrogen is protonated and a base-catalyzed one in which the hydroxyl group is ionized, the electron-withdrawing substituents in the new analogues should be stabilizing at acidic pH values but destabilizing at alkaline pH. These mechanisms account for the relative stabilities of these compounds at pH2. Indeed, the stability of compound **15** at this pH would appear to offer the possibility of an oral route of administration. However, to explain the rankings at pH 7.5, one must suppose that the base-catalyzed mechanism is promoted at this pH value only by the most electron-withdrawing substituent, namely cyanomethyl. Lastly, studies of stability at ambient temperature (Table III) afford data which indicate the likely stability of compounds under conditions of continuous infusion and again show **1** to be the least stable compound.

Solubility Studies. The solubility of **1** has been previously quoted[3] as 9.04 mg/mL from measurement of UV absorption. The experimental procedure employed to obtain this result, however, would undoubtedly have caused extensive degradation of the compound and should therefore be regarded as unrealistic. The material used in the present study was a ball-milled and lyophilized preparation used in the clinical trial of **1**.[7] Solubilities for **5** and **13** were low whereas that for **15** is at an acceptable level to consider it as suitable for iv formulation (Table IV). The solubility of **15** has been shown to be further enhanced by lyophilization and by solubilization in PEG 400 solution (data not shown).

Table III. Half-Life ($t_{1/2}$) in min for Tris(hydroxymethyl) Derivatives in Various Media at Room Temperature[a]

	compound		
medium	1	5	15
water pH 5.0	820	ND[b]	6120
water pH 7.5	ND	5100	4510
0.9% sodium chloride[c]	275	5700	8090
5% dextrose[d]	350	3000	10800

[a] Room temperature 20–24 °C. [b] ND, not done. [c] pH sodium chloride solution = 5.2. [d] pH dextrose solution = 4.0.

Table IV. Aqueous Solubilities of Tris(hydroxymethyl) Derivatives

compound	solubility (mg/mL)
1	4.5[a]
5	0.03
13	0.03
15	1.0

[a] Lyophilized, ball-milled material.

In summary, whereas the data shown in Tables II and III demonstrate the superior stability for compounds **5**, **13**, and **15** over that seen for **1**, considerations of solubility make **15** the preferred candidate for further development as a potential improvement on **1**.

Experimental Section

Chemical Synthesis. ^1H- and ^{19}F-NMR spectra were determined with a Bruker AC 250 spectrometer with Me_4Si and C_6F_6, respectively, as internal standards. The determination of HPLC retention times was conducted using a 15 cm × 4.6 mm stainless steel column packed with Spherisorb S5 ODS2. Detection was at 254 nm, and a Trilab III (Trivector, Sandy, Beds., England) was used for data processing. Infrared spectra were determined with a Perkin-Elmer 1720X spectrometer. Mass spectra were determined using a VG 7070H spectrometer and VG 2235 data system. Electron impact (EI) spectra were obtained at an ionizing voltage of 70 eV and ion-source temperature of 150 °C, and chemical ionization (CI) spectra at 50 eV with methane as ionizing gas. Fast atom bombardment (FAB) mass spectra were carried out using the Iontech saddle field gun which directed fast atoms of xenon (6 kV) at the matrix on a stainless steel probe tip.

Melting points were determined on a Kofler block (Reichert Thermovar) melting point apparatus and are uncorrected. Elemental analyses were determined by CHN Analysis Ltd., South Wigston, Leicester, England.

2-Fluoro-4,6-bis[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (2). To cyanuric fluoride (1.62 g, 12 mmol) was added with stirring at 0 °C 2,2,2-trifluoroethylamine (3.17 g, 24 mmol). When the initially vigorous reaction had subsided, the mixture was placed under vacuum (rotary evaporator) to remove excess reagents. The resulting white solid (3.41 g, 97%) gave colorless crystals from EtOAc: subl 85 °C; *m/z* 293 (M^+, 56%), 274 (40%), 273 (52%), and 224 (100%). Anal. ($C_7H_6F_7N_5$) C, H, F, N.

2,4,6-Tris[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (3). (a) In Dimethylformamide. To a stirred solution of cyanuric fluoride (2.025 g, 15 mmol) in dry DMF (12.5 mL) was added through a septum attached to the top of a reflux condenser 2,2,2-trifluoroethylamine (7.45 g, 75 mmol). When the initially vigorous reaction had subsided, CsF (5 g) was added and the mixture stirred at 40 °C for 3 days. The slurry was then poured onto ice (100 g), and **3** was recovered as a white solid (5.08 g, 91%) after washing with ice-cold water and desiccation: δ_H ($CDCl_3$) 4.05 (m, 6, CH_2), 7.41, 7.57 (2 br s, 3, NH); δ_F −70.3 (s, CF_3); *m/z* (EI) 372 (M^+). This product was used directly for the preparations of **4** and **5**.

(b) In Toluene. To a stirred solution of cyanuric fluoride (1.56 g, 0.85 mL, 11.53 mmol) in dry toluene (50 mL) was added 2,2,2-trifluoroethylamine (6.89 g, 69.2 mmol). When the initially vigorous reaction had subsided, the mixture was heated under reflux for 20 h. Solid was removed by filtration and the filtrate concentrated. Crystallization of the residue from HOAc (25 mL)/H_2O (20 mL) afforded **3** (2.24 g, 52%): mp 73–75 °C; NMR spectra identical with the product from section a above. Anal. ($C_9H_9F_9N_6$) C, H, F, N.

2-[(Hydroxymethyl)(2,2,2-trifluoroethyl)amino]-4,6-bis-[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (4). A mixture of **3** (744 mg, 2 mmol) and 40% aqueous formaldehyde adjusted to pH 8.9 (7.5 mL) was stirred for 16 h. Following complete dissolution of starting material, **4** separated subsequently and was recovered as a white solid (585 mg, 73%) after washing first with the formaldehyde solution and then with ice-cold water and then desiccation at 5 °C. Recrystallization from CH_2Cl_2 gave colorless crystals (445 mg) having a retention time (HPLC, MeCN/aqueous NH_4HCO_3, 1:1) of 1.7 min: subl 135 °C; δ_H (Me_2SO-d_6) 4.06 (app t, 4, $NHCH_2CF_3$, slight sharpening on D_2O shake to app q), 4.41 (q, J = 9.5 Hz, 2, $CH_2NCH_2CF_3$), 5.07 (d, J = 6.3 Hz, 2, CH_2OH, s on D_2O shake), 5.74 (t, 1, OH), 7.69 (br s, 2, NH); δ_F –68.4 (app s, 3, $CH_2NCH_2CF_3$), –70.32 (app s, 6, $NHCH_2CF_3$); m/z (FAB, matrix PEG) 403 ([M + H]$^+$, 35%), 385 ([M + H – H_2O]$^+$, 100%), 373 ([M + H – CH_2O]$^+$, 68%). Anal. ($C_{10}H_{11}F_9N_6O$) C, H, N.

2,4,6-Tris[(hydroxymethyl)(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (5). To a stirred solution of K_2CO_3 (829 mg, 6 mmol) in 40% aqueous formaldehyde adjusted to pH 8.9 (20 mL) was added **3** (2.231 g, 6 mmol) prepared by method a. The mixture was stirred for 3 days, and the resulting white solid was recovered by filtration, washed with aqueous formaldehyde and then with ice-cold water, and dessicated at 5 °C to give 2-[((hydroxymethoxy)methyl)(2,2,2-trifluoroethyl)amino]-4,6-bis[(hydroxymethyl)(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (**6**; 1.81 g, 61%): δ_H (Me_2SO-d_6) 4.45 (br q, 6, CH_2CF_3), 4.65 (d, 2, J = 7.6 Hz, OCH_2OH), 5.06 (br t, 4, NCH_2OH), 5.21, 5.25 (2s, total 2, NCH_2OCH_2OH), 5.90 (2t, 2, NCH_2OH), 6.39, 6.59 (2t, total 1, J = 8.5 Hz, OCH_2OH); δ_F –67.9, –68.1 (2t, $HOCH_2OCH_2NCH_2CF_3$), –68.5 (m, $HOCH_2NCH_2CF_3$). Anal. ($C_{13}H_{17}F_9N_6O_4$) C, H, N, F. A solution of **6** (1.66 g, 3.59 mmol) in acetone (10 mL) was treated with water (6.66 mL) and then set aside for 1 h at room temperature. Acetone was removed under vacuum, and the resulting white precipitate, recovered by filtration, washed with water, and dried under vacuum over $CaCl_2$, was the title compound **5** (1.54 g, 99% from **6**): δ_H (Me_2SO-d_6) 4.45 (2 overlapping q, 6, CH_2CF_3), 5.06 (2d, 6, CH_2OH), 5.87 (2t, 3, OH); δ_F –68.4 (m, CH_2CF_3). Anal. C, H, F, N.

2-[(Hydroxymethyl)(2,2,2-trifluoroethyl)amino]-4-(methylamino)-6-[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (7). A mixture of **2** (2.34 g, 8 mmol) and 30% aqueous methylamine (40 mL) was stirred for 16 h. The supernatant was decanted from the oil which separated and was then stirred with H_2O (50 mL), and the process was repeated. After two further additions of H_2O and decantation, the oil was triturated with ice–water, whereupon crude 2-(methylamino)-4,6-bis[(2,2,2-trifluoroethyl)-amino]-1,3,5-triazine (**8**) was obtained as a white solid which upon filtration and vacuum desiccation afforded a hygroscopic glass (1.26 g, 52%, m/z 304 (M$^+$, 100%)) which was used without further purification to prepare **7**. A mixture of **8** (304 mg, 1 mmol) and 40% aqueous formaldehyde adjusted to pH 8.9 (5 mL) was stirred for 1 h, whereupon **7** separated during 24 h as a white solid (446 mg, 51%): δ_H (Me_2SO-d_6) 2.75 (s, 3, CH_3), 4.07 (br s, 3, $HNCH_2CF_3$), 4.40 (m, 2, $HOCH_2NCH_2CF_3$), 4.84 (d, 2, CH_2OH), 5.74 (m, OH), 7.00 (m, 1, $NHCH_3$), 7.64 (m, 1, $NHCH_2CF_3$); δ_F –69.4 (app s, 3, $CH_2NCH_2CF_3$), –71.2 (app s, 3, $HNCH_2CF_3$). Anal. ($C_9H_9F_6N_6O$) C, H, F, N.

2,4-Bis(dimethylamino)-6-[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (9). A solution of 2,4-dichloro-6-[(2,2,2-trifluoroethyl)amino]-1,3,5-triazine[11] (3.1 g, 0.013 mol) in aqueous dimethylamine (25% w/v, 200 mL) was heated under reflux, first alone and, then when TLC indicated incomplete reaction, with addition of NaOH (1.2 g, 0.03 mol). The solid obtained on filtering the cooled reaction mixture was recrystallized from light petroleum (bp 60–80 °C) to yield **9** (1.8 g, 52%): mp 100 °C; δ_H ($CDCl_3$) 3.09 (s, 12, CH_3), 4.10 (m, 2, CH_2CF_3), 4.89 (br t, 1, NH); δ_F –73.2 (t, $J_{F,H}$ = 9.4 Hz, CF_3). Anal. ($C_9H_{16}F_3N_6$) C, H, N.

2,4-Bis(dimethylamino)-6-[(hydroxymethyl)(2,2,2-trifluoroethyl)amino]-1,3,5-triazine (10). A saturated solution of **9** (300 mg) in H_2O (1600 mL) and 0.1 M NaOH (10 mL) was prepared by overnight stirring at room temperature. Further 0.1 M NaOH (10 mL) was added to the filtered solution to bring the pH to 10.5; then 40% aqueous formaldehyde adjusted to pH 8.5 (25 mL) was added to give a final pH of 10 and the mixture stirred overnight, whereupon **10** separated as a white solid which was recovered by washing with ice-cold water and air drying (50 mg): mp 101–103 °C. Anal. ($C_{10}H_{17}F_3N_6O\cdot H_2O$) H, N. Found: C, 38.00%. Required: C, 38.46%.

2-Fluoro-4,6-bis(propargylamino)-1,3,5-triazine (11). To a stirred solution of cyanuric fluoride (810 mg, 6 mmol) in dry DMF (5 mL) was added propargylamine (660 mg, 12 mmol). After the initial reaction had subsided, further propargylamine (330 mg, 6 mmol) was added. When the further reaction had subsided, **11** had separated as a white solid (333 mg): subl 157 °C; m/z (CI) 206 ([M + H]$^+$, 100%). Anal ($C_9H_8FN_5$) C, H, N. Found: F, 8.55%. Required: F, 9.26%. Further product was recovered from the filtrate by precipitation with water (20 mL), making a total yield for **11** of 1.076 g (87%).

2,4,6-Tris(propargylamino)-1,3,5-triazine (12). (a) From Cyanuric Fluoride. To a stirred solution of cyanuric fluoride (1.35 g, 10 mmol) in toluene (20 mL) was added a solution of propargylamine (3.30 g, 60 mmol) in toluene (30 mL) during 5 min. The mixture was heated under reflux for 3 h and then filtered hot. The filtrate deposited **12** as pale buff crystals (2.225 g, 93%) of retention time (HPLC, MeCN/aqueous NH_4HCO_3, 7:3) 1.8 min: mp 125–127 °C; ν_{max} (film from CH_2Cl_2) 2116 cm^{-1} (C≡C str), 4.17 (br s, 6, CH_2), 5.04 (br s, 3, NH).

(b) From Cyanuric Chloride. To a stirred suspension of cyanuric chloride (1.84 g, 10 mmol) in dry toluene (50 mL) was added dropwise, during 5 min, propargylamine (3.30 g, 60 mmol). When the initial reaction had subsided, the mixture was heated under reflux for 20 h and then filtered hot. The solid which separated on cooling was removed by filtration, the filtrate was concentrated, and the residue was recrystallized from boiling water to give **12** (773 mg, 32%) as colorless rods identical with material prepared from cyanuric fluoride. Anal. ($C_{12}H_{12}N_6$) C, H, N.

2,4,6-Tris[(hydroxymethyl)propargylamino]-1,3,5-triazine (13). To a stirred solution of K_2CO_3 (414 mg, 3 mmol) in 40% aqueous formaldehyde adjusted to pH 8.9 was added **12** (240 mg, 1 mmol). Complete dissolution occurred in 2 h. After 3 days **13** had separated and was recovered by filtration (caution: the product is somewhat gelatinous), washing first with aqueous formaldehyde and then with ice-cold water, and then drying in vacuo at 5 °C as a white solid (251 mg, 76%) of retention time (HPLC, eluant as for **12** above) 1.5 min: ν_{max} (film from CH_2Cl_2) 2115 cm^{-1} (C≡C str); δ_H ($CDCl_3$) 2.25 (t, J = 2.5 Hz, 3, C≡CH), 4.39 (br s, 6, $C{\equiv}CCH_2$), 5.13 (s, 6, $HOCH_2$); m/z (FAB, matrix m-nitrobenzyl alcohol/PEG) 331 ([M + H]$^+$). Anal. ($C_{15}H_{18}N_6O_3$) C, H, N.

2,4,6-Tris[(cyanomethyl)amino]-1,3,5-triazine (14). A suspension of aminoacetonitrile hydrochloride (9.25 g, 0.1 mol) and $NaHCO_3$ (42 g, 0.5 mol) in dichloromethane (150 mL) was vigorously stirred for 16 h. Solid was removed by filtration, and the filtrate was concentrated at 50 °C on a rotary evaporator to give aminoacetonitrile as a mobile, colorless oil (4.62 g, 82%): δ_H (DMSO-d_6) δ 2.05 (br s, 2, NH_2), 3.48 (s, 2, CH_2), 5.73 (s, integration 0.7% of signal at δ 3.48, residual CH_2Cl_2). To a stirred solution of cyanuric fluoride (2.025 g, 14 mmol) in dry DMF (15 mL) in a flask surrounded by an ice bath was added dropwise through a septum by syringe aminoacetonitrile (5.89 g, 105 mmol, 7 mol equiv), generated by the foregoing method, during 5 min. The cooling bath was removed and the reaction mixture stirred at ambient temperature for 3 h and then treated with ice-cold water (150 mL). The resulting white solid was recovered by filtration and washed with water, and the still moist solid was recrystallized from boiling water (200 mL), a small insoluble residue being removed by filtration of the hot solution, to yield **14** as colorless needles (2.778 g, 76%): mp 232–234 °C (lit.[14] mp 216–228 °C for analytically impure compound). δ_H ($CDCl_3$) 4.21–

4.33 (m, 6, CH_2), 7.59–7.87 (m, 3, NH); *m/z* 243 (M^+; 79%), 189 ([M – NCH_2CN]$^+$, 71%). Anal. ($C_9H_9N_9$) C, H, N.

2,4,6-Tris[(cyanomethyl)(hydroxymethyl)amino]-1,3,5-triazine (15). 2,4,6-Tris[(cyanomethyl)amino]-1,3,5-triazine (14, 2.432 g, 10 mmol) was added to 40% aqueous formaldehyde (50 mL) (unbuffered, measured pH = 4.0), and the suspension was stirred at ambient temperature for 3 days. The now clear solution was extracted with ethyl acetate (150 mL and then 100 mL), the combined extracts were dried over Na_2SO_4 and concentrated, and the residual clear syrup was triturated with water (50 mL) to give a white crystalline solid, recovered by filtration, washed with water, and dried *in vacuo* over $CaCl_2$, which was the hemihydrate of 15 (1.804 g, 53%): δ_H (Me_2SO-d_6) 4.61 (s, 6, CH_2-CN), 5.12 (br d, 6, CH_2OH, J = 6.2 Hz), 5.95 (br s, OH). Anal. ($C_{12}H_{15}N_9O_3 \cdot 0.5H_2O$) C, H, N.

Cell Culture. All cell lines were grown up in continuous culture. The Walker 256 carcinoma cell line was grown in Dulbecco's minimal essential medium (DMEM) with the addition of 10% donor horse serum (DHS), 2 mM glutamine, and antibiotics; the ADJ/PC6 murine plasmacytoma cell line was grown up in DMEM with the addition of 20% DHS, 2 mM glutamine, antibiotics, hydrocortisone (0.5 μg/mL), and insulin (10 μg/mL) (Sigma, Poole, Dorset, UK). The human small cell lung cancer cell line H69 was grown up in RPMI medium with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics. The L1210 murine leukemia cell line was grown up in RPMI medium with 10% DHS, glutamine, and antibiotics. The human testicular cell lines GCT-27 and GCT-27/CisR were grown up in DMEM plus 10% FCS, glutamine, antibiotics, hydrocortisone, and insulin (as for ADJ/PC6). All cell culture media and supplements were obtained from Gibco Life Technologies unless otherwise stated.

Cell cultures were periodically checked for the presence of mycoplasma, all cultures used in the study being free from infection.

Cytotoxicity Testing. The MTT assay used was essentially that described by Mosmann,[16] with a number of modifications.[17,18] All cell lines apart from the GCT cell lines grow as suspension cultures. Prior to seeding onto 96 well tissue culture plates (Nunc, Gibco Life Technologies, Paisley, Scotland), suspension cultures were reduced to a single cell suspension (either by repeated pipetting or syringing through a 19-gauge needle). The GCT cell lines were harvested by trypsinization (0.02% EDTA/0.05% trypsin; Gibco Life Technologies, Paisley, Scotland) and then reduced to a single cell suspension by syringing as above.

Cells were plated at between 1×10^3 and 8×10^3 cells per well in a 200-μL aliquot. Drugs were initially diluted in DMSO and stored as frozen aliquots which were thawed immediately before use and diluted in tissue culture medium. Cells were drug treated in continual exposure.

The 96 well plates were left for between 72 and 144 h according to the respective growth rates of the different cell lines (as measured by doubling times), at 37 °C and 8% CO_2 in a humidifying incubator. Following this incubation period, MTT (Sigma, Poole, UK) solution (5 mg/mL in phosphate buffered saline) was added to each well in a volume of 20 μL. Plates were further incubated between 4 and 5 h at 37 °C.

The formation of formazan crystals was terminated by removal of supernatent from each well by centrifugation of plates containing suspension cultures (300 g for 5 min) followed by removal of supernatent by aspiration. The crystalline deposit of MTT formazan was then dissolved in 200 μL of DMSO and allowed to agitate gently for 10 min.

Absorbances were read on a Titertek Multiskan ELISA plate reader (Flow Laboratories, Helsinki, Finland) at a wavelength of 540 nm (with a reference wavelength of 690 nm). The absorbance values obtained were expressed as a fraction of those obtained for the control untreated wells. In all experiments 3–6 replicate wells were used for each drug concentration. Each assay was carried out at least twice.

Stability Studies (i). Decomposition of 10 in Human Plasma. To fresh human plasma (9.9 mL) maintained at 37 °C was added a solution of 10 (0.1 mL; 10 mg/mL) in dimethyl sulphoxide to give a final concentration of 100 μg/mL. At intervals of 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min, aliquots (0.5 mL) were withdrawn and added to ice-cold MeOH (1 mL). After centrifugation at 4 °C the supernatants were placed in cooled vials on a cold plate (3 °C) and aliquots (25 μL) analyzed by HPLC using an autosampler. The column used was a C6 (Spherisorb) 15 cm × 4.6 mm with a precolumn and was eluted with MeOH/0.05 M aqueous NH_4HCO_3, 55:45, at a flow rate of 1.5 mL min^{-1}. Products were monitored by absorbance at 225 nm. Retention times were, for 10, 11.0 min and, for its breakdown product 9, 9.6 min. By an essentially identical procedure, but using MeOH/40% aqueous ammonium formate, 60:40, as eluant and a flow rate of 1 mL min^{-1}, the breakdown of *N*-(hydroxymethyl)pentamethylmelamine to pentamethylmelamine (respective retention times 12.65 and 5.45 min) was studied.

Stability Studies (ii). Decomposition of Compounds 5, 13, and 15 in Various Media. The stability of compounds 5, 13, and 15 was measured by HPLC analysis. Stock preparations of compounds were made by dissolving in DMSO to a concentration of 50 mM. Aliquots (10 mL) of the appropriate medium were dispensed into polypropylene test tubes followed by the addition of drug solutions to a final concentration of 100 μM. Both 37 °C and ambient-temperature incubation conditions were controlled by incubation of test tubes in water baths.

HPLC analysis involved the use of a 15 cm × 4.6 mm steel column packed with C8 octyl 5-μm Spherisorb material (Phase Sep, Deeside, UK), with precolumn, which afforded the separation of all compounds in the study. In the case of 1 an isocratic method using 90% 0.05 M ammonium bicarbonate (pH 8.1) in HPLC grade 'Hypersolv' water (BDH) and 10% acetonitrile (Romil Chemicals, Loughborough, U.K.) mobile phase was employed at a flow rate of 1.5 mL/min. Separation of 15 from products of decomposition was also achieved isocratically by the foregoing 10% acetonitrile mobile phase at a flow rate of 1.5 mL/min. Separation of 5 and 13 from their products involved a gradient elution using 30% acetonitrile in 0.05 M ammonium bicarbonate running into 60% acetonitrile in 0.05 M ammonium bicarbonate over a period of 10 min with a flow rate of 2 mL/min. The column was housed in a cooling cabinet maintained at a temperature of 15–17 °C. Peak detection was by UV absorbance at 225 nm for all compounds. The Perkin Elmer ISS-100 sample injector module with refridgerated sample plate was set at a sample volume of 10 μL.

Half-life ($t_{1/2}$) calculations were based on the decrease in peak area from the HPLC chromatogram with time. Peak areas were computed by on-line link with the MT2 450 data acquisition system (Kontron Instruments, Watford, U.K.). Half-life values were calculated from semilog plots of peak area (y) against time (x). All measurements were performed on at least two different occasions, with a variation from between 2 and 22%. Throughout the period of analysis preparations of known concentrations of the compound of interest were run as a control for any shift in retention time and/or change in the standard curve calibration.

Measurement of Aqueous Solubility of Compounds 5, 13, and 15. Each compound (approximately 30 mg) was placed in a small glass stoppered vial. Deionized water (2 mL) was then added and the solution placed in water in a sonication bath. During this period the temperature of the bath water was maintained around 15 °C by periodic addition of iced water. This eliminated any excessive degradation of the compounds under study.

The glass stoppered vials were then centrifuged at 2000 rpm for 5 min. The supernatent was then drawn off and recentrifuged to ensure the elimination of all particulate matter. Supernatents were then analyzed by HPLC according to the methodology described under Stability Studies (ii). Peak areas obtained were then read off a standard curve and calculated as solubility in mg/mL (Table IV).

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