DNA-Targeted 1,2,4-Benzotriazine 1,4-Dioxides: Potent Analogues of the Hypoxia-Selective Cytotoxin Tirapazamine

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Tirapazamine (TPZ, 1,2,4-benzotriazin-3-amine 1,4-dioxide) is a bioreductive hypoxia-selective cytotoxin, currently in phase II/III clinical trials in combination with radiotherapy and with cisplatin-based chemotherapy. We have prepared a series of 1,2,4-benzotriazine 1,4-dioxide (BTO) analogues of TPZ where a DNA-targeting chromophore is attached at the 3-position via a flexible linker. DNA binding affinity was modified through variation of the chromophore or the pK_a of the linker chain. The association constants (K_{DNA}) for calf thymus DNA ranged from 1×10^2 to 5.6×10^5 M^{-1} (ionic strength of 0.01 M). DNA binding affinity was dependent on the presence of a positive charge, either in the linker chain or in the chromophore, and (for a series of 4-acridine carboxamide chromophore analogues) correlated strongly with linker chain pK_a. The efficacy of these BTOs in killing aerobic and hypoxic mouse SCCVII tumor cells in vitro was determined by clonogenic survival. Cytotoxicity was measured as the concentration required to reduce plating efficiency to 10% of controls (C_{10}), and the hypoxic cytotoxicity ratio (HCR) for each BTO was calculated as C_{10}(aerobic)/C_{10}(hypoxic). BTOs bearing a positive charge showed increased hypoxic cytotoxicity (1.5−56-fold) compared to TPZ and mostly modest HCRs (8−51), but some excellent (>167 and 400) values. There was a strong correlation between K_{DNA} and hypoxic cytotoxicity but no correlation between K_{DNA} and HCR. Cytotoxicity in HT-29 human colon carcinoma cells, determined using IC_{50} assays, showed similar relationships with a correlation between K_{DNA} and hypoxic cytotoxicity but no correlation between K_{DNA} and HCR. In this cell line, a higher proportion of compounds (7 of 11) had HCRs greater than or equal to that of TPZ. The data confirm that DNA targeting is a useful concept for increasing potency while maintaining hypoxic selectivity and provide a direction for the further development of DNA-targeted analogues of TPZ.

Introduction

Tirapazamine (**1**, TPZ, 1,2,4-benzotriazin-3-amine 1,4-dioxide) (Chart 1) is a bioreductive agent that is selectively toxic to hypoxic cells.[1,2] Hence, it is a useful adjunct to radiotherapy and chemotherapy, which often fail to eliminate hypoxic cells within tumors.[3,4] TPZ undergoes metabolic activation by intracellular reductases to a cytotoxic radical species which, under hypoxia, reacts with DNA to form DNA single- and double-strand breaks.[5−7] Formation of the cytotoxic radical species is inhibited by oxygen, leading to hypoxia-selective cytotoxicity.[8] Metabolism of TPZ may be effected by many intracellular reductases,[9,10] but there is evidence[11,12] that activation in the nucleus is predominantly responsible for the DNA double-strand breaks that mediate its hypoxic toxicity.[5]

TPZ is currently in phase II/III clinical trial in combination with radiotherapy and with cisplatin.[13−17] In conjunction with radiotherapy, fatigue and neutropenia are the dose-limiting toxicities,[17] which could potentially be ameliorated if more potent analogues were available.

Improving the potency of bioreductive drugs by targeting them to DNA has been the subject of a number of studies,[18] which have used various DNA-binding units to target 2-nitroimidazoles[19−23] or nitroacridines[24] and nitroquinolines.[25] However, the presence of a DNA-binding chromophore and basic side chain in a drug may limit extravascular transport through increased DNA binding or entrapment in acidic compartments within cells.[26−28] Identification of this limitation provided the impetus for a search for so-called "minimal" DNA-intercalating agents that would penetrate the extravascular compartment of tumors more effectively.[29−35] Lowering the pK_a of the chromophore or reducing the size of the intercalating chromophore reduced DNA binding, culminating in the development of the antitumor drug DACA (**2**). DACA showed superior tissue diffusion compared to its 9-aminoacridine analogue DAPA (**3**), due to its lower overall charge-state and DNA binding affinity,[36] and received a phase II clinical trial.[37,38] A similar pathway of refinement has been followed in the development of the DNA-targeted radiosensitizer NLCQ-1 (**4**)[39] from the initial analogues NLP-1[40,41] and NLA-1 (**5**).[42,43]

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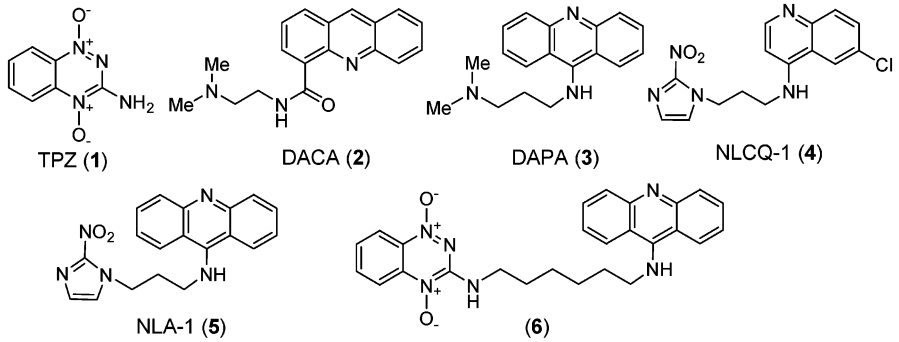
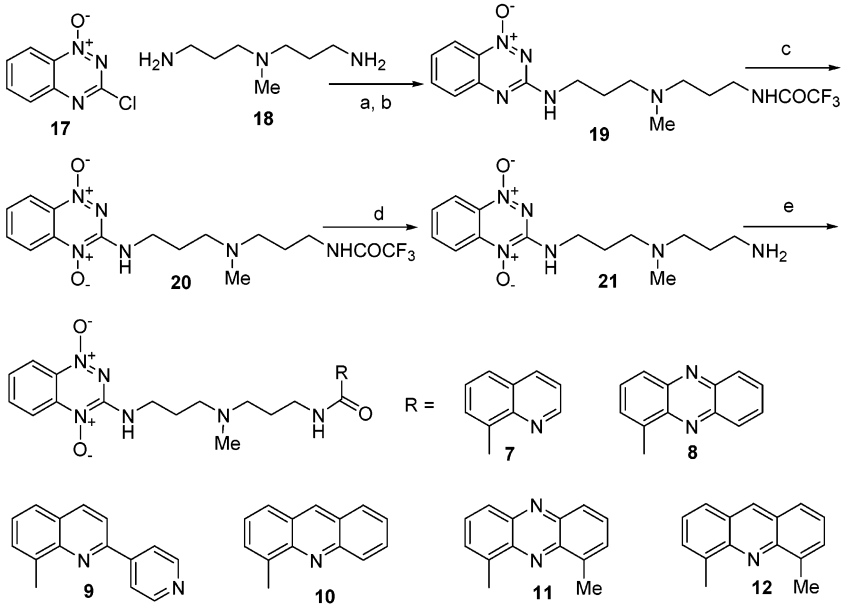
Chart 1



Scheme 1[a]



[a] Reagents: (a) Et_3N, DCM; (b) $(CF_3CO)_2O$, DCM; (c) CF_3CO_3H, DCM; (d) NH_4OH, MeOH; (e) acid **22**−**27**, CDI, DMF; then **21**, DCM.

In contrast, TPZ has received little attention as a subject for such targeting strategies. The objective of targeting TPZ to DNA is to increase the proportion of "productive" metabolism occurring sufficiently close to nuclear DNA targets to contribute to cytotoxicity. Such targeting is expected to reduce wasteful extranuclear consumption of drug (which has been shown to limit extravascular transport of TPZ[44]) and increase potency, thereby increasing therapeutic efficacy relative to TPZ. DNA targeting also has the potential to compromise extravascular transport into hypoxic regions[45,46] and to induce additional toxicities. For these reasons, one of the objectives of the present study is to identify DNA-targeted analogues with moderate binding affinities allowing efficient extravascular transport yet providing enhancement of cytotoxic potency.

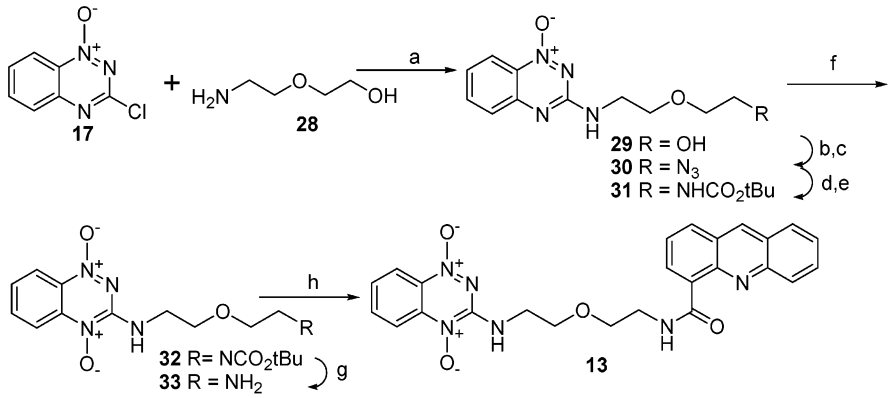
Hypoxic areas in tumor tissue are located at the end of a diffusion gradient, often distant from the microvasculature. Thus, efficient extravascular transport to the site of metabolic activation is required for hypoxia-selective bioreductive drugs to be effective. This is a particular issue for BTO analogues, since recent studies have shown that inefficient transport of TPZ itself through multicellular layer (MCL) cultures results in resistance.[44] Recently, we demonstrated that a DNA-targeted BTO analogue showed much improved in vitro efficacy relative to TPZ.[47] Thus compound **6**, a BTO linked to a 9-aminoacridine unit, showed a 20−400-fold increase in hypoxic cytotoxicity relative to TPZ in HeLa and HT-29 cells while retaining similar hypoxic selectivity. However, **6** was not active in potentiating tumor cell kill by irradiation in vivo, probably because of restricted diffusion through tumor tissue.[47] Thus, the optimization of extravascular transport properties, while retaining the benefits of DNA targeting, is one of the prime requirements for this new class of targeted bioreductive drug, exemplified by BTO **6**.

The objective of this study is to design and synthesize a range of DNA-targeted BTO analogues **7**−**16**, in which the basicity of the amine side chain and the binding strength of the DNA-affinic chromophore are varied, and to quantitate the effect of these structural modifications on the DNA-binding affinity and in vitro cytotoxicity under oxic and hypoxic conditions.

Chemistry

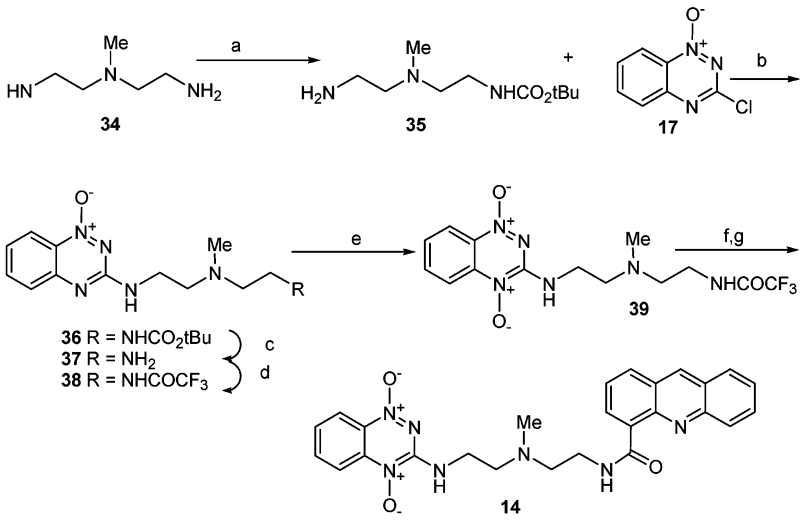
Synthesis. Chloride **17**[48] was coupled to N^1-(3-aminopropyl)-N^1-methyl-1,3-propanediamine (**18**) and protected as the trifluoracetamide **19** to facilitate purification (Scheme 1). Oxidation of **19** with trifluoroperacetic acid under acidic conditions resulted in selective aromatic *N*-oxidation to give 1,4-dioxide **20** (27%) and

Scheme 2[a]



[a] Reagents: (a) Et_3N, DCM; (b) MsCl, Et_3N, DCM; (c) NaN_3, DMF; (d) propane-1,3-dithiol, Et_3N, MeOH; (e) BOC_2O, THF; (f) MCPBA, $NaHCO_3$, DCM; (g) CF_3CO_2H, DCM; (h) **25**, CDI, DMF; then **33**, DCM.

Scheme 3[a]



[a] Reagents: (a) BOC_2O, THF; (b) **17** + **35**, Et_3N, DME; (c) HCl, MeOH; (d) CF_3CO_2Et, H_2O, MeCN; (e) CF_3CO_3H, DCM; (f) **25**, CDI, DMF; (g) **39**, THF.

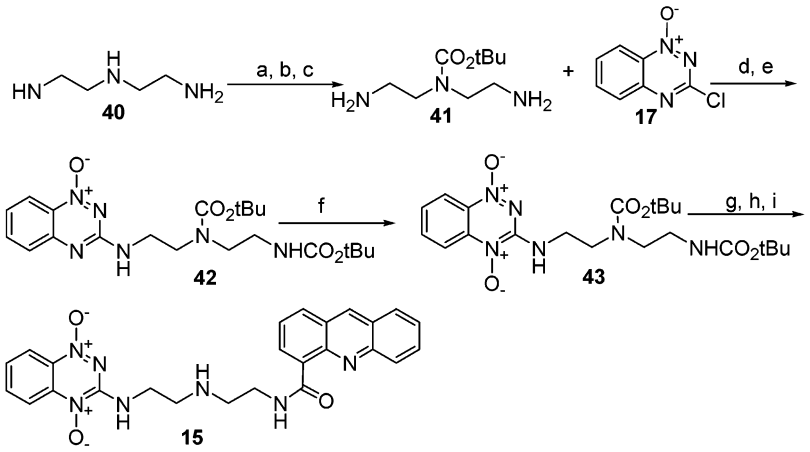
recovered starting material **19** (24%). Extended reaction times gave increased conversion to **20** but was accompanied by the formation of byproducts that complicated purification. The use of other oxidants, e.g., peracetic acid, MCPBA, and methyltrioxorhenium, gave inferior yields. Deprotection of trifluoroacetamide **20** gave amine **21**, which was coupled with the imidazolide of 8-quinolinecarboxylic acid (**22**), formed by reaction of acid **22** with CDI, to give carboxamide **7**. Similarly, reaction of **21** with imidazolides of 1-phenazinecarboxylic acid (**23**),[32] 2-(4-pyridyl)-8-quinolinecarboxylic acid (**24**),[35] 4-acridinecarboxylic acid (**25**),[49] 9-methylphenazine-4-carboxylic acid (**26**),[32] and 5-methylacridine-4-carboxylic acid (**27**)[30] gave carboxamides **8**−**12**, respectively.

Reaction of chloride **17** with 2-(aminoethoxy)ethanol (**28**) gave alcohol **29** in 63% yield, which was converted to the mesylate and displaced with sodium azide to give azide **30** in 89% yield (Scheme 2). Selective reduction of the azide group rather than the 1-oxide of **30** could not be effected by hydrogenation using palladium on charcoal or Lindlar catalyst.[50] Other chemoselective methods for reducing azides such as $NaBH_4$ under PTC,[51] BH_3·DMS,[52] or Staudinger conditions using $P(OEt)_3$[53] were ineffective. However, treatment of azide **30** with propane-1,3-dithiol and Et_3N in refluxing methanol[54] provided the intermediate amine which was protected with di-*tert*-butyl dicarbonate to give carbamate **31** in 93% yield for the two steps. Oxidation of **31** with MCPBA gave 1,4-dioxide **32** in 40% yield as well as recovered starting material **31** (50%). Deprotection of **32** with trifluoroacetic acid gave amine **33** in 91% yield. Reaction of **33** with the imidazolide of acid **25** gave carboxamide **13** in 97% yield.

Coupling of the monoprotected diamine **35**, prepared[55] from diamine **34**, gave the 1-oxide **36** in 52% yield as well as recovered starting material (25%) (Scheme 3). Deprotection and reprotection of **36** gave trifluoroacetamide **38** in 88% yield for the two steps. Oxidation of **38** with trifluoroperacetic acid gave 1,4-dioxide **39** (47%), which was deprotected, and the intermediate amine was coupled to the imidazolide of acid **25** to give carboxamide **14** in high yield.

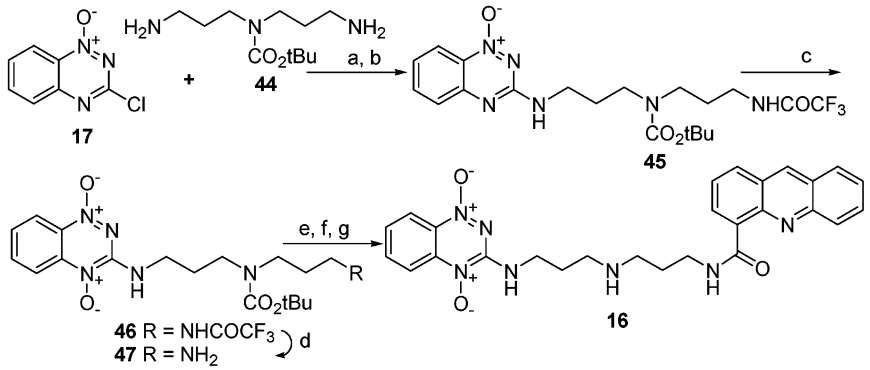
Reaction of chloride **17** with amine **41**, prepared from N^1-(2-aminoethyl)-1,2-ethanediamine (**40**), and protection of the intermediate amine to facilitate isolation gave the 1-oxide **42** in 52% yield (Scheme 4). Compound **42** was oxidized with MCPBA to give 1,4-dioxide **43**.

Scheme 4[a]



[a] Reagents: (a) CF_3CO_2Et, ether; (b) BOC_2O, THF; (c) aq NH_3, MeOH; (d) Et_3N, DME; (e) BOC_2O, DCM; (f) MCPBA, DCM; (g) HCl, MeOH; (h) **25**, CDI, DMF; (i) **43**, HCl, MeOH, then DMF.

Scheme 5[a]



[a] Reagents: (a) Et_3N, DCM; (b) $(CF_3CO)_2O$, DCM; (c) MCPBA, $NaHCO_3$, DCM; (d) K_2CO_3, aq MeOH; (e) **25**, CDI, DMF; (f) **47**, DCM; (g) HCl, MeOH.

Table 1. Physicochemical Data for BTO Analogues

				pK_a[b]		
no.	side chain	chromophore	log *D* meas[a]	chrom	side chain	K_{DNA}[c] 10^4 M^{-1}
1			−0.34 ± 0.02[d]			
6	C_6-alkyl	9-aminoacridine	1.69 ± 0.1	8.6	1.8	14.3 ± 1.5[e]
7	C_3NMeC_3	8-quinoline	0.15 ± 0.004	3.4	8.9	0.046 ± 0.018
8	C_3NMeC_3	1-phenazine	0.59 ± 0.04	-0.1	8.8	0.47 ± 0.12
9	C_3NMeC_3	2-pyridyl-8-quinoline	0.66 ± 0.02	2.9 (pyr)[f]	8.9	0.84 ± 0.17
10	C_3NMeC_3	4-acridine	1.53 ± 0.04	4.0	8.9	3.3 ± 0.6
11	C_3NMeC_3	9-methyl-1-phenazine	1.40 ± 0.05	0.5	8.9	8.5 ± 1.4
12	C_3NMeC_3	5-methyl-4-acridine	2.28 ± 0.03	4.4	8.8	56.2 ± 6.3
13	C_2OC_2	4-acridine	1.84 ± 0.02	4.0		0.010 ± 0.008
14	C_2NMeC_2	4-acridine	2.15 ± 0.02	4.1	7.5	0.75 ± 0.34
15	C_2NHC_2	4-acridine	1.36 ± 0.04	4.1	7.9	2.1 ± 0.6
16	C_3NHC_3	4-acridine	0.88 ± 0.03	4.1	9.7	6.4 ± 2.0

[a] In octanol/water at pH 7.4 (mean ± SEM). [b] Calculated using the program ACDpKa v. 4.5. [c] Equilibrium association constant for DNA binding. [d] From ref 58. [e] From ref 47. [g] Calculated for a 2-pyridyl group.

Deprotection of **43** and coupling of the intermediate amine with the imidazolide of acid **25** gave compound **15**.

Reaction of chloride **17** with *tert*-butyl bis(3-aminopropyl)carbamate (**44**) and protection of the intermediate primary amine with trifluoroacetic anhydride gave the trifluoroacetamide **45** in 39% yield for the two steps (Scheme 5). Oxidation of **45** with MCPBA gave the 1,4-dioxide **46** (8% with 65% recovered starting material). Deprotection of **46** gave amine **47** in good yield, which was coupled to the imidazolide of acid **25**, and the carbamate was deprotected with HCl to give carboxamide **16**.

Physicochemical Measurements. Octanol/water partition coefficients at pH 7.4 of TPZ (**1**) and BTOs **6**−**16** were determined by a low-volume shake flask method, with BTO concentrations in both the octanol and buffer phases analyzed by HPLC as previously described[56] (Table 1). The pK_a values of the BTOs were calculated using ACD pK_a prediction software (v. 4.5, Advanced Chemistry Development Inc., Toronto, Canada)

Table 2. Cytotoxicity Data for BTO Analogues in SCCVII Cells (clonogenic) and in HT-29 Cells (SRB IC_{50})

	clonogenic assay (SCCVII)			IC_{50} assay (HT-29)		
no.	C_{10}(hypoxic)[a] (μM)	C_{10}(aerobic)[b] (μM)	HCR[c]	IC_{50}(hypoxic)[d] (μM)	IC_{50}(aerobic)[d] (μM)	HCR[f]
1	10.2 ± 0.5 (34)[g]	2644 ± 266 (9)[g]	258 ± 21 (9)[g]	6.2 ± 0.8	387 ± 34	62.0
6	0.61	9.6	15.7	0.016 ± 0.006	0.62 ± 0.09	39.7
7	6.5	90	13.9	0.59 ± 0.13	36.3 ± 5.4	61.2
8	1.07	9.4	8.8	0.12 ± 0.03	0.59 ± 0.08	4.9
9	1.79	14	7.8	0.11 ± 0.02	12.0 ± 0.6	107
10	0.82	42	51.5	0.043 ± 0.009	5.35 ± 0.83	124
11	0.35	7.4	21.1	0.018 ± 0.007	0.56 ± 0.07	31.0
12	0.18	>30	>167	0.095 ± 0.039	8.4 ± 2.8	88.4
13	12.4	630	50.8	5.7 ± 1.4	72.5 ± 10.5	12.8
14	0.93	400	431	0.18	15.7	87.0
15	1.16	56	48.2	0.079 ± 0.024	7.8 ± 0.2	98.6
16	0.51	6.4	12.5	0.065 ± 0.021	5.7 ± 0.4	87.5

[a] Concentration of drug for a one-log cell kill under hypoxia normalized against TPZ (**1**) in the same experiment. [b] Concentration of drug for a one-log cell kill under aerobic conditions. [c] Intraexperimental hypoxic cytotoxicity ratio = C_{10}(aerobic)/C_{10}(hypoxic). [d] IC_{50} values (mean ± SEM, $n \geq 2$). [f] Intraexperimental hypoxic cytotoxicity ratio = IC_{50}(aerobic)/IC_{50}(hypoxic). [g] Mean ± SEM, with the number of measurements in parentheses.

and the apparent (macroscopic) constants for both side chain and chromophore moieties reported (Table 1). The binding of **6**−**16** to calf thymus DNA was measured by equilibrium dialysis, using a low ionic strength (0.01 M) to increase sensitivity of detection of binding. Concentrations of compound were measured on both sides of the dialysis membrane by HPLC.

Biological Assays

The efficacy of the BTOs in killing aerobic and hypoxic mouse SCCVII tumor cells in vitro was determined by clonogenic survival after 1 h drug exposure of cells under aerobic and anoxic conditions, as previously described.[57] Cytotoxicity was measured as the concentration required to reduce plating efficiency to 10% of controls (C_{10}), and the intraexperimental differential between the hypoxic and aerobic cytotoxicity for each compound was calculated as the hypoxic cytotoxicity ratio [HCR = C_{10}(aerobic)/C_{10}(hypoxic)] (Table 2). The in vitro cytotoxicity of **1** and **6**−**16** was also evaluated against HT-29 human colon carcinoma cells using a 96-well proliferation assay (sulforhodamine B; SRB)[57] to determine IC_{50} values under aerobic and anoxic conditions (Table 2). For each experiment, TPZ was included as an internal control.

Results and Discussion

log *D*. The hydrophobicities of TPZ and BTOs **6**−**16** were determined from HPLC analysis of drug distribution between octanol and aqueous buffer at pH 7.4 (Table 1). The value for TPZ using this method (−0.34 ± 0.02) has been reported previously[57] and is in good agreement with an independent literature value (−0.32).[58] Although the presence of an aromatic chromophore increases the lipophilicity of the BTOs, the presence of a moderately basic amine in the chromophore **6** or the linker chain **7**−**12** and **14**−**16** reduces the log *D* significantly (range −0.56 to 1.99), suggesting that these features should improve aqueous solubility and reduce plasma protein binding.

DNA Binding. The binding of BTOs to calf thymus DNA at 0.01 M ionic strength was determined by equilibrium dialysis as described previously,[56] and the association constants, K_{DNA}, derived from direct plots of drug bound/base pair versus free drug concentration[47,59] using the neighboring site exclusion model, are shown in Table 1. The binding site size was not well defined (range 0.7−1.9 bp), due to insufficient data points close to the asymptote. The binding of BTOs **6**−**16** ranged from the detectable limit, ca. 1×10^2 M^{-1} for **13**, through to 5.6×10^5 M^{-1} for compound **12**. Increasing the binding strength of the chromophore from 8-quinolinecarboxamide **7** through to 5-methyl-4-acridinecarboxamide **12**, while keeping the linker chain constant, gave a 1200-fold increase in K_{DNA}. Variation of the pK_a of the side chain from 7.5 (**14**) to 9.7 (**16**), while the 4-acridinecarboxamide chromophore remained fixed, provided an 8.5-fold increase in K_{DNA}. Replacement of the amine side chain with a neutral ether-linked side chain (**13**) reduced binding to close to the detectable limit. Overall, DNA binding affinity was dependent on the presence of a positive charge, either in the linker chain or in the chromophore, and correlated strongly with the pK_a of the linker chain in the small series containing the 4-acridine carboxamide chromophore.

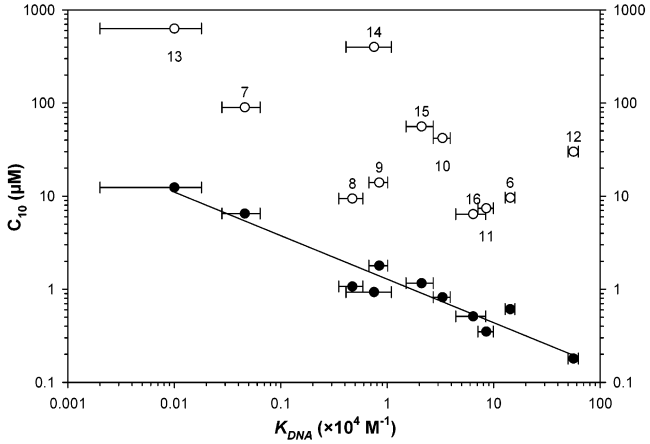


Figure 1. Cytotoxicity of targeted BTO analogues against SCCVII cells (clonogenic assay) versus K_{DNA} (equilibrium dialysis): ●, hypoxic cytotoxicity; ○, aerobic cytotoxicity.

In Vitro Cytotoxicity. Ten BTO analogues were sufficiently soluble to obtain aerobic cytotoxicity data (C_{10} in μM) in SCCVII cells, using the clonogenic assay (Table 2). The BTOs **6**−**16** were considerably more toxic than TPZ (2644 μM) under aerobic conditions, with aerobic C_{10} values ranging from 6.4 to 630 μM. There was no clear correlation between aerobic C_{10} and K_{DNA} ($r = -0.646$, $P = 0.032$; Figure 1). Hypoxic C_{10} values for analogues **6**−**16** ranged from 0.18 to 12.4 μM and

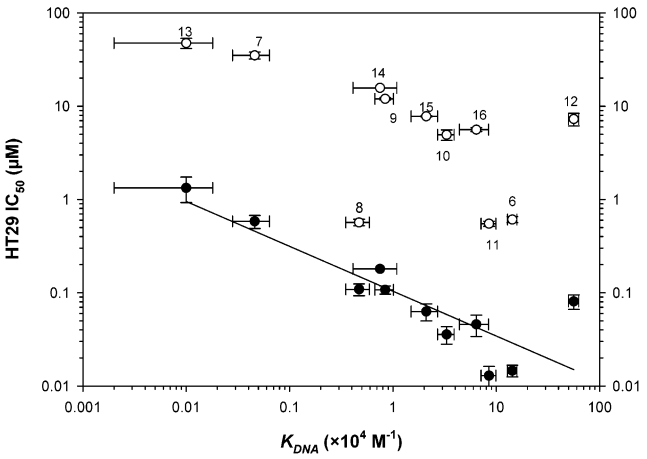


Figure 2. Cytotoxicity of targeted BTO analogues against HT-29 cells (proliferation assay) versus K_{DNA} (equilibrium dialysis): ●, hypoxic cytotoxicity; ○, aerobic cytotoxicity.

showed a strong correlation with K_{DNA} ($r = -0.975$, $P < 0.0001$; Figure 1). The strongest binder in the series, **12**, was 56 times more potent than TPZ under hypoxia. Intraexperimental hypoxic cell selectivity [HCR; C_{10}(aerobic)/C_{10}(hypoxic)] spanned a 55-fold range from 7.8 to 431 and showed no correlation with K_{DNA}. Most compounds had modest hypoxic selectivity (7.8−51), but the strongest binder **12** had an HCR of >167 (aerobic cytotoxicity limited by solubility), which compares favorably with TPZ (HCR = 258). Of particular interest was the observation that the moderate binder **14** had excellent hypoxic selectivity (431-fold), while being 11-fold more potent under hypoxia than TPZ.

BTOs **6**−**16** were all considerably more toxic than TPZ (387 μM) against HT-29 cells in the SRB proliferation assay under aerobic conditions, with IC_{50} values ranging from 0.6 to 72 μM (Table 2). Hypoxic IC_{50} values ranged from 0.016 μM for BTO **6** to 5.7 μM for the "nontargeted" neutral BTO **13**. The strongest binder, **12**, was not the most toxic compound under hypoxia, with BTOs **6** and **11** giving the largest increase in hypoxic cytotoxicity. As with SCCVII C_{10} values, a correlation between HT-29 IC_{50} values and K_{DNA} ($r = -0.861$, $P = 0.0007$; Figure 2) was seen under hypoxic but not under oxic exposure conditions ($r = -0.592$, $P = 0.055$; Figure 2). Moderate to excellent hypoxic selectivity was seen for BTOs **6**−**16**, with seven compounds showing selectivities greater than or equal to that of TPZ. There was again no relationship between HCR and K_{DNA}. As would be anticipated,[57] SCCVII and HT-29 hypoxic and oxic cytotoxicity values combined showed a strong correlation ($r = 0.927$, $P < 0.0001$), suggesting that the SAR for BTOs **6**−**16** is essentially cell line and end-point independent.

The mechanism of the increase in cytotoxic potency of the DNA-targeted analogues is not yet fully understood. The data presented here and elsewhere[47] strongly suggest that the main reason for the increase in hypoxic potency is localization of the compounds and their toxic radical metabolites in the cell nucleus. However, we cannot exclude the possibility that increased metabolism, either by nuclear or other reductive enzymes, is also involved. In addition, while the one-electron reduction potential, $E(1)$, of compound **10** was found[60] to be −444 ± 8 mV, similar to that of TPZ (−456 ± 8 mV),[57] the $E(1)$ of the neutral **13** was slightly lower (−466 ± 9 mV) and compound **16**, possessing a more basic side chain amine, was slightly higher (−421 ± 8 mV). It has been shown for nontargeted BTOs that reduction potential strongly correlates with cytotoxic potency.[57]

The lack of activity of SN 26955 (**6**) in potentiating tumor cell kill by irradiation in vivo has been postulated to be a result of its relatively strong DNA binding limiting extravascular transport.[47] The importance of extravascular transport in the efficacy of bioreductive drugs has been emphasized in a number of studies.[61−63] In particular, the resistance to TPZ in 3D cell culture has been ascribed wholly to inefficient transport rather than changes in intrinsic sensitivity.[44] The effects of DNA binding and pK_a on drug transport have been examined in detail[36] for two basic DNA intercalators, DACA (**2**) and DAPA (**3**), which bear structural similarities to the targeting units of the BTO analogues **7**−**16** and **6**, respectively. We determined the K_{DNA} of DACA to be (15.7 ± 5.6) × 10^4 M^{-1} (0.01 M ionic strength), while the K_{DNA} of DAPA was estimated by extrapolation to be ca. 6 × 10^7 M^{-1} (0.01 M ionic strength).[56] DACA was found to diffuse through multicellular layer cultures faster than DAPA as a result of its weaker basicity and lower DNA-binding affinity. These properties were also suggested to be responsible for the superior antitumor activity of related acridine-4-carboxamides relative to corresponding 9-aminoacridine derivatives.[29] The extravascular transport of the DNA-targeted BTOs is currently under investigation using the multicellular layer model, but if the same principles apply as for the above aminoacridines, then relatively high DNA-binding affinity may compromise diffusion in this series. If this is the case, then moderately DNA-affinic BTOs such as **10**, **14**, and **15**, which still possess good hypoxic selectivity, may be more promising candidates for further development than the more tightly binding analogues **6** and **12**.

Conclusions

We have prepared a series of BTO analogues of TPZ in which variation of the DNA-affinic chromophore or linker chain has provided a range in DNA association constant (K_{DNA}) of ca. 5600-fold (from 1 × 10^2 to 5.6 × 10^5 M^{-1} at an ionic strength of 0.01 M). DNA-binding affinity was dependent on the presence of a positive charge, either in the linker chain or in the chromophore. For BTOs with a fixed (acridine-4-carboxamide) chromophore, binding correlated strongly with the pK_a of the linker chain. Most DNA-targeted BTOs showed increased hypoxic cytotoxicity in both clonogenic and IC_{50} assays, and a correlation between DNA binding and hypoxic cytotoxicity was observed, but not between cytotoxicity under aerobic conditions (and consequently HCR). Despite the lack of correlation between K_{DNA} and HCR, a number of DNA-targeted BTOs showed excellent hypoxic selectivity. This study has confirmed the initial observation[47] that targeting a BTO unit to DNA by conjugation to a DNA-affinic moiety not only increases cytotoxicity relative to TPZ but also retains selective toxicity to hypoxic cells. Further development of this class of compounds, which involves optimizing extravascular transport properties (i.e., diffusion in relation to metabolic consumption),[44] is currently in progress.

Experimental Section

Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal 2300 melting point apparatus. NMR spectra were obtained on a Bruker Avance 400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra. Spectra were obtained in $CDCl_3$, unless otherwise specified, and are referenced to Me_4Si. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. Assignments were determined using COSY, HSQC, and HMBC two-dimensional experiments. Mass spectra were determined on a VG-70SE mass spectrometer using an ionizing potential of 70 eV at a nominal resolution of 1000. High-resolution spectra were obtained at nominal resolutions of 3000, 5000, or 10 000 as appropriate. All spectra were obtained using FAB with positive ionization unless otherwise stated. Solutions in organic solvents were dried with anhydrous Na_2SO_4. Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}) with visualization of components by UV light (254 nm) or exposure to I_2. Column chromatography was carried out on silica gel (Merck 230−400 mesh). All compounds designated for testing were analyzed at >99% purity by reverse phase HPLC using an Agilent 1100 liquid chromatograph, an Alltima C_{18} (5 μm) stainless steel column (150 mm × 3.2 mm i.d.), and an Agilent 1100 diode array detector. Chromatograms were run using various gradients of aqueous (0.045 M ammonium formate and formic acid at pH 3.5) and organic (80% MeCN/MilliQ water) phases. DCM refers to dichloromethane; DME refers to dimethoxyethane; DMF refers to dry dimethylformamide; ether refers to diethyl ether; EtOAc refers to ethyl acetate; EtOH refers to ethanol; MeOH refers to methanol; pet. ether refers to petroleum ether, boiling range 40−60 °C; and THF refers to tetrahydrofuran dried over sodium benzophenone ketyl. All solvents were freshly distilled.

TPZ[64] and BTO **6**[47] were synthesized as previously described.

***N*^{1}-(3-Aminopropyl)-*N*^{3}-(1,4-dioxido-1,2,4-benzotriazin-3-yl)-*N*^{1}-methyl-1,3-propanediamine (21). 2,2,2-Trifluoro-*N*-[3-(methyl{3-[(1-oxido-1,2,4-benzotriazin-3-yl)amino]propyl}amino)propyl]acetamide (19).** A solution of chloride **17**[48] (2.07 g, 11.4 mmol), *N*^{1}-(3-aminopropyl)-*N*^{1}-methyl-1,3-propanediamine (**18**) (3.31 g, 22.8 mmol), and Et_3N (3.2 mL, 22.8 mmol) in DCM (200 mL) was stirred at 20 °C for 2 days. The solvent was evaporated and the residue dissolved in MeCN (150 mL). Ethyl trifluoroacetate (5.4 mL, 45.6 mmol) and water (0.8 mL, 45.6 mmol) were added, and the solution was heated at reflux temperature for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−1%) of Et_3N/(0−10%) MeOH/DCM, to give 1-oxide **19** (1.89 g, 43%) as a yellow solid: mp (DCM) 111−115 °C; ^{1}H NMR δ 9.04 (br s, 1 H, NH), 8.25 (dd, J = 8.7, 1.4 Hz, 1 H, H-8′), 7.70 (ddd, J = 8.4, 7.1, 1.4 Hz, 1 H, H-6′), 7.57 (d, J = 8.4 Hz, 1 H, H-5′), 7.29 (ddd, J = 8.7, 7.1, 1.1 Hz, 1 H, H-7′), 6.17 (br s, 1 H, NH), 3.58 (dd, J = 6.6, 5.8 Hz, 2 H, CH_2N), 3.49 (br t, J = 6.0 Hz, 2 H, CH_2N), 2.52−2.58 (m, 4 H, 2 × CH_2N), 2.27 (s, 3 H, NCH_3), 1.84−1.90 (m, 2 H, CH_2), 1.75−1.82 (m, 2 H, CH_2); ^{13}C NMR δ 158.9, 157.3 (q, J = 36 Hz), 148.8, 135.6, 130.8, 126.4, 124.9, 120.4, 116.1 (q, J = 288 Hz), 57.1, 56.4, 41.3, 40.3 (2), 26.3, 24.4; MS m/z 387 (MH$^+$, 100%), 371 (8), 338 (30); HRMS calcd for $C_{16}H_{22}F_3N_6O_2$ (MH$^+$) m/z 387.1756, found 387.1765. Anal. ($C_{16}H_{21}F_3N_6O_2 \cdot {}^1/_2$MeOH) C, H, N.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-2,2,2-trifluoroacetamide (20).** Trifluoroacetic anhydride (4.1 mL, 29.2 mmol) was added to a stirred solution of 1-oxide **19** (1.13 g, 2.9 mmol) in $CHCl_3$ (50 mL) and the solution stirred at 20 °C for 30 min. The solution was cooled to −10 °C and 70% H_2O_2 (2 mL) (**Caution:** vigorous reaction) added dropwise. The solution was stirred at 20 °C for 3 days and partitioned between $CHCl_3$ (50 mL) and saturated aqueous $KHCO_3$ (50 mL). The aqueous fraction was extracted with $CHCl_3$ (3 × 30 mL), the combined organic fraction dried, and the solvent evaporated on to silica (**Caution**: safety shield). The residue was purified by chromatography, eluting with 10% MeOH/DCM, to give (i) starting material **19** (275 mg, 24%) and (ii) 1,4-dioxide **20** (319 mg, 27%) as a red gum: ^{1}H NMR [$(CD_3)_2SO$] δ 9.44 (br s, 1 H, NH), 8.45 (t, J = 5.9 Hz, 1 H, NH), 8.20 (d, J = 8.8 Hz, 1 H, H-8′), 8.12 (d, J = 8.6 Hz, 1 H, H-5′), 7.93 (ddd, J = 8.6, 7.1, 1.2 Hz, 1 H, H-6′), 7.57 (ddd, J = 8.8, 7.1, 1.3 Hz, 1 H, H-7′), 3.42−3.47 (m, 2 H, CH_2N), 3.21−3.25 (m, 2 H, CH_2N), 2.39 (t, J = 6.7 Hz, 2 H, CH_2N), 2.32 (t, J = 6.9 Hz, 2 H, CH_2N), 2.16 (s, 3 H, NCH_3), 1.72−1.80 (m, 2 H, CH_2), 1.61−1.68 (m, 2 H, CH_2); ^{13}C NMR [$(CD_3)_2SO$] δ 155.9 (q, J = 36 Hz), 149.7, 138.1, 135.4, 129.8, 126.7, 121.0, 116.7, 115.9 (q, J = 288 Hz), 54.9, 54.6, 41.4, 39.5, 37.6, 25.9, 25.8; MS m/z 403 (MH$^+$, 25%), 387 (5); HRMS calcd for $C_{16}H_{22}F_3N_6O_3$ (MH$^+$) m/z 403.1706, found 403.1695.

***N*^{1}-(3-Aminopropyl)-*N*^{3}-(1,4-dioxido-1,2,4-benzotriazin-3-yl)-*N*^{1}-methyl-1,3-propanediamine (21).** A solution of trifluoroacetamide **20** (175 mg, 0.44 mmol) and NH_4OH (5 mL) in MeOH (20 mL) was stirred at 30 °C for 4 h. The solvent was evaporated and the residue dried to give amine **21** (131 mg, 98%) as a red gum: ^{1}H NMR [$(CD_3)_2SO$] δ 8.43 (br s, 1 H, NH), 8.21 (d, J = 8.5 Hz, 1 H, H-8′), 8.13 (d, J = 8.4 Hz, 1 H, H-5′), 7.94 (ddd, J = 8.4, 7.1, 1.2 Hz, 1 H, H-6), 7.75 (br s, 2 H, NH_2), 7.57 (ddd, J = 8.7, 7.2, 1.3 Hz, 1 H, H-7′), 3.45 (t, J = 6.8 Hz, 2 H, CH_2N), 3.20−3.25 (m, 2 H, CH_2N), 2.88 (dd, J = 7.4, 7.2 Hz, 2 H, CH_2N), 2.40−2.46 (m, 2 H, CH_2N), 2.20 (s, 3 H, NCH_3), 1.77−1.83 (m, 2 H, CH_2), 1.68−1.75 (m, 2 H, CH_2); MS m/z 307 (MH$^+$, 2%), 291 (5); HRMS calcd for $C_{14}H_{23}N_6O_3$ (MH$^+$) m/z 307.1883, found 307.1883.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-8-quinolinecarboxamide (7).** A solution of 8-quinolinecarboxylic acid (**22**) (90 mg, 0.5 mmol) and CDI (97 mg, 0.6 mmol) in DMF (5 mL) was stirred at 55 °C for 24 h. The solution was diluted with dry benzene (10 mL), Sephadex LH-20 (300 mg) was added, and the mixture was stirred at 20 °C for 1 h. The mixture was filtered, the solvent was evaporated, and the residue was dissolved in dry THF (5 mL). A solution of amine **21** (80 mg, 0.25 mmol) in THF (5 mL) was added and the solution stirred at 20 °C for 70 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−2%) of aqueous NH_3/(0−8%) MeOH/DCM, to give compound **7** (110 mg, 91%) as a red powder: mp (DCM/pet. ether) 119−121 °C; ^{1}H NMR δ 11.39 (br s, 1 H, CONH), 8.96 (dd, J = 4.3, 1.8 Hz, 1 H, ArH), 8.74 (dd, J = 7.3, 1.5 Hz, 1 H, ArH), 8.34 (dd, J = 8.8, 1.8 Hz, 1 H, ArH), 8.25 (d, J = 8.3 Hz, 1 H, ArH), 8.17 (d, J = 8.6 Hz, 1 H, ArH), 7.98 (br s, 1 H, NH), 7.92 (dd, J = 8.1, 1.5 Hz, 1 H, ArH), 7.78 (dd, J = 8.1, 1.1 Hz, 1 H, ArH), 7.62 (t, J = 7.7 Hz, 1 H, ArH), 7.48 (dd, J = 8.3, 4.0 Hz, 1 H, ArH), 7.43 (dd, J = 7.9, 1.0 Hz, 1 H, ArH), 3.68−3.73 (m, 4 H, 2 × CH_2), 3.02−3.07 (m, 4 H, 2 × CH_2), 2.67 (s, 3 H, CH_3), 2.25−2.17 (m, 4 H, 2 × CH_2); ^{13}C NMR δ 166.4, 149.7, 149.6, 145.4, 138.2, 137.7, 135.6, 133.6, 132.0, 130.3, 128.5, 128.4, 127.1, 126.4, 121.5, 121.0, 117.3, 54.7, 54.5, 40.6, 39.4, 37.2, 25.5, 24.5; MS m/z 462 (MH$^+$, 25%), 446 (5); HRMS calcd for $C_{24}H_{28}N_7O_3$ (MH$^+$) m/z 462.2254, found 462.2249. Anal. ($C_{24}H_{27}N_7O_3$) C, H, N.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-1-phenazinecarboxamide (8).** To a solution of trifluoroacetamide **20** (283 mg, 0.7 mmol) in MeOH (10 mL) was added aqueous NH_3 (6 mL) and reaction mixture was stirred at 20 °C for 18 h. The solvent was evaporated and the residue was dissolved in DMF (5 mL). 1-(1*H*-Imidazol-1-ylcarbonyl)phenazine (283 mg, 1.0 mmol), prepared from 1-phenazinecarboxylic acid (**23**)[32] and CDI, was added and the mixture stirred at 20 °C for 48 h. The solvent was evaporated and the residue was purified by chromatography, eluting with a gradient (0−1%) of aqueous NH_3/(0−3%) MeOH/DCM to give compound **8** (293 mg, 82%) as a red solid: mp (DCM/hexane) 129−130 °C; ^{1}H NMR δ 10.85, (br s, 1 H, NH), 8.93 (dd, J = 7.1, 1.4 Hz, 1 H, ArH), 8.52 (br s, 1 H, NH), 8.33 (dd, J = 8.7, 1.4 Hz, 1 H, ArH), 8.21−8.27 (m, 2 H, ArH), 8.11 (d, J = 8.7 Hz, 1 H, ArH), 7.93 (dd, J = 8.6, 6.5 Hz, 1 H, ArH), 7.86−7.90 (m, 3 H, ArH), 7.70 (t, J = 7.8 Hz, 1 H,

ArH), 7.42 (t, $J = 7.8$ Hz, 1 H, ArH), 3.77 (q, $J = 6.4$ Hz, 2 H, CH_2), 3.66 (q, $J = 5.7$ Hz, 2 H, CH_2), 2.68 (t, $J = 7.3$ Hz, 2 H, CH_2), 2.62 (t, $J = 6.1$ Hz, 2 H, CH_2), 2.36 (s, 3 H, CH_3), 2.10 (quin, $J = 7.1$ Hz, 2 H, CH_2), 1.89 (quin, $J = 6.2$ Hz, 2 H, CH_2); ^{13}C NMR δ 165.0, 149.8, 143.4, 142.9, 141.4, 140.8, 138.2, 135.4, 135.1, 135.0, 133.4, 131.5, 130.9, 130.1, 130.1, 129.8, 129.0, 126.7, 121.6, 117.1, 56.7, 55.9, 42.1, 41.5, 38.2, 27.5, 25.6; HRMS calcd for $C_{27}H_{29}N_8O_3$ (MH^+) *m/z* 513.2363, found 513.2365. Anal. ($C_{27}H_{28}N_8O_3$) C, H, N.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-2-(4-pyridyl)-8-quinolinecarboxamide (9).** A solution of 2-(4-pyridyl)-8-quinolinecarboxylic acid (**24**)[35] (160 mg, 0.6 mmol) and CDI (150 mg, 0.9 mmol) in DMF (10 mL) was stirred at 55 °C for 24 h. The solution was cooled to 20 °C, diluted with dry benzene (15 mL), Sephadex LH-20 (300 mg) was added, and the mixture was stirred at 20 °C for 1 h. The mixture was filtered and the solvent evaporated. The residue was dissolved in dry THF (5 mL), a solution of amine **21** (90 mg, 0.3 mmol) in THF (5 mL) added, and the solution stirred at 20 °C for 4 days. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−2%) of aqueous NH_3/(0−8%) MeOH/DCM, to give compound **9** (160 mg, 94%) as a red powder: mp (DCM/pet. ether) 179−181 °C; ^{1}H NMR δ 11.08 (br s, 1 H, CONH), 8.86 (dd, $J = 4.5$, 1.6 Hz, 2 H, ArH), 8.78 (dd, $J = 7.4$, 1.5 Hz, 1 H, ArH), 8.37 (d, $J = 8.6$ Hz, 1 H, ArH), 8.21 (d, $J = 8.6$ Hz, 1 H, ArH), 8.10 (d, $J = 8.6$ Hz, 1 H, ArH), 7.95 (dd, $J = 8.2$, 1.4 Hz, 1 H, ArH), 7.92−7.90 (m, 4 H, NH, 3 × ArH), 7.98 (ddd, $J = 8.6$, 7.5, 1.3 Hz, 1 H, ArH), 7.66 (t, $J = 7.7$ Hz, 1 H, ArH) 7.40 (ddd, $J = 8.6$, 7.2, 1.2 Hz, 1 H, ArH), 3.74 (q, $J = 6.4$ Hz, 2 H, CH_2), 3.59−3.63 (m, 2 H, CH_2), 2.83−2.87 (m, 2 H, CH_2), 2.79−2.84 (m, 2 H, CH_2), 2.45 (s, 3 H, CH_3), 2.17 (q, $J = 7.2$ Hz, 2 H, CH_2), 1.96−2.00 (m, 2 H, CH_2); ^{13}C NMR δ 166.1, 154.5, 150.9 (2), 149.7, 146.2, 145.3, 139.0, 138.2, 135.5, 134.4, 131.5, 130.2, 129.4, 127.9, 127.2, 126.9, 121.7, 121.5, 118.7 (2), 117.2, 55.3, 55.2, 41.0, 40.1, 37.7, 26.6, 24.7; HRMS calcd for $C_{29}H_{31}N_8O_3$ (MH^+) *m/z* 539.2519, found 539.2527. Anal. ($C_{29}H_{30}N_8O_3 \cdot {}^1/_2H_2O$) C, H, N.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-4-acridinecarboxamide (10).** A solution of amine **21** in DCM (5 mL) was added to a stirred solution of 4-(1*H*-imidazol-1-ylcarbonyl)acridine (125 mg, 0.46 mmol), prepared from 4-acridinecarboxylic acid (**25**)[49] and CDI, in THF (20 mL), and the solution was stirred at 20 °C for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−1%) of Et_3N/(0−15%) MeOH/DCM, to give compound **10** (146 mg, 66%) as a red solid: mp (EtOAc/DCM) 169−171 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.41 (t, $J = 5.3$ Hz, 1 H, CONH), 9.31 (s, 1 H, H-9), 8.69 (dd, $J = 7.0$, 1 4 Hz, 1 H, H-3), 8.43 (t, $J = 5.6$ Hz, 1 H, NH), 8.38 (d, $J = 7.4$ Hz, 1 H, H-1), 8.32 (d, $J = 8.8$ Hz, 1 H, H-5), 8.21 (d, $J = 8.4$ Hz, 1 H, H-8), 8.16 (d, $J = 8.7$ Hz, 1 H, H-8′), 8.09 (d, $J = 8.7$ Hz, 1 H, H-5′), 7.96 (ddd, $J = 8.7$, 7.1, 1.1 Hz, 1 H, H-6′), 7.91 (dd, $J = 8.8$, 7.5 Hz, 1 H, H-6), 7.74 (dd, $J = 7.4$, 7.0 Hz, 1 H, H-2), 7.69 (br dd, $J = 8.7$, 7.1 Hz, 1 H, H-7′), 7.55 (dd, $J = 8.4$, 7.5 Hz, 1 H, H-7), 3.60−3.65 (m, 2 H, CH_2N), 3.42−3.48 (m, 2 H, CH_2N), 3.39 (s, 3 H, NCH_3), 3.00−3.08 (m, 2 H, CH_2N), 2.60−2.68 (m, 2 H, CH_2N), 2.02−2.08 (m, 2 H, CH_2), 1.92−1.98 (m, 2 H, CH_2); MS *m/z* 512 (MH^+, 25%), 496 (10); HRMS calcd for $C_{28}H_{30}N_7O_3$ (MH^+) *m/z* 512.2410, found 512.2424.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-9-methyl-4-phenazinecarboxamide (11).** A solution of 9-methylphenazine-4-carboxylic acid (**26**)[32] (130 mg, 0.53 mmol) and CDI (100 mg, 0.61 mmol) in DMF (5 mL) was stirred at 55 °C for 6 h. The solution was cooled to 20 °C, diluted with dry benzene (10 mL), Sephadex LH-20 (300 mg) was added, and the mixture was stirred at 20 °C for 1 h. The mixture was filtered and the solvent evaporated. The residue was dissolved in dry THF (5 mL), a solution of **21** (80 mg, 0.26 mmol) in THF (5 mL) added, and the solution stirred at 20 °C for 24 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−2%) of aqueous NH_3/(0−8%) MeOH/DCM, to give compound **11** (130 mg, 90%) as a red powder: mp (DCM/pet. ether) 138−142 °C; ^{1}H NMR δ 11.23 (br s, 1 H, CONH), 8.84 (d, $J = 6.6$ Hz, 1 H, ArH), 8.29 (d, $J = 7.6$ Hz, 1 H, ArH), 8.07 (d, $J = 8.5$ Hz, 1 H, ArH), 8.04 (d, $J = 8.5$ Hz, 1 H, ArH), 7.98 (d, $J = 8.6$ Hz, 1 H, ArH), 7.85 (t, $J = 7.8$ Hz, 1 H, ArH), 7.71−7.78 (m, 3 H, ArH, NH), 6.48 (t, $J = 7.6$ Hz, 1 H, ArH), 7.31 (t, $J = 7.7$ Hz, 1 H, ArH), 3.71−3.78 (m, 4 H, 2 × CH_2), 3.12−3.18 (m, 4 H, 2 × CH_2), 2.88 (s, 3 H, CH_3), 2.73 (br s, 3 H, CH_3), 2.30−2.36 (m, 2 H, CH_2), 2.19−2.24 (m, 2 H, CH_2); ^{13}C NMR δ 165.6, 149.6, 143.2, 142.9, 140.7, 139.4, 137.9, 136.4, 135.4, 135.1, 133.7, 131.3, 131.2, 130.1, 129.7, 128.5, 127.7, 127.0, 121.3, 116.9, 54.9, 54.2, 40.2, 38.9, 37.3, 25.7, 24.3, 18.1; HRMS calcd for $C_{28}H_{31}N_8O_3$ (MH^+) *m/z* 527.2519 found 527.2533. Anal. ($C_{28}H_{30}N_8O_3 \cdot 1^3/_4H_2O$) C, H, N.

***N*-{3-[{3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}(methyl)amino]propyl}-5-methyl-4-acridinecarboxamide (12).** A solution of 5-methylacridine-4-carboxylic acid (**27**)[30] (0.13 g, 0.55 mmol) and CDI (0.21 g, 1.3 mmol) in DMF (5 mL) was stirred at 55 °C for 24 h. The solution was diluted with dry benzene (10 mL), Sephadex LH-20 (300 mg) was added, and the mixture stirred at 20 °C for 1 h. The mixture was filtered and the solvent evaporated. The residue was dissolved in dry THF (5 mL), a solution of **21** (80 mg, 0.27 mmol) in THF (5 mL) added, and the solution stirred at 20 °C for 70 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−2%) of aqueous NH_3/(0−8%) MeOH/DCM, to give compound **12** (0.13 g, 88%) as a red powder: mp (DCM/pet. ether) 158−162 °C; ^{1}H NMR δ 12.08 (br s, 1 H, CONH), 8.83 (d, $J = 6.9$ Hz, 1 H, ArH), 8.76 (s, 1 H, NH), 8.06 (t, $J = 8.9$ Hz, 2 H, ArH), 7.97 (br d, $J = 8.4$ Hz, 2 H, ArH), 7.83 (d, $J = 8.4$ Hz, 1 H, ArH), 7.66 (d, $J = 6.7$ Hz, 1 H, ArH), 7.56−7.63 (m, 2 H, ArH), 7.46 (dd, $J = 7.6$, 6.5 Hz, 1 H, ArH), 7.30 (d, $J = 7.9$ Hz, 1 H, ArH), 3.77 (q, $J = 6.3$ Hz, 2 H, CH_2), 4.80−4.85 (m, 2 H, CH_2), 3.06−3.10 (m, 4 H, 2 × CH_2), 2.83 (s, 3 H, CH_3), 2.67 (br s, 3 H, CH_3), 2.30−2.35 (m, 2 H, CH_2), 2.13−2.17 (m, 2 H, CH_2); ^{13}C NMR δ 166.5, 149.6, 146.9, 145.1, 137.9 (2), 135.8, 135.3, 135.1, 132.4, 131.2, 130.0, 127.9, 126.8, 126.4, 126.3, 126.2, 125.8, 125.2, 121.3, 117.0, 55.1, 54.5, 40.5, 39.2, 37.4, 26.1, 24.5, 19.0; HRMS calcd for $C_{29}H_{32}N_7O_3$ (MH^+) *m/z* 526.2593, found 526.2582. Anal. ($C_{29}H_{31}N_7O_3 \cdot {}^1/_2H_2O$) C, H, N.

***N*-(2-{2-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]ethoxy}ethyl)-4-acridinecarboxamide (13). 3-{[2-(2-Hydroxyethoxy)ethyl]amino}-1,2,4-benzotriazine 1-Oxide (29).** A solution of chloride **17** (3.0 g, 16.5 mmol) in DCM (50 mL) was added to a stirred solution of 2-(aminoethoxy)ethanol (**28**) (2.49 mL, 24.8 mmol) and Et_3N (3.45 mL, 24.8 mmol) in DCM (80 mL) and the solution stirred at 20 °C for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with 40% EtOAc/DCM, to give 1-oxide **29** (2.62 g, 63%) as a yellow powder: mp (DCM/EtOAc) 131−131.5 °C; ^{1}H NMR δ 8.25 (dd, $J = 8.7$, 1.2 Hz, 1 H, H-8), 7.68 (ddd, $J = 8.4$, 7.2, 1.5 Hz, 1 H, H-6), 7.57 (d, $J = 8.4$ Hz, 1 H, H-5), 7.28 (ddd, $J = 8.7$, 7.2, 1.3 Hz, 1 H, H-7), 6.02 (br s, 1 H, NH), 3.74−3.80 (m, 6 H, 3 × CH_2O), 3.64−3.67 (m, 2 H, CH_2N), 2.71 (t, $J = 5.9$ Hz, 1 H, OH); ^{13}C NMR δ 158.9, 149.7, 135.5, 130.9, 126.4, 124.9, 120.4, 72.4, 69.5, 61.7, 41.9. Anal. ($C_{11}H_{14}N_4O_3$) C, H, N.

3-{[2-(2-Azidoethoxy)ethyl]amino}-1,2,4-benzotriazine 1-Oxide (30). Methanesulfonyl chloride (0.82 mL, 10.6 mmol) was added dropwise to a stirred solution of alcohol **29** (2.41 g, 9.6 mmol) and Et_3N (1.74 mL, 12.5 mmol) in DCM (100 mL) at 5 °C and the solution stirred at 20 °C for 1 h. The solution was diluted with DCM (100 mL), washed with water (3 × 50 mL) and brine (50 mL), and dried and the solvent evaporated. The residue was dissolved in DMF (50 mL), and NaN_3 (0.69 g, 10.6 mmol) was added. The mixture was heated at 100 °C for 2 h and cooled to 30 °C and the solvent evaporated. The residue was partitioned between EtOAc (100 mL) and water (100 mL). The organic fraction was washed with brine (50 mL) and dried and the solvent evaporated. The residue was purified by chromatography, eluting with 50% EtOAc/pet. ether, to give azide **30** (2.35 g, 89%) as yellow crystals: mp (EtOAc/pet. ether) 102−104 °C; ^{1}H NMR δ 8.27

(dd, J = 8.7, 1.4 Hz, 1 H, H-8), 7.70 (ddd, J = 8.6, 7.1, 1.5 Hz, 1 H, H-6), 7.59 (d, J = 8.6 Hz, 1 H, H-5), 7.29 (ddd, J = 8.6, 7.1, 1.4 Hz, 1 H, H-7), 5.70 (br s, 1 H, NH), 3.71−3.78 (m, 4 H, 2 × CH_2O), 3.69 (dd, J = 5.3, 4.8 Hz, 2 H, CH_2N_3), 3.41 (dd, J = 5.1, 4.9 Hz, 2 H, CH_2N); ^{13}C NMR δ 158.9, 148.7, 135.5, 131.1, 126.5, 125.0, 120.4, 70.0, 69.6, 50.7, 41.1. Anal. ($C_{11}H_{13}N_7O_2$) C, H, N.

3-{[2-(2-*tert*-Butyloxycarbamoylethoxy)ethyl]amino}-1,2,4-benzotriazine 1-Oxide (31). Propane-1,3-dithiol (5.7 mL, 57.0 mmol) was added dropwise to a stirred solution of azide **30** (1.57 g, 5.7 mmol) and Et_3N (8.0 mL, 57.0 mmol) in MeOH (100 mL) under N_2 and the solution heated at reflux temperature for 8 h. The solution was cooled to 20 °C and partitioned between 1 M HCl (100 mL) and Et_2O (100 mL). The aqueous fraction was adjusted to pH 12 with 7 M NaOH solution and extracted with DCM (3 × 50 mL). The organic fraction was dried and the solvent evaporated. The residue was dissolved in THF (100 mL), and a solution of di-*tert*-butyl dicarbonate (1.87 g, 8.6 mmol) in THF (50 mL) was added dropwise. The solution was stirred at 20 °C for 16 h, the solvent evaporated, and the residue purified by chromatography, eluting with 40% EtOAc/pet. ether, to give carbamate **31** (1.85 g, 93%) as a yellow solid: mp (EtOAc/pet. ether) 134−137 °C; ^{1}H NMR δ 8.26 (dd, J = 8.4, 0.9 Hz, 1 H, H-8), 7.71 (ddd, J = 8.3, 7.1, 1.4 Hz, 1 H, H-6), 7.59 (d, J = 8.3 Hz, 1 H, H-5), 7.29 (ddd, J = 8.4, 7.1, 1.3 Hz, 1 H, H-7), 5.74 (br s, 1 H, NH), 4.93 (br s, 1 H, NH), 3.67−3.73 (m, 4 H, 2 × CH_2O), 3.56 (t, J = 5.2 Hz, 2 H, CH_2N), 3.29−3.36 (m, 2 H, CH_2N), 1.45 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR δ 159.9, 155.9, 148.7, 135.5, 131.0, 126.5, 125.0, 120.4, 79.4, 70.2, 69.2, 41.1, 40.4, 28.4 (3). Anal. ($C_{16}H_{23}N_5O_4$) C, H, N.

3-{[2-(2-*tert*-Butyloxycarbamoylethoxy)ethyl]amino}-1,2,4-benzotriazine 1,4-Dioxide (32). A solution of MCPBA (1.57 g, 6.4 mmol) in DCM (50 mL) was added dropwise to a stirred solution of carbamate **31** (1.85 g, 5.3 mmol) in DCM (100 mL) and $NaHCO_3$ (0.89 g, 10.6 mmol), and the mixture was stirred at 20 °C for 6 h. The suspension was filtered through Celite, the solvent evaporated, and the residue purified by chromatography, eluting with a gradient (0−5%) of MeOH/(40−0%) EtOAc/DCM, to give (i) starting material **31** (926 mg, 50%) and (ii) 1,4-dioxide **32** (702 mg, 40%) as a red solid: mp (EtOAc) 139−140 °C; ^{1}H NMR δ 8.33 (d, J = 8.7 Hz, 1 H, H-8), 8.30 (d, J = 8.7 Hz, 1 H, H-5), 7.88 (ddd, J = 8.7, 7.2, 1.2 Hz, 1 H, H-6), 7.43−7.50 (m, 2 H, H-7, NH), 5.06 (br s, 1 H, NH), 3.78−3.83 (m, 2 H, CH_2O), 3.69 (dd, J = 5.1, 5.0 Hz, 2 H, CH_2O), 3.56 (dd, J = 5.1, 5.0 Hz, 2 H, CH_2N), 3.29−3.36 (m, 2 H, CH_2N), 1.43 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR δ 156.0, 149.8, 138.5, 135.9, 130.6, 129.5, 121.6, 117.4, 79.4, 70.3, 68.9, 41.3, 40.3, 28.3 (3); MS m/z 366 (MH^+, 40%), 350 (5) 310 (20); HRMS calcd for $C_{16}H_{24}N_5O_5$ (MH^+) m/z 366.1777, found 366.1767. Anal. ($C_{16}H_{23}N_5O_5 \cdot {}^1/_2H_2O$) C, H, N.

3-{[2-(2-Aminoethoxy)ethyl]amino}-1,2,4-benzotriazine 1,4-Dioxide (33). Trifluoroacetic acid (1.66 mL, 34.6 mmol) was added dropwise to a stirred solution of 1,4-dioxide **32** (632 mg, 1.7 mmol) in DCM (50 mL) and the solution stirred at 20 °C for 16 h. The solvent was evaporated and the residue partitioned between saturated aqueous $KHCO_3$ solution (100 mL) and $CHCl_3$ (100 mL). The aqueous phase was extracted with $CHCl_3$ (8 × 50 mL), the combined organic fraction dried, and the solvent evaporated. The residue was recrystallized to give the amine **33** (406 mg, 91%) as a red solid: mp ($CHCl_3$) 124 °C (dec); ^{1}H NMR δ 8.26 (d, J = 8.9 Hz, 1 H, H-8), 8.23 (d, J = 8.9 Hz, 1 H, H-5), 7.79 (dd, J = 8.8, 7.8 Hz, 1 H, H-6), 7.45 (dd, J = 8.9, 7.7 Hz, 1 H, H-7), 3.75 (dd, J = 5.0, 4.8 Hz, 2 H, CH_2O), 3.66 (dd, J = 5.0, 4.9 Hz, 2 H, CH_2O), 3.47 (dd, J = 5.1, 5.0 Hz, 2 H, CH_2N), 2.82 (dd, J = 5.1, 5.0 Hz, 2 H, CH_2N), NH and NH_2 not observed; ^{13}C NMR δ 149.8, 138.3, 135.8, 130.5, 127.2, 121.6, 117.4, 73.0, 68.9, 41.7, 41.3; MS m/z 266 (MH^+, 20%), 250 (5); HRMS calcd for $C_{11}H_{16}N_5O_3$ (MH^+) m/z 266.1253, found 266.1230. Anal. ($C_{11}H_{15}N_5O_3 \cdot {}^1/_4H_2O$) C, H, N.

***N*-(2-{2-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]-ethoxy}ethyl)-4-acridinecarboxamide (13).** A solution of the amine **33** (54 mg, 0.20 mmol) in THF (2 mL) was added dropwise to a stirred solution of 4-(1*H*-imidazol-1-ylcarbonyl)-acridine (58 mg, 0.21 mmol), prepared from 4-acridinecarboxylic acid (**25**) and CDI, in THF (5 mL) at 5 °C, and the solution was stirred at 20 °C for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−5%) of MeOH/DCM, to give compound **13** (93 mg, 97%) as a red solid: mp (EtOAc) 98−100 °C; ^{1}H NMR δ 12.14 (s, 1 H, CONH), 8.96 (dd, J = 7.1, 1.5 Hz, 1 H, H-3), 8.82 (s, 1 H, H-9), 8.25 (d, J = 8.4 Hz, 1 H, H-8′), 8.16 (d, J = 8.4 Hz, 1 H, H-5′), 8.11−8.13 (m, 2 H, H-1, H-5), 7.94 (d, J = 8.2 Hz, 1 H, H-8), 7.76−7.84 (m, 2 H, H-6, H-6′), 7.66 (dd, J = 8.4, 7.1 Hz, 1 H, H-2), 7.44−7.52 (m, 2 H, H-7, H-7′), 7.36 (br s, 1 H, NH), 3.85−3.95 (m, 8 H, 2 × CH_2O, 2 × CH_2N); ^{13}C NMR δ 166.1, 149.8, 147.2, 146.3, 138.1, 137.6, 135.5, 135.3, 132.4, 131.3, 130.4, 128.8, 128.3, 128.0, 127.1, 126.8, 126.2, 125.8, 125.4, 121.5, 117.3, 70.2, 68.9, 41.1, 39.5; MS m/z 471 (MH^+, 5%), 455 (4); HRMS calcd for $C_{25}H_{23}N_6O_4$ (MH^+) m/z 471.1781, found 471.1790. Anal. ($C_{25}H_{22}N_6O_4 \cdot {}^1/_2H_2O$) C, H, N.

***N*-{2-[[2-(1,4-Dioxido-1,2,4-benzotriazin-3-yl)ethyl]-(methyl)amino]ethyl}-4-acridinecarboxamide (14).** ***tert*-Butyl 2-[(2-Aminoethyl)(methyl)amino]ethylcarbamate (35).** A solution of di-*tert*-butyl dicarbonate (9.60 g, 44 mmol) in THF (50 mL) was added dropwise to a solution of bis-(diethylamino)methylamine (**34**) (10.32 g, 88 mmol) in THF (50 mL) at 5 °C, and the reaction mixture was stirred at 20 °C for 20 h. The reaction mixture was partitioned between DCM (200 mL) and saturated brine (100 mL). The organic layer was separated and the aqueous layer further extracted with DCM (3 × 25 mL). The combined organic extracts were dried, and the solvent was evaporated to give amine **35** (8.79 g, 46%) as a colorless oil, which was used directly without further purification.

***tert*-Butyl 2-[[2-(1-Oxido-1,2,4-benzotriazin-3-yl)ethyl]-(methyl)amino]ethylcarbamate (36).** A solution of chloride **17** (2.0 g, 11.0 mmol), amine **35** (2.90 g, 13.3 mmol), and triethylamine (3.0 mL, 22.1 mmol) in DME (20 mL) was heated at 90 °C for 3 h. The solvent was evaporated and the residue partitioned between DCM (100 mL) and aqueous NH_3 (50 mL). The aqueous layer was extracted further with DCM (3 × 30 mL), the combined organic fraction dried, and the solvent evaporated. The residue was purified by chromatography, eluting with a gradient (0−1%) of aqueous NH_3/(0−5%) MeOH/DCM, to give (i) starting material **35** (500 mg, 25%) and (ii) 1-oxide **36** (2.1 g, 52%) as a yellow solid: mp (DCM/hexane) 122−124 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.13 (dd, J = 8.6, 1.0 Hz, 1 H, H-8), 7.78 (ddd, J = 8.4, 7.0, 1.6 Hz, 1 H, H-6), 7.71 (br s, 1 H, NH), 7.52 (d, J = 8.4 Hz, 1 H, H-5), 7.33 (ddd, J = 7.8, 7.1, 1.2 Hz, 1 H, H-7), 6.61 (br s, 1 H, NH), 3.43 (q, J = 6.0 Hz, 2 H, CH_2), 3.01 (q, J = 6.2 Hz, 2 H, CH_2), 2.57 (t, J = 6.6 Hz, 2 H, CH_2), 2.42 (t, J = 6.7 Hz, 2 H, CH_2), 2.23 (s, 3 H, CH_3), 1.35 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 158.8, 155.4, 148.2, 135.6, 129.9, 126.0, 124.4, 119.8, 77.4, 56.4, 55. 5, 41.8, 38.5, 37.8, 28.1 (3); HRMS (CI$^+$) calcd for $C_{17}H_{27}N_6O_3$ (MH^+) m/z 363.2145, found 363.2144. Anal. ($C_{17}H_{26}N_6O_3$) C, H, N.

***N*^{1}-[2-(1-Oxido-1,2,4-benzotriazin-3-yl)ethyl]-N^1-methyl-1,2-ethanediamine (37).** Carbamate **36** (2.14 g, 5.9 mmol) was dissolved in methanolic HCl (30 mL) and stirred for 20 h at 20 °C. The solvent was evaporated and the residue partitioned between DCM (100 mL) and dilute aqueous NH_3 (100 mL), and the aqueous layer was further extracted with DCM (4 × 30 mL). The combined organic fraction was dried, and the solvent evaporated to give amine **37** (1.55 g, 100%) as yellow solid which was used directly without further purification: ^{1}H NMR [$(CD_3)_2SO$] δ 8.13 (dd, J = 8.6, 1.3 Hz, 1 H, H-8), 7.76−7.80 (m, 2 H, H-6, NH), 7.57 (d, J = 8.4 Hz, 1 H, H-5), 7.33 (ddd, J = 7.8, 7.1, 1.3 Hz, 1 H, H-7), 3.42−3.46 (m, 2 H, CH_2), 3.27−3.30 (m, 2 H, CH_2), 2.58 (q, J = 6.9 Hz, 2 H, CH_2), 2.37 (t, J = 6.5 Hz, 2 H, CH_2), 2.22 (s, 3 H, CH_3), 1.40−1.45 (m, 2 H, NH_2).

***N*-{2-[[2-(1-Oxido-1,2,4-benzotriazin-3-yl)ethyl](methyl)amino]ethyl}-2,2,2-trifluoroacetamide (38).** Ethyl trifluoroacetate (2.1 mL, 17.2 mmol) and water (0.3 mL, 17.2 mmol) were added to a solution of amine **37** (1.5 g, 5.7 mmol)

in CH_3CN (50 mL), and the reaction mixture was heated at reflux temperature for 48 h. The solvent was evaporated and the residue partitioned between DCM (100 mL) and aqueous $NaHCO_3$ solution (100 mL). The aqueous layer was further extracted with DCM (3 × 30 mL), the combined organic fraction dried, and the solvent evaporated to give trifluoroacetamide **38** (1.80 g, 88%) as a yellow solid: mp (DCM/hexane) 141−143 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.29 (br s, 1 H, NH), 8.13 (dd, J = 8.6, 1.3 Hz, 1 H, H-8), 7.78 (ddd, J = 8.4, 7.0, 1.5 Hz, 1 H, H-6), 7.68 (br s, 1 H, NH), 7.57 (d, J = 8.4 Hz, 1 H, H-5), 7.33 (ddd, J = 8.5, 7.1, 1.3 Hz, 1 H, H-7), 3.42 (q, J = 6.3 Hz, 2 H, CH_2), 3.30 (q, J = 6.8 Hz, 2 H, CH_2), 2.61 (t, J = 6.7 Hz, 2 H, CH_2), 2.56 (t, J = 6.7 Hz, 2 H, CH_2), 2.27 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 158.8, 156.1 (q, J = 36 Hz) 148.3, 135.6, 130.0, 125.9, 124.4, 119.8, 115.8 (q, J = 288 Hz), 55.4, 55.1, 41.7, 38.4, 37.2; HRMS calcd for $C_{14}H_{18}F_3N_6O_2$ (MH^+) m/z 359.1443, found 359.1451. Anal. ($C_{14}H_{17}F_3N_6O_2$) C, H, N, F.

***N*-{2-[[2-(1,4-Dioxido-1,2,4-benzotriazin-3-yl)ethyl](methyl)amino]ethyl}-2,2,2-trifluoroacetamide (39).** Hydrogen peroxide (70%, 2.0 mL, 48.8 mmol) was added dropwise to a stirred solution of trifluoroacetic anhydride (6.78 mL, 48.8 mmol) in DCM (20 mL) at 5 °C and the solution stirred at 5 °C for 15 min. The solution was added to a solution of trifluoroacetamide **38** (1.75 g, 4.9 mmol) and trifluoroacetic acid (0.80 mL, 9.8 mmol) in DCM (20 mL) and the reaction mixture stirred at 20 °C for 5 h. The reaction mixture was slowly added to a cooled solution of aqueous $NaHCO_3$ (100 mL). The organic layer was separated and the aqueous layer was further extracted with DCM (5 × 30 mL). The combined organic fraction was dried, the solvent was evaporated, and the residue was purified by chromatography, eluting with a gradient (0−4%) of MeOH/DCM, to give (i) starting material **38** (100 mg, 6%) and (ii) 1,4-dioxide **39** (859 mg, 47%) as a red solid: mp (DCM/hexane) 141−144 °C; ^{1}H NMR [$(CD_3)SO$] δ 9.28 (br s, 1 H, NH), 8.20 (d, J = 9.1 Hz, 1 H, H-5), 8.12 (d, J = 8.6 Hz, 1 H, H-8), 8.03 (t, J = 5.8 Hz, 1 H, NH), 7.96 (ddd, J = 8.6, 7.2, 1.3 Hz, 1 H, H-6), 7.56 (ddd, J = 8.6, 7.1, 1.3 Hz, 1 H, H-7), 3.48 (q, J = 6.3 Hz, 2 H, CH_2), 3.31 (q, J = 6.3 Hz, 2 H, CH_2), 2.63, (t, J = 6.6 Hz, 2 H, CH_2), 2.54 (t, J = 6.8 Hz, 2 H, CH_2), 2.27 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 156.1 (q, J = 36 Hz), 149.7, 138.0, 135.4, 129.9, 126.9, 121.0, 115.8 (q, J = 288 Hz), 116.7, 55.4, 55.0, 41.6, 38.3, 37.1; HRMS calcd for $C_{14}H_{17}F_3N_6O_3$ (MH^+) m/z 375.1393, found 375.1392. Anal. ($C_{14}H_{17}F_3N_6O_3$) C, H, N.

***N*-{2-[[2-(1,4-Dioxido-1,2,4-benzotriazin-3-yl)ethyl](methyl)amino]ethyl}-4-acridinecarboxamide (14).** Aqueous NH_3 (6 mL) was added to a solution of dioxide **39** (125 mg, 0.33 mmol) in MeOH (6 mL) and the reaction mixture stirred at 20 °C for 16 h. The solvent was evaporated, the residue was dissolved in THF (5 mL) and 4-(1*H*-imidazol-1-ylcarbonyl)acridine (180 mg, 0.66 mmol), prepared from 4-acridinecarboxylic acid (**25**) and CDI, and the reaction was stirred at 20 °C for 48 h. The solvent was evaporated, and the residue was dissolved in DCM (20 mL) and washed with water (3 × 15 mL). The organic fraction was dried, the solvent evaporated, and the residue purified by chromatography, eluting with a gradient (0−1%) of aqueous NH_3/(0−3%) MeOH/DCM, to give compound **14** (132 mg, 94%) as a red solid: mp (DCM/hexane) 160−162 °C; ^{1}H NMR δ 11.90 (br s, 1 H, NH), 8.95 (dd, J = 7.1, 1.5 Hz, 1 H, ArH), 8.72 (s, 1 H, ArH), 8.16 (d, J = 8.6 Hz, 1 H, ArH), 8.12 (d, J = 8.8 Hz, 1 H, ArH), 8.07 (dd, J = 8.4, 1.4 Hz, 1 H, ArH), 7.84 (t, J = 8.3 Hz, 2 H, ArH), 7.78 (ddd, J = 7.7, 6.7, 1.5 Hz, 1 H, ArH), 7.70 (ddd, J = 7.8, 7.1, 1.3 Hz, 1 H, ArH), 7.64 (dd, J = 8.2, 7.1 Hz, 1 H, ArH), 7.46 (ddd, J = 7.5, 7.2, 0.7 Hz, 1 H, ArH), 7.40 (ddd, J = 7.9, 7.1, 1.3 Hz, 1 H, ArH), 7.29 (br, 1 H, NH), 3.86 (q, J = 6.0 Hz, 2 H, CH_2), 3.70 (q, J = 5.6 Hz, 2 H, CH_2), 2.93 (t, J = 6.4 Hz, 2 H, CH_2), 2.89 (t, J = 6.1 Hz, 2 H, CH_2), 2.57 (s, 3 H, CH_3); ^{13}C NMR δ 166.0, 149.7, 147.4, 146.5, 137.8, 137.4, 135.3, 135.0, 132.1, 131.1, 130.1, 128.8, 128.6, 127.9, 126.8, 126.7, 126.1, 125.8, 125.5, 121.5, 117.2, 56.5, 55.9, 42.5, 39.1, 37.8; HRMS calcd for $C_{26}H_{26}N_7O_3$ (MH^+) m/z 484.2097, found 484.2102.

***N*-[2-({2-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]ethyl}amino)ethyl]-4-acridinecarboxamide (15). *tert*-Butyl Bis-(2-aminoethyl)carbamate (41).** Diethylenetriamine **40** (9.9 mL, 96 mmol) was added to a solution of ethyl trifluoroacetate (22.8 mL, 192 mmol) in dry ether (80 mL) at 5 °C, and the reaction mixture was stirred at 20 °C for 20 h. The resulting white precipitate was filtered, washed with cold ether (100 mL), and dried under vacuum to give 2,2,2-trifluoro-*N*-[2-({2-[(trifluoroacetyl)amino]ethyl}amino)ethyl]-acetamide (17.26 g, 61%): ^{1}H NMR [$(CD_3)_2SO$] δ 7.26 (br s, 2 H, 2 × CONH), 3.43 (br s, 4 H, 2 × CH_2), 2.86 (br t, J = 5.8 Hz, 4 H, 2 × CH_2); ^{13}C NMR [$(CD_3)_2SO$] δ 157.7 (q, J = 37 Hz), 115.8 (q, J = 288 Hz), 47.3 (2), 39.3 (2).

Di-*tert* butyl dicarbonate (8.26 g, 37.8 mmol) was added to a solution of acetamide (10.15 g, 34.4 mmol) in THF (100 mL) at 0 °C, and the mixture was stirred at 20 °C for 20 h. Saturated aqueous NH_4Cl (80 mL) was added and the mixture stirred at 20 °C for 5 h. The mixture was extracted with DCM (3 × 50 mL) and dried, and the solvent was evaporated to give *tert*-butyl bis{2-[(trifluoroacetyl)amino]ethyl}carbamate (13.5 g, 100%), which was used without further purification: ^{1}H NMR [$(CD_3)_2SO$] δ 9.47 (br s, 1 H, CONH), 9.40 (br s, 1 H, CONH), 3.27−3.33 (m, 8 H, 4 × CH_2), 1.38 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 156.4 (q, J = 36 Hz), 154.7, 115.8 (q, J = 288 Hz), 78.9, 45.4, 45.0, 37.7, 37.4, 27.7 (3).

Aqueous NH_3 (50 mL) was added to a solution of carbamate (14.0 g, 35.5 mmol) in MeOH (100 mL) and heated at reflux temperature for 20 h. The solvent was evaporated to give diamine **41** as a yellow foam: ^{1}H NMR [$(CD_3)_2SO$] δ 3.39 (t, J = 6.4 Hz, 4 H, 2 × CH_2), 2.94 (t, J = 6.4 Hz, 4 H, 2 × CH_2), 1.42 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 154.9, 79.9, 45.1 (2), 37.4 (2), 27.9 (3).

Di-*tert*-butyl 2-Aminoethyl{2-[(1-oxido-1,2,4-benzotriazin-3-yl)amino]ethyl}dicarbamate (42). A solution of chloride **17** (1.0 g, 5.5 mmol), diamine **41** (4.47 g, 22.0 mmol), and Et_3N (3.07 mL, 22.0 mmol) in DME (20 mL) was heated at 90 °C for 3 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−4%) of MeOH/DCM, to give (i) *tert*-butyl bis{2-[(1-oxido-1,2,4-benzotriazin-3-yl)amino]ethyl}carbamate (0.34 g, 25%) [^{1}H NMR δ 8.17 (br d, J = 8.5 Hz, 2 H, H-8, H-8′), 7.62−7.71 (m, 2 H, H-6, H-6′), 7.52 (br d, J = 8.3 Hz, 2 H, H-5, H-5′), 7.22−7.26 (m, 2 H, H-7, H-7′), 6.15 (br s, 1 H, NH), 5.95 (br s, 1 H, NH), 3.71 (q, J = 5.8 Hz, 4 H, 2 × CH_2), 3.37 (br s, 4 H, 2 × CH_2), 1.50 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR δ 158.9, 156.3 (2), 148.6 (2), 135.5 (2), 130.9 (2), 126.4 (2), 124.9 (2), 120.3 (2), 80.7, 47.7 (2), 40.9 (2), 28.4 (3).] and (ii) crude *tert*-butyl 2-aminoethyl{2-[(1-oxido-1,2,4-benzotriazin-3-yl)amino]ethyl}-carbamate (1.38 g, 72%) as a yellow foam.

Di-*tert*-butyl dicarbonate (2.7 g, 12.4 mmol) was added to a solution of the crude carbamate (1.38 g, 4.0 mmol) in THF (50 mL) and the solution stirred at 20 °C for 36 h. Water (100 mL) was added and the mixture stirred at 20 °C for 1 h. The mixture was extracted with DCM (3 × 50 mL), the organic fraction dried, and the solvent evaporated. The residue was purified by chromatography, eluting with a gradient (0−1%) of aqueous NH_3/(0−7%) MeOH/DCM, to give carbamate **42** (0.94 g, 52%) as a yellow powder: mp (DCM/hexane) 160−163 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.14 (dd, J = 8.3, 0.7 Hz, 1 H, H-8), 8.00 and 7.92 (2 × br s, 1 H, CONH), 7.79 (dd, J = 7.5, 1.2 Hz, 1 H, H-6), 7.58 (br d, J = 7.5 Hz, 1 H, H-5), 7.34 (dd, J = 7.7, 1.2 Hz, 1 H, H-7), 6.81 (br s, 1 H, NH), 3.43−3.47 (m, 2 H, CH_2), 3.37 (m, 2 H, CH_2), 3.22 (t, J = 6.2 Hz, 2 H, CH_2), 3.03−3.07 (m, 2 H, CH_2), 1.34 [s, 9 H, $C(CH_3)_3$], 1.34 and 1.27 [2 × s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 158.9, 155.5, 154.7, 148.2, 135.7, 130.1, 126.0, 124.6, 119.8, 78.4, 77.5, 47.0, 46.2, 38.5, 38.1, 28.1 (3), 27.8 (3). Anal. ($C_{21}H_{32}N_6O_5$) C, H, N.

Di-*tert*-butyl 2-Aminoethyl{2-[(1,4-dioxido-1,2,4-benzotriazin-3-yl)amino]ethyl}dicarbamate (43). MCPBA (247 mg, 1.0 mmol) was added to a solution of 1-oxide **42** (300 mg, 0.67 mmol) in DCM (10 mL) and the mixture was stirred at 20 °C for 16 h. The mixture was partitioned between dilute aqueous NH_3 (50 mL) and DCM (50 mL), and the aqueous fraction was extracted with DCM (3 × 30 mL). The combined

organic fraction was dried and the solvent evaporated. The residue was purified by chromatography, eluting with a gradient (0−2%) of MeOH/DCM, to give (i) starting material **42** (188 mg, 62%) and (ii) 1,4-dioxide **43** (122 mg, 39%): mp (DCM/hexane) 128−134 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.39 and 8.32 (2 × br s, 1 H, CONH), 8.21 (dd, J = 8.7, 0.7 Hz, 1 H, H-8), 8.14 (t, J = 8.0 Hz, 1 H, H-5), 7.94 (t, J = 7.6 Hz, 1 H, H-6), 7.57 (t, J = 7.9 Hz, 1 H, H-7), 6.77 (br s, 1 H, NH), 3.50−3.54 (m, 2 H, CH_2), 3.41−3.44 (m, 2 H, CH_2), 3.20 (t, J = 6.5 Hz, 2 H, CH_2), 3.03 (q, J = 5.6 Hz, 2 H, CH_2), 1.33 [s, 9 H, $C(CH_3)_3$], 1.33 and 1.26 [2 × s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 155.5, 154.6, 149.8, 138.1, 135.5, 129.9, 127.0, 121.0, 116.8, 78.6, 77.4, 46.8, 46.1, 38.5, 38.0, 28.8 (3), 27.7 (3); HRMS calcd for $C_{21}H_{33}H_6O_6$ (MH^+) m/z 465.2462, found 465.2456.

***N*-[2-({2-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]ethyl}amino)ethyl]-4-acridinecarboxamide (15).** A solution of carbamate **43** (252 mg, 0.54 mmol) in HCl saturated MeOH (10 mL) was stirred at 20 °C for 24 h. The solvent was evaporated and the residue partitioned between aqueous NH_3 (20 mL) and DCM (50 mL). The aqueous fraction was extracted with DCM (5 × 20 mL) and the combined organic extract dried. The solvent was evaporated to give crude N^1-(2-aminoethyl)-N^2-(1,4-dioxido-1,2,4-benzotriazin-3-yl)-1,2-ethanediamine (109 mg, 76%): ^{1}H NMR δ 8.33 (d, J = 8.7 Hz, 1 H, H-8), 8.30 (d, J = 8.8 Hz, 1 H, H-5), 7.87 (ddd, J = 8.5, 7.1, 1.0 Hz, 1 H, H-6), 7.50 (ddd, J = 8.4, 7.1, 1.2 Hz, 1 H, H-7), 3.70 (t, J = 5.9 Hz, 2 H, CH_2), 2.98 (t, J = 5.9 H, 2 H, CH_2), 2.84 (t, J = 5.6 Hz, 2 H, CH_2), 2.74 (t, J = 5.6 Hz, 2 H, CH_2), 2 × NH and NH_2 not observed.

A solution of amine (96 mg, 0.36 mmol) and 4-(1*H*-imidazol-1-ylcarbonyl)acridine (119 mg, 0.43 mmol), prepared from 4-acridinecarboxylic acid (**25**) and CDI, in DMF (5 mL) was stirred at 20 °C for 5 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−2%) of aqueous NH_3/(0−7%) MeOH/DCM, to give compound **15** (168 mg, 99%) as a red solid: mp (DCM/hexane) 151−154 °C; ^{1}H NMR δ 11.98 (t, J = 5.3 Hz, 1 H, CONH), 8.82 (dd, J = 8.2, 1.4 Hz, 1 H, ArH), 8.81 (s, 1 H, ArH), 8.08−8.17 (m, 4 H, 4 × ArH), 7.97 (d, J = 8.3 Hz, 1 H, ArH), 7.75−7.82 (m, 2 H, 2 × ArH), 7.60 (dd, J = 8.2, 7.2 Hz, 1 H, ArH), 7.54 (ddd, J = 8.3, 7.3, 0.9 Hz, 1 H, ArH), 7.40 (ddd, J = 8.6, 7.2, 1.2 Hz, 1 H, ArH), 3.86 (q, J = 5.8 Hz, 2 H, CH_2), 3.70 (t, J = 5.8 Hz, 2 H, CH_2), 3.17 (q, J = 6.3 Hz, 4 H, 2 × CH_2), 2 × NH not observed; ^{13}C NMR δ 166.5, 149.7, 147.4, 146.2, 138.0, 137.7, 135.6, 135.3, 132.5, 131.4, 130.2, 128.9, 128.0, 126.9 (2), 126.7, 126.3, 125.9, 125.4, 121.5, 117.2, 48.6, 47.8, 40.6, 39.3; HRMS calcd for $C_{25}H_{24}N_7O_3$ (MH^+) m/z 470.1941, found 470.1934. Anal. ($C_{25}H_{23}N_7O_3 \cdot 1^1/_2H_2O$) C, H, N.

***N*-[3-({3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}amino)propyl]-4-acridinecarboxamide (16).** ***tert*-Butyl 3-[(1-Oxido-1,2,4-benzotriazin-3-yl)amino]propyl-{3-[(trifluoroacetyl)amino]propyl}carbamate (45).** A solution of chloride **17** (1.34 g, 7.41 mmol) in DCM (50 mL) was added dropwise to a stirred solution of *tert*-butyl bis(3-aminopropyl)carbamate **44** (2.57 g, 11.1 mmol) and Et_3N (1.55 mL, 11.1 mmol) in DCM (200 mL) at 20 °C. The solution was stirred at 20 °C for 3 days. The solvent was evaporated and the residue purified by chromatography, eluting with 50% EtOAc/acetone, to give a crude oil. Trifluoroacetic anhydride (3.5 mL, 24.3 mmol) was added dropwise to a stirred solution of crude amine in pyridine (50 mL) at 5 °C and the solution was stirred at 20 °C for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (30−50%) of EtOAc/pet. ether, to give trifluoroacetamide **45** (0.51 g, 22%) as a yellow solid: mp (EtOAc/pet. ether) 89−90 °C; ^{1}H NMR δ 8.22−8.26 (m, 2 H, H-8, NH), 7.71 (br dd, J = 8.4, 7.0 Hz, 1 H, H-6), 7.59 (d, J = 8.4 Hz, 1 H, H-5), 7.29 (br dd, J = 8.5, 7.0 Hz, 1 H, H-7), 5.45 (br s, 1 H, NH), 4.12 (br dd, J = 6.6, 6.5 Hz, 2 H, CH_2N), 3.26−3.37 (m, 6 H, 3 × CH_2N), 1.84−1.95 (m, 2 H, CH_2), 1.71−1.77 (m, 2 H, CH_2), 1.48 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR δ 158.9, 157.3 (q, J = 37 Hz), 156.8, 148.0, 135.6, 130.9, 126.5, 125.1, 120.4, 116 (q, J = 288 Hz), 80.8, 44.5, 43.0, 38.8, 35.8, 29.7, 28.3 (3), 27.1; MS m/z 473 (MH^+, 60%), 457 (10), 373 (100); HRMS calcd for $C_{20}H_{28}F_3N_6O_4$ (MH^+) m/z 473.2124, found 473.2136. Anal. ($C_{20}H_{27}F_3N_6O_4$) C, H, N.

***tert*-Butyl 3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl{3-[(trifluoroacetyl)amino]propyl}carbamate (46).** A solution of MCPBA (2.12 g, 8.6 mmol) in DCM (50 mL) was added dropwise to a stirred solution of 1-oxide **45** (3.13 g, 6.6 mmol) in DCM (250 mL) and $NaHCO_3$ (1.1 g, 13.2 mmol). The mixture was stirred at 20 °C for 16 h and partitioned between DCM (200 mL) and saturated aqueous $KHCO_3$ solution (100 mL). The organic fraction was dried, the solvent evaporated, and the residue purified by chromatography, eluting with a gradient (0−4%) of MeOH/40% EtOAc/DCM, to give (i) starting material **45** (2.04 g, 65%) and (ii) 1,4-dioxide **46** (252 mg, 8%) as a red solid: ^{1}H NMR δ 8.34 (d, J = 8.7 Hz, 1 H, H-8), 8.30 (d, J = 8.4 Hz, 1 H, H-5), 8.25 (br s, 1 H, NH), 7.88 (br dd, J = 8.4, 7.0 Hz, 1 H, H-6), 7.52 (br dd, J = 8.7, 7.0 Hz, 1 H, H-7), 7.20 (br s, 1 H, NH), 3.62 (dt, J = 6.8, 6.7 Hz, 2 H, CH_2N), 3.26−3.38 (m, 6 H, 3 × CH_2N), 1.92−1.98 (m, 2 H, CH_2), 1.73−1.79 (m, 2 H, CH_2), 1.49 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR δ 157.3 (q, J = 37 Hz), 156.8, 149.8, 138.2, 135.9, 130.5, 127.4, 121.7, 117.4, 116.1 (q, J = 288 Hz), 80.9, 44.4, 43.2, 38.9, 31.9, 29.7, 28.4 (3), 22.7; MS m/z 489 (MH^+, 10%), 473 (12), 373 (15); HRMS calcd for $C_{20}H_{28}F_3N_6O_5$ (MH^+) m/z 489.2073, found 489.2086.

***tert*-Butyl 3-Aminopropyl{3-[(1,4-dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}carbamate (47).** A mixture of trifluoroacetamide **46** (541 mg, 1.1 mmol) and K_2CO_3 (0.77 g, 5.5 mmol) in MeOH (20 mL) and water (5 mL) was heated at reflux temperature for 1 h. The mixture was partitioned between $CHCl_3$ (50 mL) and water (30 mL). The aqueous fraction was extracted with $CHCl_3$ (3 × 30 mL), the combined organic fraction dried, and the solvent evaporated to give amine **47** (322 mg, 74%) as a red oil: ^{1}H NMR [$(CD_3)_2SO$] δ 10.50 (br s, 1 H, NH), 8.21 (d, J = 8.7 Hz, 1 H, H-8), 8.13 (d, J = 8.6 Hz, 1 H, H-5), 7.94 (br dd, J = 8.6, 7.5 Hz, 1 H, H-6), 7.56 (br dd, J = 8.6, 7.5 Hz, 1 H, H-7), 7.20 (br s, 2 H, NH_2), 3.39 (t, J = 6.9 Hz, 2 H, CH_2N), 3.11−3.21 (m, 6 H, 3 × CH_2N), 1.78−1.86 (m, 2 H, CH_2), 1.49−1.58 (m, 2 H, CH_2), 1.39 [s, 9 H, $C(CH_3)_3$]; ^{13}C NMR [$(CD_3)_2SO$] δ 154.7, 149.7, 138.1, 135.4, 129.8, 127.9, 121.0, 116.7, 78.3, 44.3, 43.9, 38.8, 38.4, 32.2, 31.6, 27.9 (3); MS m/z 393 (MH^+, 15%), 377 (9), 338 (3); HRMS calcd for $C_{18}H_{29}N_6O_4$ (MH^+) m/z 393.2250, found 393.2249.

***N*-[3-({3-[(1,4-Dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}amino)propyl]-4-acridinecarboxamide (16).** A solution of 4-acridinecarboxylic acid **25** (846 mg, 4.35 mmol) and CDI (846 mg, 5.21 mmol) in DMF (20 mL) was stirred at 50 °C for 1 h. The solvent was evaporated and the residue recrystallized from DCM/pet. ether to give 4-(1*H*-imidazol-1-ylcarbonyl)acridine (746 mg, 63%), which was used directly without characterization. A solution of the amine **47** (320 mg, 0.82 mmol) in DCM (10 mL) was added dropwise to a stirred solution of imidazolide (234 mg, 0.86 mmol) in THF (25 mL) at 5 °C and the solution was stirred at 20 °C for 16 h. The solvent was evaporated and the residue purified by chromatography, eluting with a gradient (0−5%) of MeOH/DCM, to give *tert*-butyl 3-[(4-acridinylcarbonyl)amino]propyl{3-[(1,4-dioxido-1,2,4-benzotriazin-3-yl)amino]propyl}carbamate (330 mg, 67%) as a red gum: ^{1}H NMR δ 11.92 (br s, 1 H, CONH), 8.98 (dd, J = 7.2, 1.5 Hz, 1 H, H-3), 8.89 (s, 1 H, H-9), 8.26−8.32 (m, 3 H, H-5, H-5′, H-8′), 8.16 (d, J = 8.3 Hz, 1 H, H-1), 8.07 (d, J = 8.8 Hz, 1 H, H-8), 7.82−7.89 (m, 3 H, H-3, H-6, H-6′), 7.65−7.69 (m, 1 H, H-7′), 7.58−7.62 (m, 1 H, H-7), 7.48 (br s, 1 H, NH), 3.72 (dt, J = 6.6, 6.0 Hz, 2 H, CH_2N), 3.61 (dt, J = 6.6, 6.4 Hz, 2 H, CH_2N), 3.38−3.50 (m, 4 H, 2 × CH_2N), 2.04−2.08 (m, 2 H, CH_2), 1.88−1.94 (m, 2 H, CH_2), 1.40 [s, 9 H, $C(CH_3)_3$]; MS m/z 598 (MH^+, 8%), 582 (6); HRMS calcd for $C_{32}H_{36}N_7O_5$ (MH^+) m/z 598.2778, found 598.2772.

HCl-saturated MeOH (30 mL) was added to a solution of the carbamate (328 mg, 0.55 mmol) in MeOH (30 mL) and the solution stirred at 20 °C for 16 h. The solution was evaporated, the residue dissolved in water (20 mL), and the solution neutralized with $KHCO_3$ and extracted with $CHCl_3$ (5 × 50 mL). The combined organic fraction was dried and the solvent

evaporated to give compound **16** (247 mg, 90%) as a red solid: ^{1}H NMR [$(CD_3)_2SO$] δ 11.38 (t, J = 5.5 Hz, 1 H, CONH), 10.50 (br s, 1 H, NH), 9.28 (s, 1 H, H-9), 8.71 (dd, J = 7.1, 1.5 Hz, 1 H, H-3), 8.35 (dd, J = 8.4, 1.5 Hz, 1 H, H-1), 8.24 (d, J = 8.7 Hz, 1 H, H-5), 8.19 (d, J = 8.3 Hz, 1 H, H-8), 8.14 (d, J = 8.5 Hz, 1 H, H-8′), 8.03 (d, J = 8.5 Hz, 1 H, H-5′), 7.92−7.96 (m, 1 H, H-6), 7.83−7.88 (m, 1 H, H-6′), 7.75 (dd, J = 8.3, 7.1 Hz, 1 H, H-2), 7.65−7.68 (m, 1 H, H-7), 7.48−7.54 (m, 1 H, H-7′), 7.38 (s, 1 H, NH), 3.64 (dt, J = 6.9, 5.9 Hz, 2 H, CH_2N), 3.46 (t, J = 6.7 Hz, 2 H, CH_2N), 2.79 (t, J = 6.9 Hz, 2 H, CH_2N), 2.70 (t, J = 6.5 Hz, 2 H, CH_2N), 1.88−1.94 (m, 2 H, CH_2), 1.76−1.82 (m, 2 H, CH_2); ^{13}C NMR [$(CD_3)_2SO$] δ 164.7, 149.6, 147.0, 145.4, 138.5, 138.0, 135.3, 134.4, 132.6, 131.8, 129.7, 128.5, 128.4, 128.3, 126.7, 126.4, 126.3, 125.5, 125.2, 121.0, 116.7, 47.1, 46.9, 39.6, 37.2, 29.3, 28.2; MS m/z 498 (MH^+, 15%), 482 (5); HRMS calcd for $C_{27}H_{28}N_7O_3$ (MH^+) m/z 498.2254, found 498.2258. Anal. ($C_{27}H_{27}N_7O_3 \cdot 2H_2O$) C, H; N, calcd 18.4, found 17.1%.

Partition Coefficients. The octanol−water partition coefficient (P) was measured using the shake flask method,[56] using GPR-grade octanol (BDH Laboratory Supplies). Briefly, lipophilic drugs were dissolved directly in octanol-saturated PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, pH 7.4) and hydrophilic drugs in PBS-saturated octanol, to 25−100 μM. Equal volumes of PBS and octanol were mixed on a Bellco roller drum (Bellco Glass, Inc., New Jersey) at 20 rpm for 3 h at ambient temperature. The two solvent layers were separated after a brief spin and analyzed by HPLC directly (aqueous layer), or after addition of 4 volumes of methanol (organic layer).

DNA Binding Assay. DNA binding was measured by equilibrium dialysis using a Dianorm dialyzer (Diachema AG, Rüschlikon-Zürich, Switzerland) with 20 two-cell dialysis chambers (1 mL each) as described previously.[56] Solutions of BTO (15 μM) with calf thymus DNA (Sigma type I) (25−1000 μM in base pairs) in KHE buffer (10 mM KCl, 2 mM NaHEPES, 10 μM Na_2EDTA, pH 7.0) were dialyzed (2 h at 37 °C) against free BTO (15 μM) in KHE buffer using a Cuprophan dialysis membrane (Medicell International Ltd., London, UK) with a MW cutoff of 10 000 Da. After equilibrium was reached, the contents of the dialysis chambers with KHE buffer were collected and analyzed directly by HPLC. To the samples containing DNA, 9 volumes of ice-cold ethanol were added and the mixture was kept on ice for 1 h followed by a brief spin to precipitate the DNA. Subsequently, the volume of the aqueous ethanol was reduced using a Speed-Vac concentrator and reconstituted with mobile phase followed by injection onto an HPLC. BTO concentrations were calculated using TPZ as internal standard, and the equilibrium association constants (K_{DNA}) and standard errors were obtained from the best fit obtained by nonlinear regression analysis of direct plots of the ratio of drug bound/base pair versus free drug concentration[47,59] using the neighboring site exclusion model.[65]

Clonogenic Assays. Clonogenic survival of mouse SCCVII tumor cells in vitro was determined for 1 h drug exposure of cells under aerobic and hypoxic conditions as previously described.[57] Hypoxic C_{10} values are corrected for interexperimental variation in hypoxia by using TPZ as an internal standard: C_{10}(BTO,calc) = C_{10}(BTO,meas) × [C_{10}(TPZ,mean)/C_{10}(TPZ,meas)].

IC_{50} Assays. IC_{50} assays were determined for BTOs under aerobic and hypoxic conditions as previously described.[57] For each experiment, compounds were simultaneously tested under both oxic and hypoxic conditions against the HT-29 cell line and included TPZ as an independent internal control at the front and back of the assay (n = 2). Final data were pooled from a series of seven independent experiments and are calculated using interexperimental means. In all cases, 8-methyl-5-nitroquinoline was used as a second internal control to confirm that strict hypoxia was present during the experiment.[66] Plates were stained as described previously[67] and IC_{50} values determined. Results were averaged for two to six independent experiments for each compound.

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