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Hypoxic selectivity and solubility—investigating the properties of A-ring substituted nitro *seco*-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-ones (nitroCBIs) as hypoxia-activated prodrugs for antitumor therapy

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ABSTRACT

Nitro *seco*-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-ones (nitroCBIs) are a new class of prodrugs for antitumor therapy that undergo hypoxia-selective metabolism to form potent DNA minor groove alkylating agents. Although hindered by poor aqueous solubility, several examples have shown activity against hypoxic tumor cells in vivo. Here we investigate structural properties that influence hypoxic selectivity in vitro, and show that for high hypoxic selectivity nitroCBIs should combine an electron-withdrawing group of H-bond donor capacity on the A-ring, with a basic substituent on the minor groove-binding side chain. Substitution on the A-ring is compatible with the introduction of functionality that can improve water solubility.

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1. Introduction

We recently reported[1] the first examples of a new class of hypoxia-activated prodrugs (HAPs), compounds that are designed to be selectively toxic to cells at low oxygen concentrations. Since hypoxia is rarely found in normal tissues, but is a common occurrence in solid tumors where it contributes to treatment failure, HAPs represent a promising avenue to selective tumor therapy.[2–4] Several examples of HAPs, relying on either bioreduction of an *N*-oxide or nitro group to achieve the hypoxia-selective step, are in current or recent clinical trial.[5–8] Our new class, termed nitroCBIs[1] (nitro *seco*-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-ones, **1**), also utilizes a nitro prodrug strategy (Fig. 1), but is unique in that the reduction product (the aminoCBI, **2**) is a highly potent DNA minor groove alkylating agent,[9,10] structurally and functionally related to a small family of naturally occurring antitumor antibiotics.[11,12] The prototype nitroCBI examples **3** and **4** (bearing respectively the 5,6,7-trimethoxyindole (TMI) side chain found in the duocarmycins,[11] or a basic 5-[(dimethylamino)ethoxy]indole (DEI) side chain) were found to be up to 300-fold more cytotoxic to cultured tumor cells under hypoxic compared to aerobic conditions, and provided selective killing of hypoxic cells in murine RIF-1 tumors in vivo.[1] However the rate of reduction under hypoxic conditions appeared to be quite low, and high hypoxic cytotoxicity ratios (HCRs) were only achieved in some cell lines.

To address this issue we investigated analogues of **3** and **4** in which the A-ring bears an electron-withdrawing group (EWG) designed to raise the one-electron reduction potential, $E(1)$.[13] A subset of compounds was identified, those with the DEI side chain and a primary sulfonamide or carboxamide A-ring substituent (particularly in the 7- or 8-position) that generated high HCRs in each cell line examined. For this subset there was a correlation between HCR and $E(1)$, with the highest hypoxic selectivity being observed for the substituent which generated the highest one-electron reduction potential. The most promising analogue **5** displayed HCRs of 19-330 across an 11-cell line panel, was shown to be efficiently and selectively metabolized to the corresponding aminoCBI, and demonstrated activity against hypoxic tumor cells in a human tumor xenograft in vivo.[13] In vitro comparison to the established HAPs tirapazamine[5] and PR-104A[6] showed that **5** exhibited similar levels of hypoxic selectivity but was several hundred-fold more potent than either agent under hypoxic conditions.[13]

Abbreviations: AUC, area under the concentration–time curve; CBI, *seco*-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one; DEI, 5-[(dimethylamino)ethoxy]-indole; EDCI, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; EWG, electron-withdrawing group; HAP, hypoxia-activated prodrug; HCR, hypoxic cytotoxicity ratio; MTD, maximum tolerated dose; TMI, 5,6,7-trimethoxyindole.

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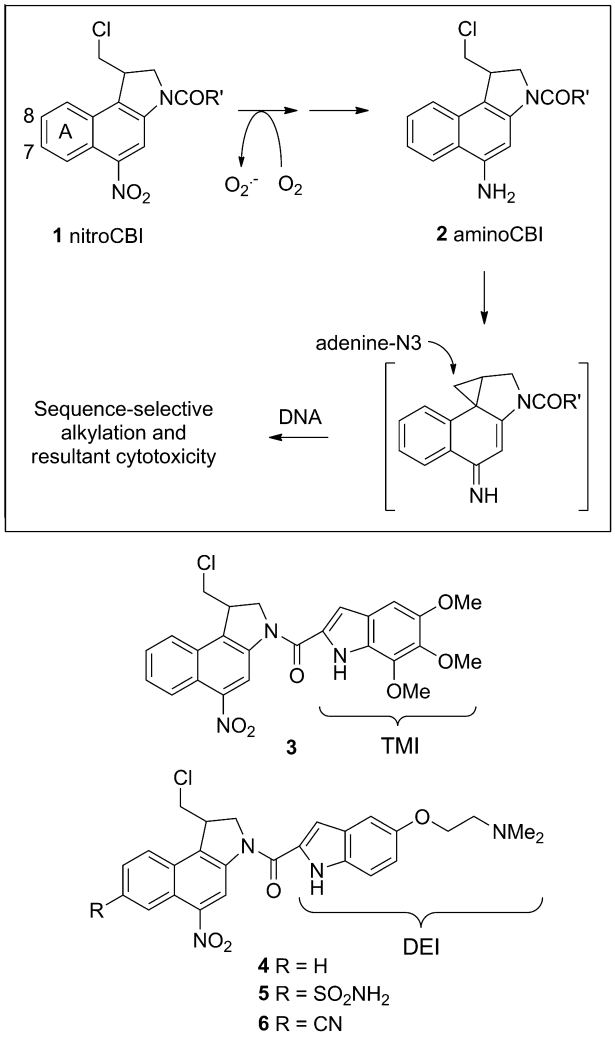
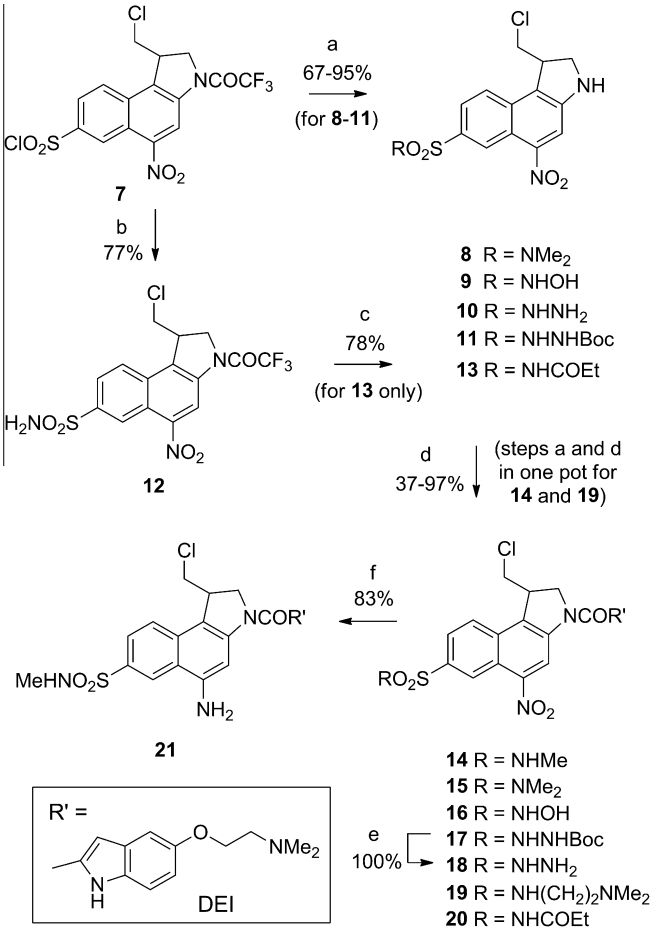


Figure 1. Proposed mechanism of action of nitroCBI hypoxia-activated prodrugs, and structures of some previously reported nitroCBIs. If **1** is reduced to **2** via an initial 1-electron step this process can be reversed in the presence of oxygen. R′ is a side chain that can bind in the minor groove of DNA.



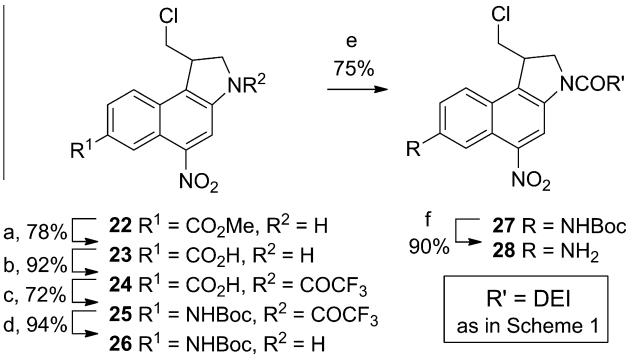
Scheme 1. Synthesis of nitroCBIs with a 7-sulfonamide substituent. Reagents and conditions: (a) excess amine (RH), THF, 0 °C then Cs_2CO_3, MeOH when required to complete deprotection of trifluoroacetamide; (b) NH_3, −78 °C; (c) $(EtCO)_2O$, Et_3N, DMAP then Cs_2CO_3, MeOH; (d) 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid, EDCI, TsOH; (e) HCl, dioxane; (f) H_2, PtO_2.

The major limitation remaining for the application of nitroCBIs like **5** as HAPs is their poor water solubility. Here we investigate the effect of introducing substituents onto the sulfonamide or carboxamide groups, with a view to incorporating solubilizing functional groups (such as tertiary amines). Our previous work had shown that reduction potential alone is not the sole determinant of hypoxic selectivity—the nitrile **6** for example, with the same $E(1)$ as **5**, exhibits almost no hypoxic selectivity, and the hypoxic selectivity of **5** is reduced more than 25-fold simply by switching from the DEI to TMI side chain.[13] A key question in this current study was therefore to determine which combination of A-ring substituent and side chain are compatible with introducing solubilizing functional groups, while at the same time retaining high hypoxic selectivity.

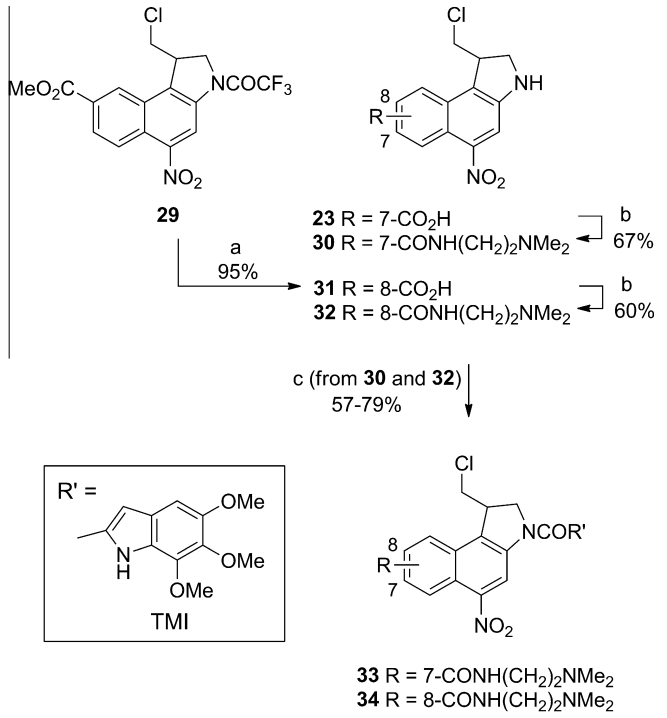
2. Results and discussion

2.1. Chemistry

The new analogues based on a 7-sulfonamide substituent were prepared as shown in Scheme 1, beginning from our previously reported sulfonyl chloride intermediate **7**.[13] Reaction with a variety of amine nucleophiles in THF at 0 °C produced the corresponding substituted sulfonamides, accompanied by cleavage of the trifluoroacetamide protecting group, leading to indolines **8**–**11**. If the reaction of **7** with ammonia was conducted at −78 °C it was possible to isolate **12** (with the trifluoroacetamide intact), thus allowing the subsequent preparation of acyl sulfonamide **13**. The indolines **8**, **9**, **11**, and **13** were coupled with the DEI side chain using EDCI [*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride]



Scheme 2. Synthesis of a nitroCBI with a 7-amino substituent. Reagents and conditions: (a) concd. HCl; (b) $(CF_3CO)_2O$; (c) $(COCl)_2$, DMF then NaN_3 then *tert*-BuOH, toluene reflux; (d) Cs_2CO_3; (e) 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid, EDCI, TsOH; (f) TFA.



Scheme 3. Synthesis of nitroCBIs with 7- and 8-carboxamide substituents. Reagents and conditions: (a) concd. H_2SO_4 then NH_3; (b) $Me_2N(CH_2)_2NH_2$, $(EtO)_2P(O)CN$; (c) HCl then 5,6,7-trimethoxyindole-2-carboxylic acid, EDCI.

as previously described.[13] It was not necessary to isolate the intermediate indolines, and in two cases (in the preparation of **14** and **19**) the direct conversion from **7** was achieved in good overall yield (69–75%). EDCI coupling of the sulfonylhydrazine **10** was not successful, presumably because of competing reaction on the hydrazine, so the Boc-protected derivative **11** was prepared and coupled instead, followed by HCl-mediated deprotection to give **18**. Hydrogenation of **14** in THF over PtO_2 gave the corresponding aminoCBI **21**, but surprisingly this was not successful for **15**—the poor solubility of this nitroCBI necessitated the use of a mixed THF/DMF solvent, and under these conditions a mixture of products was obtained from which the desired aminoCBI could not be isolated.

A nitroCBI analogue with a 7-NH_2 substituent was prepared as shown in Scheme 2. The ester **22**[13] was hydrolyzed under acidic conditions, the indoline protected as the corresponding trifluoroacetamide, and the acid converted via Curtius rearrangement of the corresponding acyl azide to the Boc-protected intermediate **25**. Cleavage of the trifluoroacetamide and coupling with the DEI side chain as above gave **27**, from which the Boc protecting group was removed on acid treatment.

Scheme 3 illustrates the preparation of two nitroCBIs bearing a substituted carboxamide at either the 7- or 8-position of the A-ring. The syntheses began from the acids **23** and **31**, the latter prepared by acidic hydrolysis of the previously reported methyl ester **29**.[13] Each acid was coupled to 2-(dimethylamino)ethylamine using diethyl cyanophosphonate, which allowed selective reaction in the presence of the weakly nucleophilic indoline nitrogen, followed by EDCI coupling with the TMI side chain to give **33** and **34**.

2.2. Physical properties and in vitro cytotoxicity

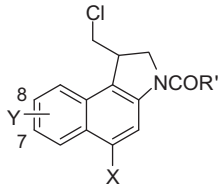
Our previous work had shown that there is a very large difference in hypoxic selectivity between sulfonamide **5** and nitrile **6**. It was therefore of interest to observe the effect of alkylation of the primary sulfonamide, progressively removing the H-bond donor capacity of the A-ring substituent. Oxic and hypoxic cytotoxicities were determined as previously, as IC_{50} values after 4 h of drug exposure in two human tumor cell lines—the ovarian carcinoma Skov3 and the colon carcinoma HT29. Table 1 and Figure 2 clearly show that in moving from **5** to **14** to **15** there is an erosion of hypoxic selectivity, to the point where **15** is no more hypoxia-selective than **6**. This is largely a consequence of decreasing toxicity of the nitroCBI under hypoxic conditions, rather than any loss of potency of the aminoCBI (at least for **14** where the corresponding aminoCBI **21** was available for comparison). The extra methyl substituents are only weakly electron donating, and would not be expected to have any significant impact on $E(1)$ of the nitroCBI. In contrast, the *N*-hydroxysulfonamide **16** and the sulfonylhydrazine **18** bear much more electron-donating sulfonamide substituents, but these compounds clearly retain high hypoxic selectivity in both of the tested cell lines. These observations highlight the importance of the H-bond donor capacity of the A-ring substituent, and are consistent with our previous suggestion that this property makes the nitroCBI a better substrate for the reductase(s) responsible for hypoxic activation.[13]

In comparison we also investigated the nitroCBI **28** where the 7-amino group has H-bond donor capacity but electron-donating character. The question was whether H-bond donor capacity could over-ride the requirement for an electronegative substituent, but **28** proved to have little hypoxia-selective activity. If the correlation between σ_p and $E(1)$[13] can be extrapolated to strongly electron-donating groups, then the unprotonated 7-amino substituent ($\sigma_p = -0.66$) will generate a nitroCBI with $E(1) = -639$ mV. This is well below the range we previously observed for nitroCBIs with significant hypoxic selectivity (−390 to −481 mV).

From the above comparison the primary sulfonamide **5** emerges as the best HAP on the basis of hypoxic selectivity and potency under hypoxic conditions, but is compromised by poor water solubility. Previously **5** was formulated in a DMSO–lactate buffer–PEG vehicle for administration to mice, but ip administration led to precipitation in the peritoneum at the site of injection, precluding determination of a maximum tolerated dose (MTD).[13] In the present study the solubility of **5** was found to be in the low μM range in culture medium (Table 1), even in the presence of 5% fetal calf serum, which tends to increase the apparent solubility of nitroCBIs by protein binding. A prodrug approach that has been successfully applied to improve the water solubility of other primary sulfonamides is the formation of the corresponding acyl sulfonamides. When applied to COX-2 inhibitors the sodium salts of the acyl sulfonamides were found to be several hundred-fold more water soluble than the parent drugs, and the prodrugs (particularly the propionyl and butyryl sulfonamides) underwent rapid hydrolysis in vivo to regenerate the parent sulfonamide.[14,15] Unfortunately, propionyl sulfonamide **20** proved to be very insoluble and difficult to purify and handle, presumably because of its zwitterionic nature. Since a basic side chain had been shown to be required for high hypoxic selectivity, no further attempts were made to prepare nitroCBI acyl sulfonamide prodrugs.

An alternative approach is to accept some loss in hypoxic selectivity in the formation of a secondary sulfonamide, but to use the extra substituent as an opportunity to introduce a more soluble functional group, such as a tertiary amine. Following this approach we prepared **19** as shown in Scheme 1. We were also interested in whether the tertiary amine of the DEI side chain, apparently necessary for high hypoxic selectivity, could be transposed to the A-ring substituent and provide the same activity. To this end we prepared the TMI compounds **33** and **34** (Scheme 3), where a carboxamide in either the 7- or 8-position was used to append a dimethylaminoethyl side chain. While **33** and **34** showed moderate HCRs in the Skov3 cell line (16–24, compared to 6 for the TMI compound with a primary carboxamide in the 7-position[13]) hypoxic selectivity in

Table 1
Structure, solubility, and in vitro cytotoxicity data for NitroCBIs with various A-ring substituents and side chains



Compd	X	Y	R′[a]	Sol.[b] (μM)	Skov3			HT29		
					IC_{50}[c] (μM)		HCR[d]	IC_{50}[c] (μM)		HCR[d]
					Oxic	Hypoxic		Oxic	Hypoxic	
5[e]	NO_2	7-SO_2NH_2	DEI	46	6.9 ± 1.5	0.028 ± 0.002	275 ± 57	4.6 ± 0.6	0.018 ± 0.004	330 ± 110
35[e]	NH_2	7-SO_2NH_2	DEI	105	0.023 ± 0.002	0.016 ± 0.001	1.4 ± 0.1	0.035 ± 0.006	0.023 ± 0.011	1.8 ± 0.6
14	NO_2	7-SO_2NHMe	DEI	8	2.6 ± 0.1	0.12 ± 0.01	21 ± 1	2.7 ± 0.3	0.13 ± 0.04	24 ± 7
21	NH_2	7-SO_2NHMe	DEI	42	0.020 ± 0.002	0.011 ± 0.001	1.8 ± 0.2	0.024 ± 0.004	0.020 ± 0.001	1.2 ± 0.3
15	NO_2	7-SO_2NMe_2	DEI	6	0.50 ± 0.03	0.21 ± 0.02	2.4 ± 0.2	1.1 ± 0.1	0.37 ± 0.13	3.6 ± 0.8
16	NO_2	7-SO_2NHOH	DEI	135	24 ± 2	0.45 ± 0.04	55 ± 5	24 ± 3	0.15 ± 0.02	160 ± 20
18	NO_2	7-SO_2NHNH_2	DEI	31	33 ± 4	0.21 ± 0.01	160 ± 10	31 ± 3	0.16 ± 0.04	220 ± 40
28	NO_2	7-NH_2	DEI	47	2.5 ± 0.4	1.3 ± 0.2	2.0 ± 0.6	1.8 ± 0.1	0.76 ± 0.11	2.4 ± 0.2
19	NO_2	7-$SO_2NH(CH_2)_2NMe_2$	DEI	155	0.83 ± 0.22	0.0066 ± 0.0005	130 ± 40	0.73 ± 0.22	0.0090 ± 0.0020	110 ± 50
33	NO_2	7-$CONH(CH_2)_2NMe_2$	TMI	40	2.0 ± 0.4	0.14 ± 0.04	24 ± 15	1.5 ± 0.2	0.30 ± 0.10	5.9 ± 1.4
34	NO_2	8-$CONH(CH_2)_2NMe_2$	TMI	66	2.5 ± 0.2	0.18 ± 0.04	16[f]	1.3 ± 0.2	0.54 ± 0.04	2.4 ± 0.4

[a] Structures of the side chains are shown in Schemes 1 and 3.
[b] Solubility in culture medium (αMEM) containing 5% FCS.
[c] Drug concentration to reduce cell density to 50% of that of controls following 4 h exposure. Values are the mean ± SEM for 2–7 experiments.
[d] Hypoxic cytotoxicity ratio = IC_{50}(oxic)/IC_{50}(hypoxic). Values are the mean of intraexperiment ratios ± SEM for 2–6 experiments.
[e] Previously reported.[13]
[f] Single determination.

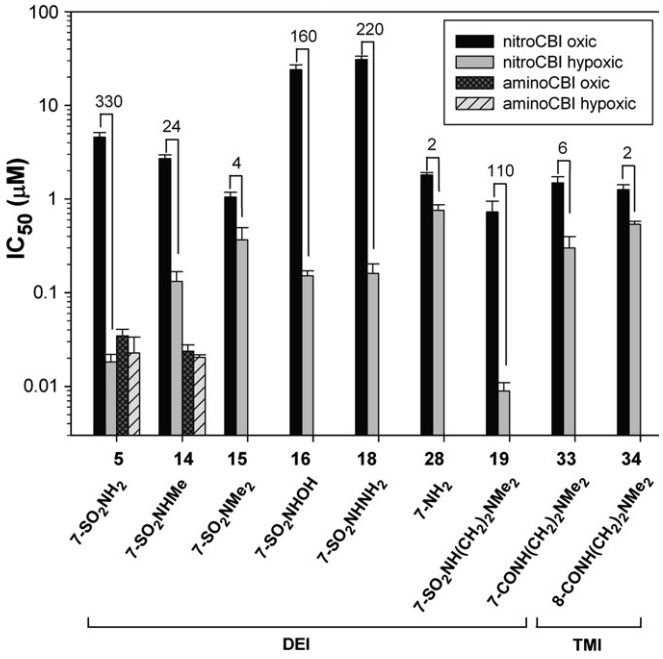


Figure 2. Cytotoxicity of the nitroCBI and aminoCBI compounds of Table 1 under oxic and hypoxic conditions in the HT29 cell line. Numbers above the bars are the hypoxic cytotoxicity ratios (HCRs) for the nitroCBI compounds. Bold numbers below the bars refer to the nitroCBI compound number.

the HT29 cell line was poor. In contrast **19** showed good hypoxic selectivity and potency in both cell lines, performing better than expected in comparison to **14**. This may be due to the higher one-electron reduction potential of **19**, which at a measured value of −357 ± 8 mV is more than 30 mV higher than that recorded for **5**. This elevation in $E(1)$ may be due to an inductive effect from the protonated dimethylamino group (reduction potentials were determined at pH 7), but the magnitude of the effect was surprisingly large, and the same was not observed for **33** ($E(1) = -426 \pm 11$ mV, the same within experimental error as for a nitroCBI bearing a primary carboxamide at the 7-position[13]).

Given the favorable cytotoxicity profile of **19**, we investigated metabolism of this compound, in comparison to **5**, using subcellular fractions prepared from mouse livers. As previously described, post-mitochondrial S9 fractions were supplemented with NADPH and incubated with the nitroCBIs under either oxic or hypoxic conditions (Fig. 3).[13] Under hypoxic conditions the only significant metabolite was the corresponding aminoCBI (identified using authentic **35** as previously described[13] or by UV–vis spectroscopy and mass spectrometry for the aminoCBI derived from **19**), with more extensive reductive metabolism of nitroCBI **19**, consistent with the higher reduction potential. Under oxic conditions formation of the aminoCBI was completely suppressed, and a new metabolite formed (mass spectrum 16 *m/z* units higher than respective parent), probably resulting from N-oxidation of a dimethylamino side chain.

2.3. In vivo evaluation

Of the new analogues in Table 1, **19** appears the most promising for hypoxia-selective activity. It carries an additional basic side chain compared to **5** and is about threefold more soluble in aqueous solvents (Table 1). This is sufficient to allow formulation in lactate buffer (pH 4) for administration to mice and to allow determination of an MTD (23.7 μmol/kg for a single ip dose). At this dose however there was no significant activity in HT29 xenografts in combination with radiation, as judged by excision and clonogenic assay of tumors 18 h after treatment. Preliminary analysis of pharmacokinetic parameters, using serial bleeds from 4 mice and LC–MS analysis of plasma samples 15–180 min after ip administration, suggested low bioavailability and large inter-animal variability. The highest plasma concentration observed (15 min

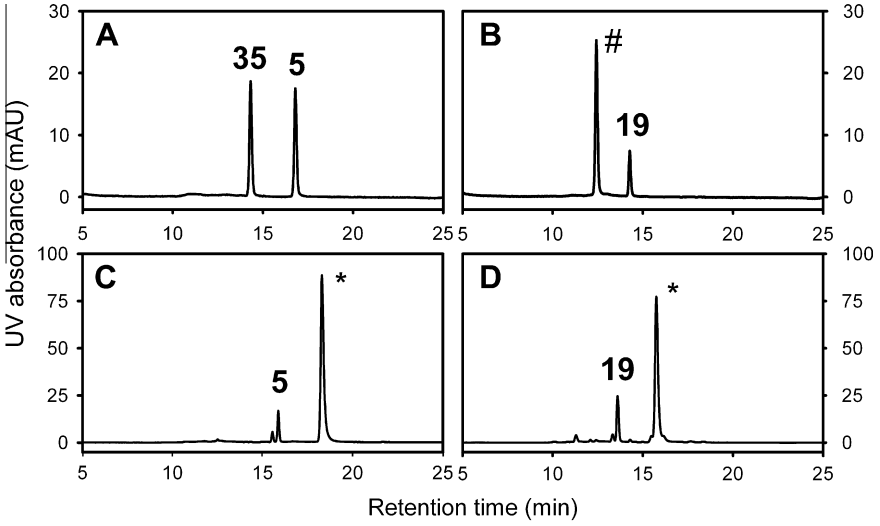


Figure 3. HPLC chromatograms following incubation of 10 μM nitroCBI **5** (panels A and C)[13] and **19** (B and D) with mouse liver S9 (protein concentration 8 mg/mL) at 37 °C for 30 min under either hypoxic (A and B) or oxic (C and D) conditions. HPLC conditions were slightly different between the oxic and hypoxic samples. On the basis of absorbance spectroscopy, mass spectrometry, and comparison with previous results,[13] the peak marked with # is identified as the aminoCBI corresponding to **19**, and the peaks marked with ∗ are identified as the products of N-oxidation of a dimethylamino side chain.

post-dose) ranged from <0.01 to 1.2 μM, and the area under the concentration–time curve ($AUC_{0-180\ min}$) ranged from <1.8 to 70 μM min. Although the highest AUC theoretically equates to more than sufficient exposure to achieve IC_{50} in hypoxic HT29 cell culture (2.2 μM min according to Table 1 where the nitroCBI exposure time is 4 h), the lack of antitumor activity in the excision assay suggests that the actual exposure of hypoxic tumor cells in vivo was significantly lower than this. Possible contributing factors include binding of the nitroCBI to plasma proteins, or slow diffusion within the extravascular tumor compartment.

3. Conclusions

The current study was performed to investigate structure–activity relationships influencing hypoxic selectivity for a new class of promising hypoxia-activated prodrugs. The general conclusion is that for high hypoxic selectivity, nitroCBIs **1** should carry an EWG on the A-ring that is also capable of acting as a H-bond donor, and further that a basic substituent appears necessary, which seems to be more effective when attached to the minor groove-binding side chain than the A-ring substituent. The new examples **16**, **18**, and **19** fit this description and provide HCRs of 55–220 in the two cell lines tested.

Of these examples **19** also carries a second substituent (attached via the sulfonamide side chain) with the potential to improve water solubility. In the event the extra tertiary amine provided only a small increase in water solubility (**19** cf. **5**), and the bis-basic nature of the compound may have hindered in vivo distribution and antitumor activity. Although this particular compound has not provided in vivo activity, the study has indicated a clear strategy for the incorporation of alternative solubilizing functional groups, via secondary sulfonamides or carboxamides at the 7-position of the A-ring of a nitroCBI. Such compounds have the potential to translate the excellent in vitro profile of nitroCBI HAPs like **5** to in vivo application.

4. Experimental

4.1. Chemistry

All final products were analysed by reverse-phase HPLC (Alltima C18 5 μm column, 150 × 3.2 mm; Alltech Associated, Inc., Deerfield, IL) using an Agilent HP1100 equipped with a diode-array detector. Mobile phases were gradients of 80% acetonitrile/20% H_2O (v/v) in 45 mM ammonium formate at pH 3.5 and 0.5 mL/min. Purity was determined by monitoring at 330 ± 50 nm and was >95% except where noted. Final product purity was also assessed by combustion analysis carried out in the Campbell Microanalytical 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 ^1H and 100 MHz for ^{13}C spectra.

4.1.1. 1-(Chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-7-dimethylsulfonamide (8)

Dimethylamine (40% w/w aqueous solution, 0.12 mL, 0.9 mmol) was added to a solution of 1-(chloromethyl)-5-nitro-3-(trifluoroacetyl)-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonyl chloride (**7**)[13] (104 mg, 0.23 mmol) in THF (5 mL) at 0 °C. After 10 min the cooling bath was removed and Cs_2CO_3 (0.15 g, 0.46 mmol) and MeOH (2 mL) were added. After 30 min the mixture was diluted with water and extracted with CH_2Cl_2 (×2). The combined extracts were dried and evaporated and the resulting red oil was crystallized from EtOAc to give to give **8** (57 mg, 68%) as a red powder: mp 170–172 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.54 (d, J = 1.6 Hz, 1H), 8.05 (d, J = 9.0 Hz, 1H), 7.82 (s, 1H), 7.73 (dd, J = 8.9, 1.8 Hz, 1H), 6.81 (s, 1H), 4.30–4.22 (m, 1H), 3.95–3.86 (m, 2H), 3.83–3.73 (m, 2H), 2.66 (s, 6H). Anal. Calcd for $C_{15}H_{16}ClN_3O_4S$: C, 48.72; H, 4.36; N, 11.36. Found: C, 48.84; H, 4.38; N, 11.27. The mother liquor was evaporated and the residue purified by chromatography, eluting with EtOAc/petroleum ether (3:7) to give more **8** (18 mg, 21%).

4.1.2. 1-(Chloromethyl)-*N*-hydroxy-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (9)

A solution of hydroxylamine hydrochloride (55 mg, 0.8 mmol) in water (1 mL) and then a solution of $NaHCO_3$ (132 mg, 1.6 mmol) in water (2 mL) were added to a solution of **7** (90 mg, 0.20 mmol) in THF (5 mL) at 0 °C. The orange solution was stirred at 0 °C for 10 min, and then Cs_2CO_3 (0.12 g, 0.4 mmol) and MeOH (3 mL) were added. The cooling bath was removed and the mixture was stirred for a further 1h. The mixture was diluted with brine and extracted with CH_2Cl_2 (×4). The combined extracts were dried and evaporated

to give **9** (43 mg, 61%) as a red-brown solid. A sample was recrystallized from EtOAc/petroleum ether as an orange solid: mp 170–175 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 9.63–9.58 (m, 2H), 8.63 (d, J = 1.6 Hz, 1H), 8.04 (d, J = 8.9 Hz, 1H), 7.82 (dd, J = 8.9, 1.7 Hz, 1H), 7.78 (s, 1H), 6.80 (s, 1H), 4.30–4.22 (m, 1H), 3.95–3.87 (m, 2H), 3.82 (dd, J = 11.0, 8.2 Hz, 1H), 3.75 (dd, J = 10.5, 3.1 Hz, 1H). Anal. Calcd for $C_{13}H_{12}ClN_3O_5S$: C, 43.64; H, 3.38; N, 11.75. Found: C, 43.90; H, 3.49; N, 11.99.

4.1.3. 1-(Chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-7-sulfonohydrazide (10)

A solution of **7** (108 mg, 0.24 mmol) in THF (5 mL) was added slowly to a solution of hydrazine hydrate (0.11 mL, 2.4 mmol) in THF (5 mL) at 0 °C and the mixture was stirred at this temperature for 10 min. The ice bath was removed and the mixture was stirred for a further 10 min. The mixture was diluted with water, the THF was removed under reduced pressure, and the resulting precipitate was filtered off and dried to give **10** (80 mg, 95%) as a red solid: mp 230–235 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.59 (d, J = 1.5 Hz, 1H), 8.43 (br s, 1H), 8.03 (d, J = 8.9 Hz, 1H), 7.78 (dd, J = 8.9, 1.8 Hz, 1H), 7.76 (s, 1H), 6.74 (s, 1H), 4.28–4.21 (m, 1H), 4.14–4.09 (m, 2H), 3.95–3.86 (m, 2H), 3.81 (dd, J = 11.0, 8.3 Hz, 1H), 3.78–3.72 (m, 1H). HRMS (FAB) calcd for $C_{13}H_{13}{}^{35}ClN_4O_4S$ (M^+) m/z 356.03460, found 356.03456.

4.1.4. *tert*-Butyl 2-{[1-(chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indol-7-yl]sulfonyl}hydrazinecarboxylate (11)

tert-Butyl carbazate (86 mg, 0.65 mmol) was added to a solution of **7** (107 mg, 0.23 mmol) in THF (5 mL) and the mixture was stirred at room temperature for 16 h. Cs_2CO_3 (150 mg, 0.46 mmol) and MeOH (2 mL) were added and the mixture was stirred for a further 2 h. The mixture was diluted with water and extracted with CH_2Cl_2 (×2). The combined extracts were dried and evaporated and the residue was purified by chromatography, eluting with EtOAc/petroleum ether (1:4 then 2:3). The product was recrystallized from EtOAc/petroleum ether to give **11** (72 mg, 67%) as an orange crystalline solid: mp 179 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 9.60 (br s, 1H), 9.18 (v br s, 1H), 8.54 (d, J = 1.4 Hz, 1H), 8.00 (d, J = 8.9 Hz, 1H), 7.75 (s, 1H), 7.74 (dd, J = 8.9, 1.8 Hz, 1H), 6.74 (s, 1H), 4.29–4.22 (m, 1H), 3.94–3.85 (m, 2H), 3.80 (dd, J = 11.0, 8.0 Hz, 1H), 3.74 (dd, J = 10.5, 3.0 Hz, 1H), 1.10 (br s, 9 H). HRMS (FAB) calcd for $C_{18}H_{21}{}^{35}ClN_4O_6S$ (M^+) m/z 456.0870, found 456.0877.

4.1.5. 1-(Chloromethyl)-5-nitro-3-(trifluoroacetyl)-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (12)

Concentrated aqueous NH_3 (0.32 mL, 4.7 mmol) was added to a solution of **7** (215 mg, 0.47 mmol) in THF (10 mL) at −78 °C. After 10 min water (10 mL), aqueous HCl (2 N, 5 mL, 9.4 mmol), and EtOAc (20 mL) were added and the mixture was allowed to warm to room temperature. Brine was added and the mixture was extracted with EtOAc (×2). The combined extracts were washed with brine and dried, and the EtOAc solution was evaporated onto silica. Chromatography eluting with EtOAc/petroleum ether (1:10 then 1:3 then 2:1) gave **12** (158 mg, 77%) as a pale yellow solid: mp (EtOAc) 274–278 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 9.11 (s, 1H), 8.89 (d, J = 1.6 Hz, 1H), 8.50 (d, J = 8.9 Hz, 1H), 8.11 (dd, J = 8.9, 1.7 Hz, 1H), 7.66 (s, 1H), 4.73–4.64 (m, 2H), 4.57–4.49 (m, 1H), 4.24–4.11 (m, 2H). Anal. Calcd for $C_{15}H_{11}ClF_3N_3O_5S$: C, 41.16; H, 2.53; N, 9.60. Found: C, 41.45; H, 2.62; N, 9.45.

4.1.6. 1-(Chloromethyl)-5-nitro-*N*-propionyl-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (13)

Propionic anhydride (83 μL, 0.64 mmol) was added to a solution of **12** (141 mg, 0.32 mmol) and DMAP (4 mg, 0.03 mmol) in THF (10 mL) and Et_3N (0.18 mL, 1.3 mmol) and the mixture was stirred at room temperature for 1.5 h. Cs_2CO_3 (0.21 g, 0.64 mmol) and MeOH (10 mL) were added and the mixture was stirred for a further 16 h. Aqueous HCl (2 N, 4 mL) was added, and the organic solvents were evaporated under reduced pressure. The aqueous residue was diluted with brine and extracted with EtOAc (×2). The combined extracts were washed with brine, dried, and evaporated. The residue was triturated with EtOAc/petroleum ether to give **13** (100 mg, 78%) as a red-brown solid: mp 173–177 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 12.06 (s, 1H), 8.73 (d, J = 1.6 Hz, 1H), 8.03 (d, J = 9.0 Hz, 1H), 7.86 (dd, J = 9.0, 1.8 Hz, 1H), 7.80 (s, 1H), 6.85 (s, 1H), 4.28–4.21 (m, 1H), 3.95–3.86 (m, 2H), 3.81 (dd, J = 11.1, 8.2 Hz, 1H), 3.75 (dd, J = 10.5, 3.1 Hz, 1H), 2.22 (q, J = 7.5 Hz, 2H), 0.88 (t, J = 7.5 Hz, 3H). Anal. Calcd for $C_{16}H_{16}ClN_3O_5S{\cdot}½EtOAc$: C, 48.93; H, 4.56; N, 9.51. Found: C, 48.93; H, 4.66; N, 9.61.

4.1.7. General method for amide formation using indole-2-carboxylic acids and EDCI. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-*N*-hydroxy-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (16)

A mixture of **9** (28 mg, 0.078 mmol), 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid hydrochloride[16] (29 mg, 0.10 mmol), EDCI (60 mg, 0.31 mmol), and TsOH (3 mg, 0.016 mmol) in DMA (2 mL) was stirred at room temperature for 4 h and then cooled to 0 °C. Ice-cold aqueous $NaHCO_3$ was added and the mixture was extracted with EtOAc (×3). The combined extracts were washed with water and dried, and the EtOAc solution was evaporated onto silica. Chromatography, eluting with EtOAc/MeOH (9:1 then 4:1 then 3:2), gave crude **16** (24 mg, 52%). The crude product was suspended in CH_2Cl_2 (4 mL) and MeOH (4 mL) and treated with methanolic HCl (1 mL). After 90 min the precipitate was filtered off and dried to give the hydrochloride salt **16**·HCl (18 mg, 37%) as a yellow solid: mp 260–265 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 11.83 (s, 1H), 9.90 (br s, 1H), 9.86 (d, J = 3.3 Hz, 1H), 9.76 (d, J = 3.2 Hz, 1H), 9.31 (s, 1H), 8.92 (d, J = 1.6 Hz, 1H), 8.47 (d, J = 8.9 Hz, 1H), 8.05 (dd, J = 8.9, 1.7 Hz, 1H), 7.47 (d, J = 8.9 Hz, 1H), 7.28 (d, J = 2.2 Hz, 1H), 7.25 (d, J = 1.7 Hz, 1H), 7.05 (dd, J = 8.9, 2.4 Hz, 1H), 5.04–4.96 (m, 1H), 4.72 (dd, J = 10.9, 2.4 Hz, 1H), 4.70–4.64 (m, 1H), 4.40–4.34 (m, 2H), 4.20–4.11 (m, 2H), 3.59–3.50 (m, 2H), 2.89 (s, 6H). Anal. Calcd for $C_{26}H_{26}ClN_5O_7S{\cdot}HCl{\cdot}H_2O$: C, 48.60; H, 4.55. Found: C, 48.72; H, 4.55. HRMS (FAB) calcd for $C_{26}H_{27}{}^{35}ClN_5O_7S$ (MH^+) m/z 588.1320, found 588.1334. HPLC analysis showed this material to have a purity of 87% with multiple small impurities of 6.0%, 2.7%, and <1%.

4.1.8. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-*N*,*N*-dimethyl-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-7-sulfonamide (15)

Reaction of **8** with 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid using the general method gave **15** (97%), which was immediately converted to the hydrochloride salt **15**·HCl: mp >350 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.83 (s, 1H), 9.88 (br s, 1H), 9.33 (s, 1H), 8.82 (d, J = 1.7 Hz, 1H), 8.46 (d, J = 8.9 Hz, 1H), 7.97 (dd, J = 8.9, 1.7 Hz, 1H), 7.47 (d, J = 8.8 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.24 (d, J = 1.7 Hz, 1H), 7.04 (dd, J = 8.9, 2.4 Hz, 1H), 5.02–4.93 (m, 1H), 4.77–4.62 (m, 2H), 4.38–4.31 (m, 2H), 4.20–4.08 (m, 2H), 3.56–3.46 (m, 2H), 2.87 (s, 6H), 2.73 (s, 6H). ^{13}C NMR δ 160.6, 152.1, 147.0, 142.9, 138.0, 133.9, 132.3, 130.8, 130.1, 127.2, 125.7, 125.0, 124.3, 120.7, 116.5, 116.3, 113.4, 106.3, 104.0, 62.8, 55.6. 54.8, 47.5, 42.9, 41.4, 37.4. Anal. Calcd for $C_{28}H_{30}ClN_5O_6S{\cdot}HCl{\cdot}½H_2O$: C, 52.10; H, 5.00; N, 10.85. Found: C, 52.20; H, 5.15; N, 10.73.

4.1.9. *tert*-Butyl 2-{[1-(chloromethyl)-3-({5-[2-(dimethylamino)-ethoxy]indol-2-yl}carbonyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indol-7-yl]sulfonyl}hydrazinecarboxylate (17)

Reaction of **11** with 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid using the general method, and isolation of the product

as a precipitate after dilution of the reaction mixture with ice-cold aqueous $NaHCO_3$ gave **17** (94%) as a yellow solid: mp 175–180 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 11.72 (d, J = 1.8 Hz, 1H), 9.85 (br s, 1H), 9.30 (v br s, 1H), 9.29 (s, 1H), 8.83 (d, J = 1.5 Hz, 1H), 8.44 (d, J = 8.9 Hz, 1H), 7.98 (dd, J = 8.9, 1.7 Hz, 1H), 7.42 (d, J = 8.9 Hz, 1H), 7.21 (d, J = 1.6 Hz, 1H), 7.18 (d, J = 2.4 Hz, 1H), 6.95 (dd, J = 8.9, 2.4 Hz, 1H), 5.02–4.96 (m, 1H), 4.72 (dd, J = 10.9, 2.4 Hz, 1H), 4.70–4.64 (m, 1H), 4.17–4.09 (m, 2H), 4.07 (t, J = 5.9 Hz, 2H), 2.66 (t, J = 5.7 Hz, 2H), 2.25 (s, 6H), 1.10 (br s, 9 H). HRMS (FAB) calcd for $C_{31}H_{36}{}^{35}ClN_6O_8S$ (MH^+) m/z 687.2004, found 687.2002.

4.1.10. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-5-nitro-*N*-propionyl-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (20)

Compound **13** was reacted with 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid using the general method. Addition of ice-cold aqueous $NaHCO_3$ caused a fine precipitate to separate. The mixture was centrifuged at 0 °C (3000 rpm, 10 min) and the resulting pellet resuspended and recentrifuged, firstly using aqueous $NaHCO_3$ and then water. The resulting solid was dried and then triturated with EtOAc to give **20** (83%) as an orange solid: mp 221–225 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.75 (d, J = 2.0 Hz, 1H), 9.21 (s, 1H), 8.78 (d, J = 1.1 Hz, 1H), 8.26 (d, J = 8.8 Hz, 1H), 8.04 (dd, J = 8.8, 1.6 Hz, 1H), 7.43 (d, J = 8.9 Hz, 1H), 7.22 (d, J = 2.3 Hz, 1H), 7.20 (d, J = 1.8 Hz, 1H), 6.97 (dd, J = 8.9, 2.4 Hz, 1H), 4.98–4.91 (m, 1H), 4.70 (dd, J = 10.9, 2.4 Hz, 1H), 4.65–4.58 (m, 1H), 4.19 (t, J = 5.5 Hz, 2H), 4.17–4.09 (m, 2H), 3.06 (br s, 2H), 2.54 (s, 6H), 2.03 (q, J = 7.5 Hz, 2H), 0.87 (t, J = 7.5 Hz, 3H). HRMS (FAB) calcd for $C_{29}H_{31}{}^{35}ClN_5O_7S$ (MH^+) m/z 628.1633, found 628.1634. HPLC analysis showed this material to have a purity of 84% with multiple small impurities of 5.4%, 1.4%, and <1%. Purification by trituration was hampered by the poor solubility of this zwitterionic material, while an attempt to prepare the hydrochloride salt resulted in decomposition.

4.1.11. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-*N*-[2-(dimethylamino)ethyl]-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (19)

A solution of **7** (50 mg, 0.11 mmol) in CH_2Cl_2/THF (1:1, 20 mL) was treated with *N,N*-dimethylethylenediamine (25 mg, 0.28 mmol), and stirred at room temperature for 30 min. Cs_2CO_3 (0.5 g, 1.5 mmol) was then added, and the mixture was stirred at room temperature for another 15 min, then poured in water (100 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried, HCl/MeOH (10 mL) was added, and the solution was evaporated under reduced pressure. The residue, 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid hydrochloride (80 mg, 0.28 mmol), EDCI (100 mg, 0.52 mmol), anhydrous TsOH (20 mg, 0.12 mmol) and DMA (3 mL) were mixed and stirred at room temperature overnight. The mixture was poured into dilute $NaHCO_3$ at 0 °C and extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with water (3 × 30 mL) and brine, dried, and evaporated. The residue was crystallized from CH_2Cl_2/MeOH to give **19**. This was dissolved in CH_2Cl_2/MeOH (1:1, 20 mL) and HCl/MeOH (2 mL) was added. Precipitation with petroleum ether gave the hydrochloride salt **19**·2HCl (54 mg, 69%): mp >350 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.83 (s, 1H), 10.25 (br s, 1H), 10.11 (br s, 1H), 9.31 (s, 1H), 8.90 (d, J = 1.7 Hz, 1H), 8.48 (d, J = 8.9 Hz, 1H), 8.44–8.38 (m, 1H), 8.06 (dd, J = 8.9, 1.7 Hz, 1H), 7.47 (d, J = 8.8 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.25 (d, J = 1.7 Hz, 1H), 7.04 (dd, J = 8.9, 2.4 Hz, 1H), 5.03–4.95 (m, 1H), 4.77–4.64 (m, 2H), 4.41–4.33 (m, 2H), 4.19–4.10 (m, 2H), 3.59–3.50 (m, 2H), 3.19–3.16 (m, 4 H), 2.89 (s, 3H), 2.87 (s, 3H), 2.78 (s, 6H), 2.77 (s, 3H); ^{13}C NMR δ (one C not observed) 160.6, 152.1, 147.0, 143.0, 138.0, 132.3, 130.8, 130.0, 127.3, 126.0, 124.4, 123.4, 120.6, 116.3, 116.2, 113.4, 106.3, 104.0, 62.7, 55.6, 55.4, 54.8, 47.6, 42.7, 42.3, 41.3, 37.5. Anal. Calcd for $C_{30}H_{35}ClN_6O_6S·2HCl·2½H_2O$: C, 47.34; H, 5.56; N, 11.04. Found: C, 47.28; H, 5.13; N, 10.80.

4.1.12. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-*N*-methyl-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonamide (14)

Treatment of **7** with aqueous methylamine, followed by treatment with 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid, EDCI, and TsOH as for the synthesis of **19** above, gave the free base of **14**, which was immediately converted to the hydrochloride salt **14**·HCl (75%); mp >350 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.81 (s, 1H), 9.9 (br s, 1H), 9.29 (s, 1H), 8.85 (d, J = 1.7 Hz, 1H), 8.44 (d, J = 8.9 Hz, 1H), 8.01 (dd, J = 8.9, 1.7 Hz, 1H), 7.76 (m, 1H), 7.47 (d, J = 8.8 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.24 (d, J = 1.7 Hz, 1H), 7.04 (dd, J = 8.9, 2.4 Hz, 1H), 5.02–4.93 (m, 1H), 4.74–4.61 (m, 2H), 4.39–4.32 (m, 2H), 4.17–4.12 (m, 2H), 3.55–3.50 (m, 2H), 2.87 (s, 6H), 2.48 (s, 3H); ^{13}C NMR δ (one C not observed) 160.7, 152.2, 147.0, 142.8, 138.0, 132.4, 130.7, 130.1, 127.3, 125.9, 124.7, 123.3, 120.7, 116.4, 116.3, 113.4, 106.4, 104.1, 62.8, 55.6, 54.9, 47.7, 42.9, 41.4, 28.6. Anal. Calcd for $C_{27}H_{28}ClF_3N_5O_6S·HCl·¾H_2O$: C, 50.98; H, 4.68; N, 11.01. Found: C, 50.94; H, 4.51; N, 10.74.

4.1.13. 1-(Chloromethyl)-3-{5-[2-(dimethylamino)ethoxy]indol-2-carbonyl}-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-sulfonohydrazide (18)

Compound **17** (77 mg, 0.11 mmol) was stirred with HCl/dioxane (4 M, 2.5 mL) for 16 h, and the solvent was evaporated. The residue was triturated with EtOAc to give the product as the dihydrochloride salt **17**·2HCl (74 mg, 100%) as a yellow solid: mp 280–285 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ (also showing the presence of dioxane) 11.82 (d J = 1.7 Hz, 1H), 10.02 (br s, 1H), 9.29 (s, 1H), 8.88 (d, J = 1.7 Hz, 1H), 8.74 (br s, 1H), 8.45 (d, J = 8.9 Hz, 1H), 8.02 (dd, J = 8.9, 1.7 Hz, 1H), 7.47 (d, J = 8.9 Hz, 1H), 7.28 (d, J = 2.3 Hz, 1H), 7.24 (d, J = 1.7 Hz, 1H), 7.04 (dd, J = 8.9, 2.4 Hz, 1H), 5.02–4.96 (m, 1H), 4.73 (dd, J = 10.8, 2.4 Hz, 1H), 4.69–4.64 (m, 1H), 4.37 (t, J = 5.0 Hz, 2H), 4.20–4.11 (m, 2H), 3.51 (t, J = 5.0 Hz, 2H), 2.88 (d, J = 4.9 Hz, 6H). Anal. Calcd for $C_{26}H_{27}ClN_6O_6S·2HCl·½C_4H_8O_2$: C, 47.77; H, 4.73; N, 11.93. Found: C, 47.93; H, 4.90; N, 11.63. HPLC analysis showed this material to have a purity of 85% with multiple small impurities of 5.6%, 2.9%, 2.8%, 1.6%, 1.2%, and <1%.

4.1.14. 5-Amino-1-(chloromethyl)-3-{5-[2-(dimethylamino)-ethoxy]indol-2-carbonyl}-*N*-methyl-1,2-dihydro-3*H*-benzo[*e*]-indole-7-sulfonamide (21)

14·HCl (23 mg, 0.037 mmol) was suspended in THF (4 mL) and a solution of $NaHCO_3$ (60 mg, 0.71 mmol) in water (3 mL) was added. The mixture was stirred at room temperature until all the solid had dissolved. The mixture was diluted with water and extracted with EtOAc (×2), and the combined extracts were washed with water and then dried and evaporated. The residue was dissolved in THF (30 mL), PtO_2 (35 mg) was added, and the mixture was hydrogenated at 50 psi for 35 min. The mixture was filtered through Celite and the filtrate was evaporated under reduced pressure. The residue was triturated with EtOAc to give **21** (83%) as a yellow solid: mp 260–265 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 11.57 (s, 1H), 8.53 (d, J = 1.5 Hz, 1H), 7.94 (d, J = 8.9 Hz, 1H), 7.82 (s, 1H), 7.71 (dd, J = 8.8, 1.7 Hz, 1H), 7.40 (d, J = 8.9 Hz, 1H), 7.34–7.29 (m, 1H), 7.16 (d, J = 2.3 Hz, 1H), 7.09 (s, 1H), 6.92 (dd, J = 8.9, 2.4 Hz, 1H), 6.31 (s, 2H), 4.76 (dd, J = 10.8, 9.1 Hz, 1H), 4.53 (dd, J = 10.8, 1.8 Hz, 1H), 4.21–4.15 (m, 1H), 4.07 (t, J = 5.9 Hz, 2H), 4.00 (dd, J = 11.0, 3.0 Hz, 1H), 3.81 (dd, J = 11.0, 7.7 Hz, 1H), 2.65 (t, J = 5.9 Hz, 2H), 2.45 (br d, J = 4.2 Hz, 3H), 2.24 (s, 6H). Anal. Calcd for $C_{27}H_{30}ClN_5O_4S·½H_2O·½$EtOAc: C, 57.18; H, 5.79; N, 11.50. Found: C, 57.24; H, 5.69; N, 11.64.

4.1.15. 1-(Chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-7-carboxylic acid (23)

A solution of methyl 1-(chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-carboxylate (**22**)[13] (142 mg, 0.44 mmol) in concd. HCl (15 mL) was heated at reflux for 1 h, then evaporated to dryness under reduced pressure and re-evaporated after addition of water. The residue was triturated with water and the collected solid was dissolved in EtOAc. The solution was filtered through a column of silica gel and the product was recrystallized twice from EtOAc/hexane to give **23** (106 mg, 78%) as a red solid: mp 214–217 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 13.0 (v br s, 1H) 8.75 (d, J = 1.1 Hz, 1H), 7.96 (dd, J = 8.8, 1.6 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H), 7.71 (s, 1H), 6.68 (s, 1H), 4.27–4.18 (m, 1H), 3.94–3.83 (m, 2H), 3.78 (dd, J = 11.1, 8.6 Hz, 1H), 3.73 (dd, J = 10.5, 3.1 Hz, 1H). Anal. Calcd for $C_{14}H_{11}ClN_2O_4$: C, 54.82; H, 3.62; N, 9.14. Found: C, 55.58; H, 3.80; N, 9.10.

4.1.16. 1-(Chloromethyl)-5-nitro-3-(trifluoroacetyl)-1,2-dihydro-3*H*-benzo[*e*]indole-7-carboxylic acid (24)

A stirred solution of **23** (400 mg, 1.30 mmol) in THF (20 mL) was treated with $(CF_3CO)_2O$ (0.74 mL, 5.24 mmol) and stirred at 20 °C for 30 min. Concentration under reduced pressure left a residue which was shaken with water and the resulting solid was collected and crystallized from EtOAc/*i*-Pr_2O to give **24** (484 mg, 92%) as a tan solid: mp 246–247 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 13.3 (br s, 1H), 9.06 (s, 1H), 9.02 (d, J = 1.1 Hz, 1H), 8.37 (d, J = 8.8 Hz, 1H), 8.20 (dd, J = 8.8, 1.5 Hz, 1H), 4.72–4.62 (m, 2H), 4.56–4.48 (m, 1H), 4.20 (dd, J = 11.2, 2.6 Hz, 1H), 4.17–4.09 (m, 1H). Anal. Calcd for $C_{16}H_{10}ClF_3N_2O_5$: C, 47.72; H, 2.50; N, 6.96. Found: C, 47.86; H, 2.62; N, 6.84.

4.1.17. *tert*-Butyl 1-(chloromethyl)-5-nitro-3-(trifluoroacetyl)-1,2-dihydro-3*H*-benzo[*e*]indole-7-carbamate (25)

A suspension of **24** (410 mg, 1.02 mmol) in CH_2Cl_2 (15 mL) containing DMF (one drop) was treated with oxalyl chloride (0.27 mL, 3.10 mmol) and stirred at room temperature for 30 min. The mixture was evaporated under reduced pressure and azeotroped dry with benzene. The resulting acid chloride was dissolved in acetone (5 mL) and treated at 0 °C with a solution of NaN_3 (300 mg, 4.6 mmol) in water (1 mL). The mixture was shaken at room temperature for 1 min, and the precipitate was collected, dried, and stirred in toluene (15 mL) at reflux for 1.5 h. After addition of *tert*-BuOH (1.0 mL, 10 mmol) the mixture was heated at reflux for 5 min then concentrated under reduced pressure. The residue was purified by chromatography, eluting with CH_2Cl_2, followed by crystallization from CH_2Cl_2/hexane to give **25** (347 mg, 72%) as an orange solid: mp 219–220 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.93 (s, 1H), 8.94 (s, 1H), 8.71 (d, J = 1.9 Hz, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.82 (dd, J = 9.2, 2.0 Hz, 1H), 4.65–4.42 (m, 3H), 4.17 (dd, J = 11.3, 2.8 Hz, 1H), 4.09 (dd, J = 11.3, 5.2 Hz, 1H), 1.52 (s, 9 H). Anal. Calcd for $C_{20}H_{19}ClF_3N_3O_5$: C, 50.70; H, 4.04; N, 8.87. Found: C, 50.81; H, 4.10; N, 8.83.

4.1.18. *tert*-Butyl 1-(chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-carbamate (26)

A suspension of **25** (218 mg, 0.46 mmol) in dioxane (5 mL) was treated at room temperature with a solution of Cs_2CO_3 (0.33 g, 1.0 mmol) in water (1 mL) and MeOH (9 mL). The mixture was stirred at room temperature for 5 min and then treated with AcOH (0.15 mL) and diluted with water. The precipitate was collected and crystallized from CH_2Cl_2/hexane to give **26** (164 mg, 94%) as a red solid: mp 162–163 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 9.57 (s, 1H), 8.46 (d, J = 1.7 Hz, 1H), 7.81 (d, J = 9.1 Hz, 1H), 7.65–7.57 (m, 2H), 6.09 (d, J = 2.1 Hz, 1H), 4.19–4.10 (m, 1H), 3.88 (dd, J = 10.9, 3.7 Hz, 1H), 3.81–3.63 (m, 3H), 1.50 (s, 9 H). Anal. Calcd for $C_{18}H_{20}ClN_3O_4$: C, 57.22; H, 5.34; N, 11.12. Found: C, 57.46; H, 5.34; N, 11.16.

4.1.19. *tert*-Butyl 1-(chloromethyl)-3-{5-[2-(dimethylamino)-ethoxy]indol-2-carbonyl}-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-7-carbamate (27)

A mixture of **26** (75 mg, 0.20 mmol), 5-[2-(dimethylamino)ethoxy]indole-2-carboxylic acid hydrochloride (73 mg, 0.26 mmol), EDCI (152 mg, 0.79 mmol) and TsOH (5 mg, 0.03 mmol) in DMA (1.5 mL) was stirred at room temperature for 1 h, then poured into dilute aqueous NH_3. The precipitate was collected, washed with water, and dissolved in CH_2Cl_2 (250 mL). The solution was dried, filtered, concentrated under reduced pressure to a small volume, and then diluted with *i*-Pr_2O to give **27** (91 mg, 75%) as a yellow solid: mp (THF/CH_2Cl_2/hexane) >250 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.67 (d, J = 1.6 Hz, 1H), 9.85 (s, 1H), 9.12 (s, 1H), 8.69 (d, J = 1.9 Hz, 1H), 8.14 (d, J = 9.2 Hz, 1H), 7.79 (dd, J = 9.2, 2.0 Hz, 1H), 7.41 (d, J = 8.9 Hz, 1H), 7.18 (d, J = 2.3 Hz, 1H), 7.15 (d, J = 1.7 Hz, 1H), 6.93 (dd, J = 8.9, 2.4 Hz, 1H), 4.90 (t, J = 10.2 Hz, 1H), 4.67 (dd, J = 10.9, 2.4 Hz, 1H), 4.58–4.51 (m, 1H), 4.16–4.03 (m, 4 H), 2.66 (t, J = 5.8 Hz, 2H), 2.24 (s, 6H), 1.53 (s, 9 H). Anal. Calcd for $C_{31}H_{34}ClN_5O_6$: C, 61.23; H, 5.64; N, 11.52. Found: C, 60.84; H, 5.64; N, 11.40.

4.1.20. 7-Amino-1-(chloromethyl)-3-{5-[2-(dimethylamino)-ethoxy]indol-2-carbonyl}-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole (28)

A suspension of **27** (72 mg, 0.12 mmol) in TFA (3 mL) was stirred at room temperature for 30 min and the resulting solution was evaporated to dryness under reduced pressure below 30 °C. The residue was stirred in dilute aqueous NH_3 at room temperature for 30 min, and the resulting base was collected, washed with water, and dried. This was dissolved in DMF (0.2 mL) and the solution was diluted with excess CH_2Cl_2, clarified by filtration, and then refrigerated to provide **28** (54 mg, 90%) as a red solid: mp >300 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.62 (d, J = 1.4 Hz, 1H), 9.09 (s, 1H), 7.93 (d, J = 9.1 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.40 (d, J = 8.9 Hz, 1H), 7.21–7.13 (m, 2H), 7.11 (d, J = 1.7 Hz, 1H), 6.93 (dd, J = 8.9, 2.4 Hz, 1H), 6.12 (s, 2H), 4.83 (dd, J = 10.8, 9.4 Hz, 1H), 4.62 (dd, J = 11.0, 2.2 Hz, 1H), 4.50–4.42 (m, 1H), 4.13–3.97 (m, 4 H), 2.66 (t, J = 5.9 Hz, 2H), 2.24 (s, 6H). Anal. Calcd for $C_{26}H_{26}ClN_5O_4 \cdot 1½H_2O$: C, 58.37; H, 5.46. Found: C, 58.14; H, 5.00.

4.1.21. 1-(Chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]-indole-8-carboxylic acid (31)

A suspension of methyl 1-(chloromethyl)-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-8-carboxylate (**29**)[13] (314 mg, 0.75 mmol) in a mixture of concd. H_2SO_4 (4.5 mL) and water (0.5 mL) was stirred at 90 °C for 3 h, then cooled and diluted with water (80 mL). The solution was clarified by filtration and adjusted to pH 4 with aqueous NH_3. The resulting precipitate was collected, dissolved in EtOAc and the solution was then filtered, concentrated under reduced pressure to a small volume and diluted with hexane to give **31** (226 mg, 95%) as a red solid: mp 205–208 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 13.3 (v br s, 1H), 8.38 (d, J = 1.3 Hz, 1H), 8.19 (d, J = 9.1 Hz, 1H), 7.82 (dd, J = 9.1, 1.6 Hz, 1H), 7.76 (s, 1H), 6.45 (s, 1H), 4.35–4.25 (m, 1H), 3.91–3.80 (m, 2H), 3.76 (dd, J = 11.2, 8.5 Hz, 1H), 3.72 (dd, J = 10.3, 2.8 Hz, 1H). Anal. Calcd for $C_{14}H_{11}ClN_2O_4$: C, 54.82; H, 3.62; N, 9.14. Found: C, 54.75; H, 3.87; N, 9.11.

4.1.22. 1-(Chloromethyl)-*N*-[2-(dimethylamino)ethyl]-5-nitro-1,2-dihydro-3*H*-benzo[*e*]indole-7-carboxamide (30)

A stirred solution of **23** (124 mg, 0.40 mmol) in dry DMF (1.5 mL) was treated at 0 °C with *N*,*N*-dimethyl-1,2-ethanediamine (111 μL, 1.01 mmol), followed by drop wise addition of diethyl cyanophosphonate (132 μL, 93%, 0.81 mmol). The mixture was warmed to room temperature for 30 min, then poured into dilute aqueous NH_3 saturated with NaCl. The precipitated solid was

collected, washed with water, and recrystallized twice from CH_2Cl_2/*i*-Pr_2O to give **30** (102 mg, 67%) as a red solid: mp 155–158 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.57 (d, J = 0.7 Hz, 1H), 8.51 (t, J = 5.6 Hz, 1H), 7.93 (dd, J = 8.9, 1.5 Hz, 1H), 7.90 (d, J = 8.7 Hz, 1H), 7.66 (s, 1H), 6.54 (s, 1H), 4.26–4.18 (m, 1H), 3.91 (dd, J = 11.0, 3.8 Hz, 1H), 3.86 (td, J = 9.8, 2.3 Hz, 1H), 3.78 (dd, J = 11.0, 8.6 Hz, 1H), 3.72 (dd, J = 10.2, 2.8 Hz, 1H), 3.39 (q, J = 6.5 Hz, 2H), 2.42 (t, J = 6.9 Hz, 2H), 2.49 (s, 6H). Anal. Calcd for $C_{18}H_{21}ClN_4O_3 \cdot \frac{1}{2}H_2O$: C, 56.03; H, 5.75; N, 14.52. Found: C, 56.22; H, 5.63; N, 14.69.

4.1.23. 1-(Chloromethyl)-*N*-[2-(dimethylamino)ethyl]-5-nitro-1,2-dihydro-3*H*-benzo[e]indole-8-carboxamide (32)

A stirred solution of **31** (120 mg, 0.39 mmol) in dry DMF (1.5 mL) was treated at 0 °C with *N*,*N*-dimethyl-1,2-ethanediamine (107 μL, 0.97 mmol), followed by the drop wise addition of diethyl cyanophosphonate (128 μL, 93%, 0.78 mmol). The mixture was warmed to room temperature for 45 min, then poured into dilute aqueous NH_3 saturated with NaCl. The resulting solid was collected, washed with water, and recrystallized twice from CH_2Cl_2/*i*-Pr_2O to give **32** (88 mg, 60%) as a red solid: mp 178–180 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.68 (t, J = 5.7 Hz, 1H), 8.22 (d, J = 1.2 Hz, 1H), 8.16 (d, J = 9.1 Hz, 1H), 7.77 (dd, J = 9.1, 1.7 Hz, 1H), 7.72 (s, 1H), 6.41 (d, J = 1.7 Hz, 1H), 4.28–4.18 (m, 1H), 3.98 (dd, J = 10.9, 3.7 Hz, 1H), 3.84 (td, J = 9.7, 2.4 Hz, 1H), 3.75 (dd, J = 11.0, 9.0 Hz, 2H), 3.49–3.37 (m, 2H), 2.45 (t, J = 7.0 Hz, 2H), 2.21 (s, 6H). Anal. Calcd for $C_{18}H_{21}ClN_4O_3$: C, 57.37; H, 5.62; N, 14.87. Found: C, 57.32; H, 5.46; N, 14.82.

4.1.24. 1-(Chloromethyl)-*N*-[2-(dimethylamino)ethyl]-5-nitro-3-(5,6,7-trimethoxyindol-2-carbonyl)-1,2-dihydro-3*H*-benzo[*e*]-indole-7-carboxamide (33)

A suspension of **30** (45 mg, 0.12 mmol) in dioxane (10 mL) was treated at 20 °C with HCl gas until colorless, then evaporated to dryness under reduced pressure. To the resulting dihydrochloride salt was added 5,6,7-trimethoxyindole-2-carboxylic acid (36 mg, 0.14 mmol), EDCI (92 mg, 0.48 mmol), and dry DMA (1 mL), and the mixture was stirred at room temperature for 2 h and then poured into saturated aqueous $KHCO_3$. The precipitated solid was collected, dissolved in CH_2Cl_2, and the solution was washed with water, dried, and concentrated under reduced pressure below 25 °C. The residue was triturated with EtOAc/*i*-Pr_2O to give crude **33**. Treatment of a solution of the free base in CH_2Cl_2 with HCl(g)/EtOAc/hexane, followed by crystallization from MeOH/EtOAc gave the hydrochloride salt **33**·HCl (61 mg, 79%) as a yellow solid: mp 246–248 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 11.58 (d, J = 1.8 Hz, 1H), 9.84 (br s, 1H), 9.14 (s, 1H), 9.07 (t, J = 5.5 Hz, 1H), 8.86 (d, J = 1.4 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H), 8.19 (dd, J = 8.9, 1.6 Hz, 1H), 7.19 (d, J = 2.2 Hz, 1H), 6.98 (s, 1H), 4.93 (t, J = 10.6 Hz, 1H), 4.68–4.57 (m, 2H), 4.18–4.07 (m, 2H), 3.94 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.69 (q, J = 5.8 Hz, 2H), 3.23 (after D_2O exchange, t, J = 5.7 Hz, 2H), 2.85 (s, 6H). Anal. Calcd for $C_{30}H_{32}ClN_5O_7 \cdot HCl$: C, 55.73; H, 5.14; N, 10.83. Found: C, 55.62; H, 5.15; N, 10.77.

4.1.25. 1-(Chloromethyl)-*N*-[2-(dimethylamino)ethyl]-5-nitro-3-(5,6,7-trimethoxyindol-2-carbonyl)-1,2-dihydro-3*H*-benzo[*e*]-indole-8-carboxamide (34)

A suspension of **32** (72 mg, 0.19 mmol) in dioxane (15 mL) was treated at 20 °C with HCl gas until colorless, then evaporated to dryness under reduced pressure. To the resulting dihydrochloride salt was added 5,6,7-trimethoxyindole-2-carboxylic acid (58 mg, 0.23 mmol), EDCI (148 mg, 0.77 mmol), and dry DMA (2.0 mL), and the mixture was stirred at room temperature for 1.5 h. The mixture was poured into saturated aqueous $KHCO_3$ and the precipitated solid was collected and dissolved in CH_2Cl_2. The solution was washed with water, dried, concentrated under reduced pressure below 25 °C, and then diluted with hexane to give crude **34**. Treatment of a solution of **34** in CH_2Cl_2 with HCl(g)/EtOAc/hexane, followed by crystallization from MeOH/EtOAc, gave the hydrochloride salt **34**·HCl (71 mg, 57%) as a yellow solid: mp 228–229 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 11.58 (d, J = 1.7 Hz, 1H), 9.87 (v br s, 1H), 9.28–9.14 (m, 2H), 8.71 (s, 1H), 8.46 (d, J = 9.1 Hz, 1H), 8.14 (dd, J = 9.1, 1.5 Hz, 1H), 7.19 (d, J = 2.2 Hz, 1H), 6.98 (s, 1H), 4.94 (t, J = 10.7 Hz, 1H), 4.72–4.61 (m, 2H), 4.25–4.15 (m, 2H), 3.95 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.76–3.68 (m, 2H), 3.26 (after D_2O exchange, t, J = 5.7 Hz, 2H), 2.87 (br s, 6H). Anal. Calcd for $C_{30}H_{32}ClN_5O_7 \cdot HCl$: C, 55.73; H, 5.14; N, 10.83. Found: C, 55.52; H, 5.21; N, 10.73.

4.2. Solubility in culture medium

A stock solution in DMSO (5 mM) was diluted 100-fold with αMEM containing 5% FCS (equilibrated with 5% CO_2/air) and allowed to stand at 22 °C for 10 min. The precipitate was separated by centrifugation (13,000 rpm for 6 min) and the supernatant (pH 7.6–8.2) was diluted 1:1 with more αMEM-5% FCS. The concentration in the supernatant was determined by HPLC as described above, using diluted stock solution as a comparison. If no precipitate formed (solubility $\geqslant$50 μM) a more concentrated solution was prepared by sonication in αMEM-5% FCS.

4.3. One-electron reduction potentials

Pulse radiolysis experiments were performed as previously described.[13] One-electron reduction potentials, $E(1)$, were determined at pH 7 in deaerated solutions containing 2-propanol (0.5 M) and phosphate buffer (2.5 mM) (**33**) or imidazole buffer (3 mM) (**19**), using methylviologen (1,1′-dimethyl-4,4′-bipyridinium dichloride), −447 ± 7 mV, as reference compound.

4.4. In vitro cytotoxicity

Inhibition of proliferation of log-phase monolayers was assessed in 96-well plates as previously described.[17] The drug exposure time was 4 h under aerobic (20% O_2) or anoxic (<20 ppm O_2) conditions followed by sulforhodamine B staining 5 days later. The IC_{50} was determined by interpolation as the drug concentration required to inhibit cell density to 50% of that of the controls on the same plate.

4.5. In vitro metabolism

Studies were performed as previously described.[13] Briefly, liver S9 homogenates (protein concentration 8 mg/mL) were prepared from female homozygous nude mice and incubated for 30 min at 37 °C in the presence of 1 mM NADPH and 10 μM of nitroCBI under oxic or hypoxic conditions. After protein precipitation with ice-cold MeCN the samples were analysed by HPLC with UV–vis spectroscopy and mass spectrometry.

4.6. MTD and excision assay

Studies were performed as previously described.[1] In the excision assay a single whole-body radiation dose of 20 Gy to HT29 tumor-bearing CD1 nude mice caused a log cell kill of 1.45 ± 0.10 clonogens/g tumor tissue, which was not significantly different to that when radiation was combined with **19** (administered ip at its MTD of 23.7 μmol/kg) either 30 min before (1.45 ± 0.10) or 5 min after (1.60 ± 0.10) irradiation. In the same experiment the bioreductive drug tirapazamine administered at its MTD (270 μmol/kg) 5 min after radiation produced a log cell kill of 3.40 ± 0.20 clonogens/g tumor tissue.

4.7. Pharmacokinetic analysis

19 was administered ip at 23.7 μmol/kg to non-tumor-bearing male CD1 nude mice and serial blood samples were collected from the tail vein at 15, 30, 60, 120, and 180 min, as described previously.[18] Samples were analysed by HPLC as described above. The area under the drug concentration–time curve (AUC_{0-180}), and the maximum concentration (C_{max}) were calculated using noncompartmental analysis (WinNonLin version 4.0, Pharsight Corp.).

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