Bioreductive Activation of a Series of Indolequinones by Human DT-Diaphorase: Structure−Activity Relationships

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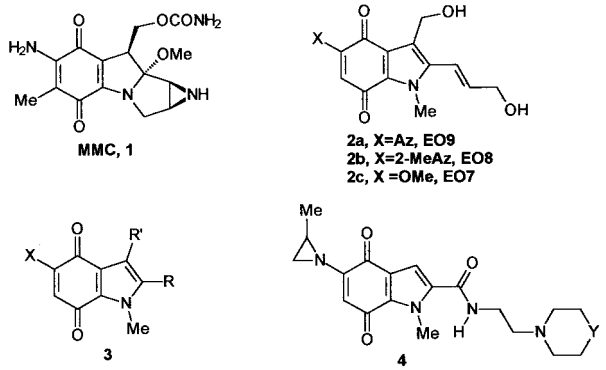
A series of indolequinones including derivatives of EO9 bearing various functional groups and related indole-2-carboxamides have been studied with a view to identifying molecular features which confer substrate specificity for purified human NAD(P)H:quinone oxidoreductase (DT-diaphorase), bioreductive activation to DNA-damaging species, and selectivity for DT-diaphorase-rich cells in vitro. A broad spectrum of substrate specificity exists, but minor changes to the indolequinone nucleus have a significant effect upon substrate specificity. Modifications at the 2-position are favorable in terms of substrate specificity as these positions are located at the binding site entrance as determined by molecular modeling studies. In contrast, substitutions at the (indol-3-yl)methyl position with bulky leaving groups or a group containing a chlorine atom result in compounds which are poor substrates, some of which inactivate DT-diaphorase. Modeling studies demonstrate that these groups sit close to the mechanistically important amino acids Tyr 156 and His 162 possibly resulting in either alkylation within the active site or disruption of charge-relay mechanisms. An aziridinyl group at the 5-position is essential for potency and selectivity to DT-diaphorase-rich cells under aerobic conditions. The most efficient substrates induced qualitatively greater single-strand DNA breaks in cell-free assays via a redox mechanism involving the production of hydrogen peroxide (catalase inhibitable). This damage is unlikely to form a major part of their mechanism of action in cells since potency does not correlate with extent of DNA damage. In terms of hypoxia selectivity, modifications at the 3-position generate compounds which are poor substrates for DT-diaphorase but have high hypoxic cytotoxicity ratios.

Introduction

The enzyme DT-diaphorase (NAD(P)H:quinone acceptor oxidoreductase, EC 1.6.99.2) is a cytosolic flavoprotein which catalyzes the two-electron reduction of a broad range of substrates (particularly quinones) using either NADH or NADPH as electron donors.[1,2] Its physiological function is believed to be the detoxification of quinones (such as menadione), although certain quinones are reduced to metabolites which are more toxic than the parent compound via a process termed bioreductive activation.[3,4] The ability to activate certain quinones in conjunction with the fact that certain tumor types (particularly NSCLC) contain elevated levels of DT-diaphorase suggests that DT-diaphorase is a good candidate for enzyme-directed bioreductive drug development.[5,6]

The role of DT-diaphorase in the activation of quinones is complex and in some cases (e.g. mitomycin C, **1**) (Chart 1) controversial.[2,6,7] A much clearer role for DT-diaphorase in the bioreductive activation of the indolequinone EO9 (5-aziridinyl-1-methyl-2-[1*H*-indole-4,7-dione]prop-*β*-en-α-ol, **2a**) has been established, although cellular responses depend on a complex interaction between DT-diaphorase activity and oxygenation status. EO9 is a good substrate for both human and rodent DT-diaphorase and is activated to DNA-damaging species in cell-free assays.[8−10] Under aerobic conditions, a good correlation exists between DT-diaphorase activity and chemosensitivity.[11,12] Under hypoxic conditions however, this relationship changes with significant potentiation (i.e. high hypoxic cytotoxicity ratios, HCR) of EO9 activity seen only in cell lines which have low levels of DT-diaphorase activity.[12−14] EO9 is therefore an unusual bioreductive drug in that it has the potential to kill either the aerobic fraction of DT-diaphorase-rich tumors or the hypoxic fraction of DT-diaphorase-deficient tumors.[6] EO9 has been evaluated in the clinic,

Chart 1



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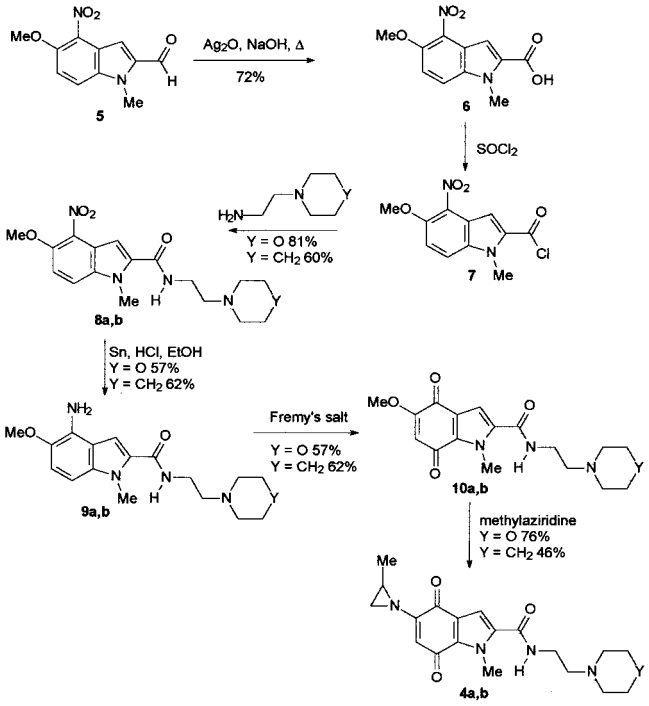
Scheme 1[a]



[a] For compounds **4** and **8**−**10**: **a**, Y = O; **b**, Y = CH_2.

but despite reports of three partial responses in phase I clinical trials, no activity was reported in subsequent phase II clinical trials in NSCLC, breast, pancreatic, colorectal, and gastric cancers.[15−17] Recent studies have suggested that the failure of EO9 in the clinic was unlikely to be caused by poor pharmacodynamic interactions but rather by poor drug delivery to tumors.[18] As part of a broad approach to developing analogues of EO9 which retain the desirable characteristics of EO9 (i.e. bioreductive activation by DT-diaphorase) but have better pharmacological properties in terms of drug delivery, a series of indolequinone derivatives of EO9 have been synthesized.[19−22] The principal aim of this study is to identify indolequinone derivatives (of type **3**) and related novel indole-2-carboxamides (**4**) which are both good substrates for and bioactivated to DNA-damaging species by purified human DT-diaphorase. Structure−activity relationships are also reported in conjunction with molecular modeling studies of indolequinone binding to DT-diaphorase with a view to identifying key components of the indolequinone molecule which confer good substrate specificity. Finally, chemosensitivity studies using DT-diaphorase-rich and -deficient tumor cell lines are also reported with the aim of identifying structural features of molecules which confer selectivity toward DT-diaphorase-rich cells under aerobic conditions and hypoxia selectivity.

Results and Discussion

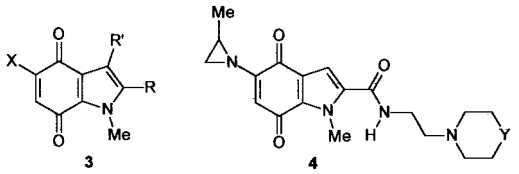
Chemistry. The synthesis of indolequinones **3b**−**3d** has been reported previously by us,[20] and the preparation of compounds **3a**, **3e**, and **3f**−**3s** has also been described previously.[19,20,22] EO8 (**2b**) and EO7 (**2c**) were synthesized as described by Speckamp and Oostveen.[23] The novel indole-3-carboxamides analogues **4a** and **4b** were prepared as shown in Scheme 1, in which 5-methoxy-1-methyl-4-nitroindole-2-carboxaldehyde (**5**)[21] was reacted with silver oxide in the presence of base to give the corresponding carboxylic acid **6**. Treatment of **6** with thionyl chloride (to give the acid chloride **7**) and reaction with 4-(2-aminoethyl)morpholine or 1-(2-aminoethyl)-piperidine gave the nitroindole-2-carboxamides **8a** and **8b**, respectively. Reduction of the nitroindoles **8a** and **8b** with tin and HCl gave the corresponding aminoindoles **9a** and **9b**. Immediate oxidation (with Fremy's salt) gave the desired indolequinonecarboxamides **10a** and **10b**. Reaction of **10a** and **10b** with 2-methylaziridine afforded the target compounds **4a** and **4b**, respectively.

Biology. A broad spectrum of substrate specificity for purified human DT-diaphorase exists within the panel of compounds evaluated (Table 1). The most efficient substrate for DT-diaphorase was **3a** which has a phenyl group at the 2-position of the indolequinone nucleus. These results are consistent with the fact that streptonigrin (a 2-pyridyl-substituted quinolinedione) is a good substrate for DT-diaphorase[24] and confirm the recent findings of Beall et al.[25] which demonstrate that 2-phenylindolequinone derivatives are good substrates for DT-diaphorase. Phenyl substitution of simpler aziridinylbenzoquinones has also been reported to increase DT-diaphorase substrate specificity.[26] The results of this and other studies clearly demonstrate that various modifications to the 2-position of the indolequinone nucleus can be made without adversely affecting substrate specificity for DT-diaphorase and may indeed substantially increase it. In this study, compounds with phenyl, cyclopropyl, various carboxamide groups, etc., at the 2-position can all form good substrates for DT-diaphorase.

In contrast to these findings, very few changes at the 3-position (R′) can be made without major effects upon substrate specificity. In general terms, modifications at R′ result in compounds which not only are poor substrates for DT-diaphorase but also inactivate the enzyme (Table 1). As in previous studies[25,27] inactivation of the enzyme was NADH-dependent, suggesting that the compounds are reduced by DT-diaphorase to generate metabolites which either alkylate within the active site or disrupt charge-relay mechanisms. Several of these analogues are very potent inhibitors of DT-diaphorase activity (Figure 1) with ID_{50} values (the concentration of drug required to reduce DT-diaphorase activity by 50%) ranging from 1.02 nM (**3l**) to 7.42 nM (**3o**). While these compounds are of little interest therapeutically, they may be useful experimental tools for obtaining proof of principle, and further studies are required to determine whether these inhibitors can be used to identify DT-diaphorase-activated prodrugs. Further studies to determine whether these compounds are selective inhibitors of DT-diaphorase with respect to other one-electron reductases are required before studies of this nature are conducted.

With regard to the 5-position (X), previous studies have demonstrated that an aziridinyl function is a desirable but not an essential requirement for substrate specificity.[10,28] The results of this study support this conclusion in that aziridinyl, methylaziridinyl, and methoxy groups at X do not necessarily affect substrate specificity (Table 1). In some cases however, substrate specificity is reduced by replacing an aziridinyl group

Table 1. Structure and Metabolism of a Series of Indolequinones by Purified Human DT-Diaphorase



compd	X	R′	R	substrate specificity (μmol/min/mg)	% inhibition[a]
3a	OCH_3	CH_2OH	Ph	127.4 ± 3.2	2.75
3b	Az	CH_2OH	H	52.7 ± 0.93	7.14
3c	Az	H	$CH{=}CHCH_2OH$	41.6 ± 1.08	12.78
3d	OCH_3	CH_2OH	H	25.3 ± 0.9	0.59
EO9	Az	CH_2OH	$CH{=}CHCH_2OH$	19.9 ± 0.5	4.72
4a (Y = O)				16.9 ± 1.2	17.80
3e	Az	CH_2OH	*c*-Pr	14.9 ± 0.2	2.55
4b (Y = CH_2)				12.8 ± 0.1	0
3f	Az	CH_2OH	CH_3	9.9 ± 0.3	11.08
3g	2-MeAz	CH_2OH	Ph	9.6 ± 0.3	0
3h	2-MeAz	CH_2OH	CH_3	7.4 ± 0.25	4.06
3i	OCH_3	OH	CH_3	6.0 ± 0.1	0.59
3j	2,3-Me_2Az	OH	CH_3	3.1 ± 0.08	0
EO7	OCH_3	CH_2OH	$CH{=}CHCH_2OH$	2.6 ± 0.05	0
EO8	2-MeAz	CH_2OH	$CH{=}CHCH_2OH$	1.5 ± 0.1	ND
3k	OCH_3	CH_2OCONH_2	$CH(CH_3)_2$	0.82 ± 0.08	0
3l	OCH_3	CH_2OCO-*o*-F-Ph	H	0.66 ± 0.02	98.91
3m	OCH_3	CH_2O-2,4,6-tri-Cl-Ph	H	0.50 ± 0.12	99.12
3n	OCH_3	CH_2OCONH_2	*c*-Pr	0.34 ± 0.02	46.23
3o	OCH_3	CH_2OCOPh	H	0.29 ± 0.09	99.56
3p	OCH_3	CH_2OCOPh-*p*-F	H	0.26 ± 0.06	99.15
3q	OCH_3	CH_2Cl	H	0.17 ± 0.03	95.01
3r	OCH_3	CH_2OCOPh-*p*-F	CH_3	0.00	ND
3s	OCH_3	CH_2CO-*p*-FPh	Ph	0.00	ND

[a] Drug concentrations used for inhibition studies were 50 μM (30-min drug incubation).

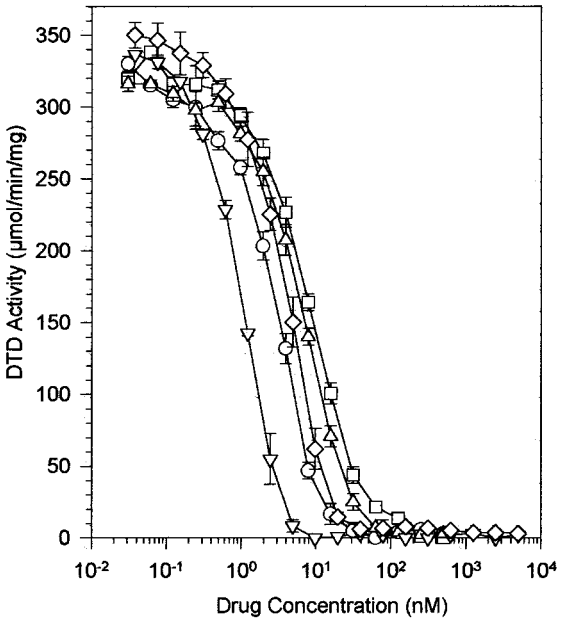


Figure 1. Inactivation of purified human DT-diaphorase activity by **3l** (▽), **3m** (○), **3o** (□), **3p** (△), and **3q** (◇). Each point represents the mean of three independent experiments ± standard deviations.

with a methylaziridine (compare EO9 with EO8) or replacing a methoxy group with a methylaziridinyl group at the 5-position (compare **3a** with **3g**). These changes are likely to be due to steric rather than electronic effects since there is little difference between methylaziridine and aziridine in terms of the latter. The distortion of the strained three-membered ring is likely to present a significant steric constraint compared to the methoxy group.

A molecular model of human DT-diaphorase was constructed with homology to the known rat structure,[29] and models of substrates bound to the enzyme were produced. All the quinone-based substrates moved with respect to their initial positions in order to allow for steric interactions at the 5-position (X) of the molecule. Analysis of this region showed that Trp 106 provides a 'wall' against which the aziridinyl group can produce a hydrophobic contact. The distance is enough to allow the aziridine or an −OMe to interact favorably. The addition of a methyl group to the aziridine, or ring opening, evidently provides a steric interaction with Trp 106 which forces the quinone oxygen atoms to be unfavorably positioned for reduction, and hence the rate is significantly or totally reduced. Figure 2a shows EO9 in its position in the final model with the aziridine group next to Trp 106. The substitution at the 3-position (R′) with a bulky group (e.g. **3p**) generates compounds which typically inactivate DT-diaphorase (Table 1). In addition, substitution with a CH_2Cl group (compound **3q**) results in the inactivation of DT-diaphorase (Table 1). The model (Figure 2b) shows that this group will be within close proximity to the residues Tyr 156 and His 162 which have been shown to be essential components in the reduction mechanism.[29] It has been postulated that proton transfer occurs between Tyr 156 and FAD, and the presence of the electronegative chlorine atom may prevent or disrupt the charge-relay mechanism. In contrast, alterations at the 2-position (R), located in the binding site entrance, tend to be tolerated (e.g. **3a**, Figure 2c,d). It is of interest to note that the indole N-Me

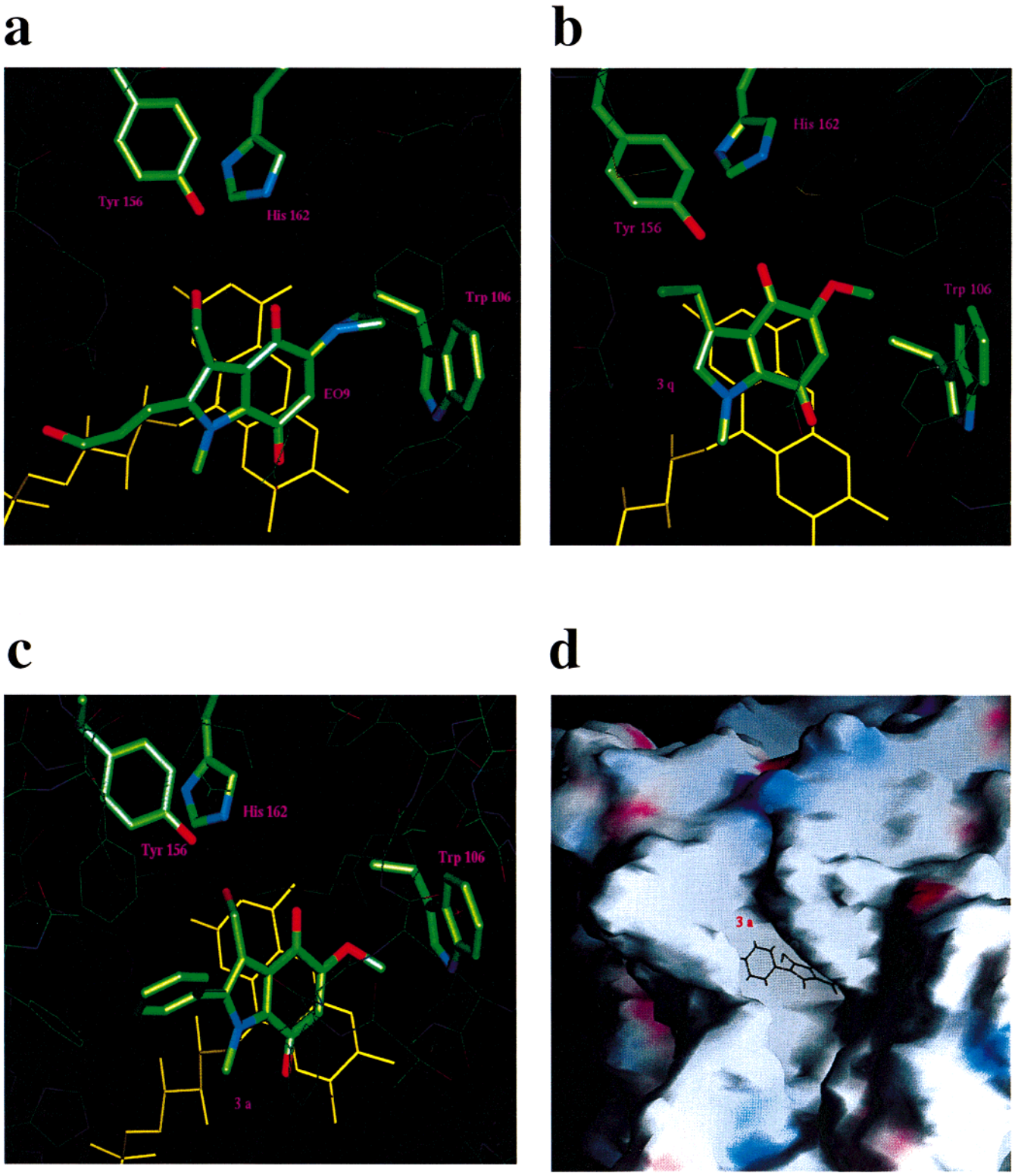


Figure 2. (a) Molecular model of EO9 docked into the binding site of DT-diaphorase. The amino acid Trp 106 forms the back of the binding pocket, and the mechanistically important amino acids Tyr 156 and His 162 are located at the top of the binding pocket. The FAD cofactor is shown in yellow. (b) Molecular model of compound **3q** (which inactivates DT-diaphorase) docked into the binding site of DT-diaphorase. (c) Molecular model of compound **3a** docked into the binding site of DT-diaphorase. (d) Molecular surface of DT-diaphorase with compound **3a** docked into the binding site. The indole N-Me is shown pointing out of the entrance to the binding site. The molecular surface was generated using GRASP[37] and is color-coded by electrostatic potential (positive as blue, negative as red).

group is also located at the entrance to the binding site (Figure 2d) suggesting that modifications to the methyl group may generate compounds which are good substrates for DT-diaphorase. The models suggest that the small modifications that result in significant differences in the ability of quinone-based compounds to be reduced do so due to steric interactions in the active site. In particular Trp 106 provides a 'wall' at the rear of the

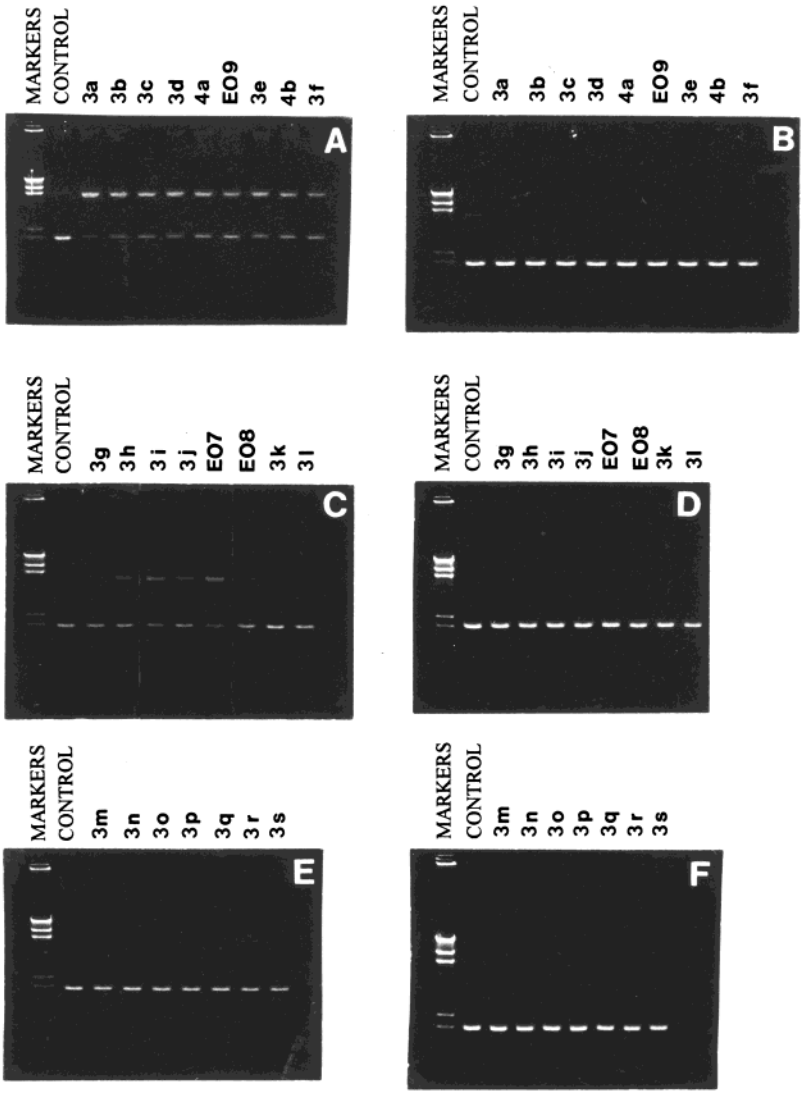


Figure 3. Single-strand breaks in supercoiled pRSETB plasmids induced by a series of indolequinones following reduction by human DT-diaphorase (panels A, C, and E) and in the absence of DT-diaphorase (panels B, D, and F). Molecular weight markers are λDNA digested with *Hin*dIII (Sigma).

active site which limits substitutions at the 5-position (X). In addition, certain substitutions at the 3-position (R′) of the indole ring can disrupt mechanistically essential amino acids, although substitutions at the 2-position can be more favorably accommodated. These models therefore indicate structural restraints on potential bioreductive quinone-based antitumor compounds which are metabolized by DT-diaphorase. Further work using MM/QM calculations is needed to study the mechanistic implications of these models, although they do suggest explanations for a large number of experimental data and should also significantly assist rational drug design of novel substrates for this enzyme.

In terms of bioactivation to DNA-damaging species following reduction by DT-diaphorase, there is a general trend between substrate specificity and a qualitative assessment of single-strand breaks in plasmid DNA (Figure 3). No DNA damage was observed when compounds were incubated with DNA in the absence of DT-diaphorase (Figure 3). The DNA-damaging species is likely to be hydrogen peroxide as co-incubation with the enzyme catalase significantly reduces the conversion of supercoiled to open circular plasmid (Figure 4). These

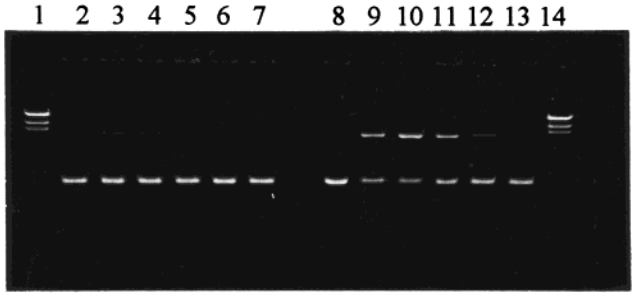


Figure 4. Single-strand breaks in plasmid DNA following the reduction of selected indolequinones by human DT-diaphorase in the presence (lanes 2−7; 1 200 units/reaction) and absence (lanes 8−13) of the enzyme catalase. Lanes 1 and 14, molecular weight markers (λDNA digested with *Hin*dIII); lanes 2 and 8, control plasmid DNA; lanes 3 and 9, **3c**; lanes 4 and 10, **3b**, lanes 5 and 11, EO9; lanes 6 and 12, EO7; lanes 7 and 13; EO8.

findings are consistent with studies by Butler et al.[30] which demonstrate that the hydroquinone form of EO9 is unstable in air resulting in the production of hydrogen peroxide. Chemosensitivity studies using cell lines with high (A549), intermediate (HT-29), and low (BE) levels

Table 2. Response of DT-Diaphorase-Rich (A549 and HT-29) and -Deficient (BE) Cells to Selected Indolequinones

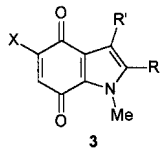
	IC_{50} (μM)[a]			selectivity ratio IC_{50}	
compd	A549	HT-29	BE	BE/A549	BE/HT-29
3a	14.77 ± 1.09	26.0 ± 2.05	24.11 ± 8.88	1.63	0.92
3b	0.033 ± 0.014	0.039 ± 0.01	21.71 ± 9.44	657.8	556.6
3c	0.28 ± 0.01	1.00 ± 0.21	18.84 ± 6.39	67.28	18.84
3d	>40	>40	>40		
EO9	0.059 ± 0.021	0.124 ± 0.06	1.25 ± 0.11	21.1	10.08
4a	0.787 ± 0.207	1.19 ± 0.25	13.95 ± 6.75	17.7	11.68
3e	0.18 ± 0.13	0.715 ± 0.12	13.77 ± 11.12	76.5	19.25
4b	0.741 ± 0.186	2.17 ± 0.54	1.83 ± 0.62	2.46	0.84
3f	0.069 ± 0.029	0.062 ± 0.01	18.69 ± 10.84	270.8	301.4
3g	>40	>40	35.44 ± 2.57	<1	<1
3h	0.8 ± 0.05	1.38 ± 0.32	>40	>50	>28.9
3i	>40	>40	>40		
3j	>40	13.53 ± 2.1	>40		>2.95
EO7	>40	>40	>40		
3k	1.34 ± 0.23	2.07 ± 1.05	3.81 ± 0.78	2.84	1.84
3n	2.10 ± 0.15	2.43 ± 0.89	1.70 ± 0.52	0.81	0.69
EO8	1.84 ± 0.65	6.84 ± 1.25	9.98 ± 1.65	5.42	1.45
3q	>40	>40	>40		
3l	0.50 ± 0.10	0.90 ± 0.11	0.31 ± 0.12	0.62	0.34
3p	1.02 ± 0.15	1.53 ± 0.21	0.36 ± 0.09	0.35	0.23
3o	0.73 ± 0.12	0.78 ± 0.22	0.61 ± 0.21	0.83	0.78
3m	3.53 ± 0.76	5.73 ± 0.29	1.04 ± 0.15	0.29	0.18

[a] DT-diaphorase activity for A549, HT-29, and BE cells was 1800 ± 122, 377.1 ± 52.1, and 0 nmol of DCPIP reduced/min/mg of protein, respectively. Each value represents the mean of three independent experiments ± standard deviations.

of DT-diaphorase activity demonstrate two key points in terms of structure−activity relationships. First, there is a poor correlation between substrate specificity (and extent of DNA damage) and potency in vitro. The most potent compounds against DT-diaphorase-rich cell lines in vitro are those which have the aziridinyl function at R (i.e. **3b**, **3c**, EO9, **3e, 3f**). The inclusion of a methylaziridinyl group generally results in a decrease in potency, whereas a methoxy group at R results in a significant decrease in potency (Table 2). These results do not correlate with the extent of single-strand breaks induced following reduction by DT-diaphorase (Figure 3) suggesting that single-strand breaks in the DNA form a minor component of the compounds mechanism of action in cells. This discrepancy between substrate specificity, single-strand breaks, and potency in vitro suggests that alkylation of DNA may be important, and further studies to characterize the type of DNA damage induced in DT-diaphorase-rich cells are required. Second, the data in Table 2 also suggests that the aziridinyl group at the 5-position (X) has a significant bearing upon selectivity for DT-diaphorase-rich cells in vitro. Selectivity ratios are generally high for compounds which have an aziridinyl function at R with **3b** and **3f** having particularly high selectivity ratios compared with EO9 (Table 2). Selectivity ratios for compounds with the 5-methylaziridinyl are relatively low, whereas compounds with the 5-methoxy have poor selectivity ratios (Table 2). Based upon these studies, therefore, it would appear that while the aziridinyl group at the 5-position is not essential in order for the compound to act as a substrate for DT-diaphorase, it is desirable in terms of conferring both potency and selectivity for DT-diaphorase-rich cells in vitro under aerobic conditions. The increased reactivity of the aziridine moiety due to increased pK_a following reduction is a likely key factor.

As stated previously, EO9 is an unusual bioreductive drug in that it has the ability to kill either the aerobic fraction of DT-diaphorase-rich tumors or the hypoxic fraction of DT-diaphorase-deficient tumors.[6] While this feature of EO9's biological properties may be considered desirable, it is difficult to determine whether activity in vivo is due to killing of the aerobic or hypoxic fraction. In terms of obtaining proof of principle, it would be beneficial if compounds were developed which did not have this 'dual personality' and targeted either the aerobic or hypoxic fraction of tumors exclusively. Previous studies[19,21] have focused on the ability of indolequinones to act as hypoxia-selective agents, and the structural features of selected compounds which are hypoxia-selective or selective for DT-diaphorase-rich cells under aerobic conditions are presented in Table 3. In terms of hypoxia selectivity, the presence of a 5-aziridinyl or a 5-(2-methylaziridinyl) and a combination of 5-methoxy with either 3-(carbamoyloxy)methyl or 2-cyclopropyl groups are desirable.[19] The most effective compounds were the 2-cyclopropyl and 5-(2-methylaziridinyl) derivatives, two of which (**3e** and **3h**) demonstrated activity against RIF-1 and KHT tumors in vivo both as single agents and in combination with radiotherapy.[19] Both compounds are however good substrates for DT-diaphorase, and **3e** in particular is selectively toxic toward DT-diaphorase-rich cell lines in vitro under aerobic conditions (Table 2). These compounds therefore have similar characteristics to EO9, and activity in vivo will depend on both oxygenation status and DT-diaphorase activity. In terms of hypoxia selectivity, modifications at the 3-position are desirable since increasing the steric bulk significantly reduces substrate specificity for DT-diaphorase. Compounds **3k** and **3n** which have 3-(carbamoyloxy)methyl substituents are very poor substrates for DT-diaphorase, are not selectively toxic toward DT-diaphorase-rich cells, but have high HCR values compared with EO9 (Table 3). These compounds should therefore behave as classical bioreductive drugs and have the potential to target the hypoxic fraction of solid tumors, irrespective of DT-diaphorase activity. Substitutions at the 5-position may also have a bearing upon hypoxia selectivity. In the case of EO9 and EO8, for example, replacing the 5-aziridinyl

Table 3. Structural Requirements That Determine Selectivity for DT-Diaphorase-Rich Cells under Aerobic Conditions and Hypoxia Selectivity of Selected Indolequinones



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compd	X	R′	R	substrate specificity	selectivity ratio for aerobic cells[a]	HCR[b]
3b	Az	CH_2OH	H	52.7	657.8	15.4[c]
3c	Az	H	$CH{=}CHCH_2OH$	41.6	67.3	4.2[d]
EO9	Az	CH_2OH	$CH{=}CHCH_2OH$	19.9	21.1	47.5[d]
3e	Az	CH_2OH	*c*-Pr	14.9	76.5	103.5[c]
3f	Az	CH_2OH	CH_3	9.9	270.8	12.8[c]
3h	2-MeAz	CH_2OH	CH_3	7.4	>50	188[c]
3k	OMe	$CHOCONH_2$	$CH(CH_3)_2$	0.82	2.84	178.6[c]
3n	OMe	$CHOCONH_2$	*c*-Pr	0.34	0.81	65.9[c]
EO8	2-MeAz	CH_2OH	$CH{=}CHCH_2OH$	1.5	5.42	5245[d]

[a] Selectivity ratio = IC_{50} BE/IC_{50} A549. [b] HCR = hypoxic cytotoxicity ratio determined in V79-379A cells where. [c] Naylor et al.[19] [d] Jaffar et al.[21]

group of EO9 with a 5-(methylaziridinyl) group significantly reduces substrate specificity for DT-diaphorase and selectivity toward aerobic DT-diaphorase-rich cells but results in a dramatic increase in HCR values. Similarly, comparisons **3f** and **3h** (both of which are good substrates for DT-diaphorase) suggest that the presence of a 5-aziridinyl group confers selectivity for DT-diaphorase-rich cells under aerobic conditions (SR = 270.8 and HCR = 12.8, **3f**), whereas a 5-(2-methylaziridinyl) group confers both selectivity for DT-diaphorase-rich cells and hypoxia selectivity (SR = >28.9 and HCR = 188, **3h**). Structural requirements which confer good selectivity for DT-diaphorase-rich cells in air and have low HCR values are not as clear, although **3d**, **3c**, and **3f** have high selectivity ratios in vitro but have low HCR values (HCR < 15) compared with EO9 (HCR = 47.5). In terms of identifying compounds which should preferentially target either the aerobic fraction of DT-diaphorase-rich tumors, compounds such as **3d**, **3c**, and **3f** would be most appropriate. Compounds **3k**, **3n**, and EO8 on the other hand should be selected as potential hypoxia-selective agents. The distinction between targeting either the hypoxic or aerobic fraction is desirable not only in terms of obtaining proof of principle but also for the design of subsequent in vivo studies (i.e. hypoxia-selective agents should be tested in combination with radiotherapy).

In conclusion, the results of this study have identified several novel analogues of EO9 which are good substrates for DT-diaphorase, bioactivated to DNA-damaging species following reduction by DT-diaphorase, and selectively toxic toward DT-diaphorase-rich cell lines in vitro. As in previous studies,[10,21,25] minor changes in structure have a significant impact upon substrate specificity and chemosensitivity with the aziridine ring at R playing a particularly prominent role in determining both potency and selectivity toward DT-diaphorase-rich cells in vitro. Compounds **3c**−**3f** have better properties than EO9 in terms of selectivity for DT-diaphorase-rich cells in vitro, and further evaluation of these compounds is warranted. In terms of identifying compounds which exclusively target DT-diaphorase-rich cells under aerobic conditions, **3d**, **3c**, and **3f** have high selectivity ratios with low HCR values. To exclusively target the hypoxic fraction of tumors, modifications at the 3-position result in compounds which are poor substrates for DT-diaphorase (e.g. **3k** and **3n**) but have high HCR values. Based upon these studies, it is possible to identify compounds which are distinct from EO9 in that they have the potential to kill either aerobic, DT-diaphorase-rich cells or hypoxic cells (where DT-diaphorase activity is irrelevant) with very little overlap between the two properties. Further evaluation of these compounds to determine whether they are pharmacologically superior to EO9 in terms of drug delivery is required.

Experimental Section

Chemistry. Melting points were recorded on Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 270 MHz on a JEOL JNMR-EX270 270-MHz spectrometer using tetramethylsilane as the internal standard. Elemental analyses were determined by Butterworth Laboratories Ltd., U.K. Thin-layer chromatography and flash column chromatography were performed on silica gel aluminum-backed plates (60 F_{256}) and silica gel 60 (230−400 mesh, Merck grade), respectively. High-resolution mass spectra on chromatographically homogeneous compounds were recorded on a fusons VG Trio 2000 mass spectrometer. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

5-Methoxy-1-methyl-4-nitroindole-2-carboxylic Acid (6). Aqueous sodium hydroxide (0.20 g, in 2 mL H_2O) was added to silver(I) oxide (0.23 g, 1 mmol) and the suspension was heated at 50 °C. The indole-3-carboxaldehyde (**5**) (0.73 g, 1 mmol) was then added and heating was continued for 0.25 h. The cooled mixture was then filtered and the filtrate acidified with HCl (3 M, 10 mL). The product was extracted with EtOAc (2 × 75 mL), washed with brine (50 mL), dried ($MgSO_4$), and evaporated in vacuo to give a yellow solid which was chromatographed (eluting with hexane/EtOAc, 1:1, and then EtOAc) to give **6** (180 mg, 72%) as a yellow solid: mp 285−286 °C; ^{1}H NMR (DMSO-d_6) δ 7.93 (1H, s, 3-*H*), 7.77 (1H, d, J = 9.0 Hz, 7-*H*), 7.28 (1H, d, J = 9.0 Hz, 6-*H*), 4.11 (3H, s, OCH_3), 4.01 (3H, s, NCH_3).

5-Methoxy-1-methyl-4-nitroindole-2-carbonyl Chloride (7). To a suspension of **6** (500 mg, 2.0 mmol) in THF (10 mL) was added $SOCl_2$ (4 mL) and the mixture was heated at reflux for 0.25 h. Excess $SOCl_2$ was removed to give the acid chloride **7** (400 mg, 75%) as a yellow solid: mp 176−178 °C; HRMS found (M^+) 268.0250, $C_{11}H_9N_2O_4Cl$ requires M 268.0251.

***N*-(2-Morpholin-4-ylethyl)-5-methoxy-1-methyl-4-nitroindole-2-carboxamide (8a).** To a stirred solution of crude **7** (400 mg, 1.5 mmol) in THF (10 mL) was added 4-(2-

aminoethyl)morpholine (2.0 g, 1.5 mmol). After 2 min the mixture was reduced in vacuo and the product was chromatographed (eluting with EtOAc/MeOH, 1:1) to give **8a** (440 mg, 81%) as a yellow solid: mp 186−187 °C; R_f = 0.64 (EtOAc/MeOH, 2:1); ^{1}H NMR ($CDCl_3$) δ 7.58 (1H, d, J = 9.0 Hz., 7-*H*), 7.18 (1H, s, 3-*H*), 7.12 (1H, d, J = 9.0 Hz, 6-*H*), 6.92 (1H, br s, CON*H*), 4.08 (3H, s, OCH_3), 4.03 (3H, s, NCH_3), 3.76 (4H, t, morpholine-OCH_2), 3.56 (2H, dt, J = 6.0 Hz, CON(H)CH_2), 2.62 (2H, t, J = 6.0 Hz, NCH_2), 2.53 (4H, t, J = 4.0 Hz, 2 × morpholine NCH_2); HRMS found (M^+) 362.1590, $C_{17}H_{22}N_4O_5$ requires M 362.1590.

When 1-(2-aminoethyl)piperidine was used, this gave *N*-(2-piperidin-1-ylethyl)-5-methoxy-1-methyl-4-nitroindole-2-carboxamide (**8b**) (60%) as an orange solid: mp 135−136 °C; ^{1}H NMR ($CDCl_3$) δ 7.61 (1H, d, J = 8.1 Hz, 6-*H*), 7.12 (2H, m, 7-*H*, 3-*H*), 4.08 (s, 3H, OCH_3), 4.02 (3H, s, NCH_3), 3.63 (2H, dd, J = 6.3 Hz, CON(H)CH_2), 2.66 (6H, m, 3 × NCH_2), 1.64 (6H, m, 3 × piperidine CH_2).

***N*-Ethyl-*N*-(2-morpolin-4-ylethyl)-4-amino-5-methoxy-1-methylindole-2-carboxamide (9a).** To **8a** (360 mg, 1.0 mmol) in EtOH (120 mL) was added tin powder (820 mg, 7.0 mmol) followed by HCl (2M, 20 mL). The mixture was stirred at room temperature for 1.5 h. Excess tin was removed by filtration; the filtrate was neutralized (NaOH, 150 mL), extracted with EtOAc (3 × 150 mL), dried ($MgSO_4$), and evaporated in vacuo to give **9a** (320 mg, 99%). This material was used in the next step without further purification.

***N*-(2-Piperidin-1-yl)-4-amino-5-methoxy-1-methylindole-2-carboxamide (9b).** The title compound (99%) was synthesized as described for **9a**.

***N*-(2-Morpholin-4-ylethyl)-5-methoxy-1-methyl-4,7-dioxoindole-2-carboxamide (10a).** To crude **9a** (320 mmol) dissolved in acetone (50 mL) was added a solution of potassium nitrosodisulfonate (Fremys salt) (2.0 g, 7 mmol) in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 6, 25 mL). The mixture was stirred for 0.5 h at room temperature. Excess acetone was removed and the residue neutralized with saturated sodium bicarbonate (100 mL), extracted with EtOAc (3 × 100 mL), washed with brine (50 mL), and evaporated to dryness. The product was purified by chromatography (eluting with EtOAc/MeOH, 1:1) and recrystallized from EtOAC to give **10a** (190 mg, 57%) as an orange solid: mp 206−207 °C; ^{1}H NMR ($CDCl_3$) δ 6.94 (1H, s, 3-*H*), 6.69 (1H, br s, CON*H*) 5.77 (2H, s, 5-*H*), 4.29 (3H, s, OCH_3), 3.86 (3H, s, NCH_3), 3.77 (4H, t, J = 4.6 Hz, 2 × morpholine OCH_2), 3.53 (2H, q, J = 5.28 Hz, CON(H)CH_2), 2.61 (2H, t, J = 5.94 Hz, NCH_2), 2.59 (4H, m, 2 × morpholine NCH_2); HRMS found (M^+) 347.1478, requires $C_{17}H_{21}N_3O_5$ M 347.1481.

***N*-(2-Piperidin-4-ylethyl)-5-methoxy-1-methyl-4,7-dioxoindole-2-carboxamide (10b).** The title compound (63 mg, 62%) was prepared from **9b** as described for **10a** as an orange solid: mp 197−198 °C; ^{1}H NMR ($CDCl_3$) δ 6.89 (1H, s, 3-*H*), 5.75 (2H, m, CON*H*, 5-*H*), 4.28 (3H, s, OCH_3), 3.84 (3H, s, NCH_3), 3.55 (2H, m, CON(H)CH_2), 2.57 (6H, m, 3 × NCH_2), 1.75 (6H, m, 3 × piperidineCH_2). Anal. ($C_{18}H_{23}N_3O_4$·0.33H_2O) C, H, N.

***N*-(2-Morpholin-4-ylethyl)-5-(2-methylaziridin-1-yl)-1-methyl-4,7-dioxoindole-2-carboxamide (4a).** Compound **10a** (80 mg, 0.23 mmol) was dissolved in 2-methylaziridine (0.5 mL, ca. 11 mmol; **CAUTION!**) and the solution stirred at room temperature for 2.5 h, evaporated, and redissolved in EtOAc (5 mL). The solution was evaporated to dryness and chromatographed (MeOH/EtOAc, 1:1). The residue was recrystallized from EtOAc to give **4a** (60 mg, 70%) as a red solid: mp 145−146 °C; ^{1}H NMR ($CDCl_3$) δ 6.88 (1H, s, 3-*H*), 6.62 (1H, br s, CON*H*) 5.86 (2H, s, 5-*H*), 4.27 (3H, s, NCH_3), 3.74 (4H, t, J = 4.0 Hz, 2 × morpholine OCH_2), 3.49 (2H, q, J = 6.0 Hz, CON(H)CH_2), 2.58 (2H, t, J = 5.94 Hz, NCH_2), 2.50 (4H, t, 2 × morpholine NCH_2), 2.25 (1H, m, aziridine-*H*), 2.13 (2H, dd, aziridine-CH_2), 1.43 (3H, d, J = 5.0 Hz, aziridine-CH(CH_3)); HRMS found (M^+) 372.1807, requires $C_{19}H_{24}N_4O_4$ M 372.1797.

***N*-(2-Piperidin-1-ylethyl)-5-(2-methylaziridin-1-yl)-1-methyl-4,7-dioxoindole-2-carboxamide (4b).** The title compound (46%) was prepared as described for **4a** as an orange solid: mp 128−129 °C; ^{1}H NMR ($CDCl_3$) δ 7.18 (1H, br s, CON*H*), 6.90 (1H, s, 3-*H*), 6.00 (1H, s, 5-*H*), 4.23 (3H, s, NCH_3), 3.66 (2H, q, CON(H)CH_2), 2.79 (2H, t, NCH_2), 2.56 (4H, m, 2 × piperidine NCH_2), 2.36 (1H, m, aziridine C*H*), 1.62 (4H, m, 2 × piperidine CH_2), 1.48 (2H, m, aziridine CH_2), 1.44 (3H, d, J = 5.6 Hz, aziridine CH(CH_3)). Anal. ($C_{20}H_{26}N_4O_3$·0.5H_2O) C, H, N.

Biology. Recombinant human DT-diaphorase derived from full length H460 NSCLC cDNA inserted into the pKK-233 plasmid and its subsequent purification by Cibacron Blue affinity chromatography are described elsewhere.[10] NADH, cytochrome *c*, EDTA, and DCPIP (2,6-dichlorophenol-indophenol) were obtained from Sigma (Sigma Aldrich, Poole, U.K.). All cell culture material was obtained from Life Technologies (Paisley, U.K.). All drugs were dissolved in DMSO at a concentration of 5 mM and stored at −80 °C.

Substrate Specificity. The ability of compounds to serve as substrates for human DT-diaphorase was determined by measuring the reduction of cytochrome *c* at 550 nm on a Beckman DU 650 spectrophotometer, details of which are described elsewhere.[10] Briefly, all enzyme assays contained cytochrome *c* (70 μM), NADH (2 mM), purified DT-diaphorase (0.48 μg), and the test compound (25 μM) in a final volume of 1 mL of Tris-HCL buffer (50 mM, pH 7.4) containing 0.14% bovine serum albumin. The final concentration of DMSO in the cuvette was 0.5%. Reactions were carried out at room temperature and started by the addition of NADH. Rates of reduction were calculated from the initial linear part of the reaction curve (30 s) and results were expressed in terms of μmol of cytochrome *c* reduced/min/mg of protein using a molar extinction coefficient of 21.1 mM^{-1} cm^{-1} for cytochrome *c*. All reactions were carried out in triplicate and background rates of cytochrome *c* reduction (in the presence of 0.5% DMSO) were subtracted from test results.

Inhibition of DT-Diaphorase by Indolequinones. The ability of indolequinones to inhibit human DT-diaphorase was determined spectrophotometrically as described elsewhere.[31] Briefly, compounds (at a range of concentrations) were incubated with DT-diaphorase (1 μg) in Tris-HCl (25 mM, pH 7.4) plus Tween 20 (2 mg/mL) buffer containing NADH (100 μM) at room temperature for 30 min (reaction volume was 0.5 mL). Reactions were stopped by the addition of 2 mL of ice-cold Tris-HCl (25 mM, pH 7.4) containing sucrose (250 mM) and DT-diaphorase activity was determined using a standard DT-diaphorase assay.[32] In this assay 1 μL of the above stopped reaction mix was added to 1 mL of buffer (Tris HCl, 25 mM, pH 7.4, plus 0.7 mg/mL BSA) containing 40 μM DCPIP and 200 μM NADH. The reaction was performed at room temperature and DCPIP reduction was determined at 600 nm over 30 s. Inactivation of DT-diaphorase was determined by calculating the difference between ΔA/min for reactions containing drug and control (no drug).

DNA-Damaging Assays: Single-Strand Breaks. Bioreductive activation of EO9 to metabolites capable of inducing single-strand breaks in DNA was determined by measuring the conversion of supercoiled plasmid DNA to the open circular form as described elsewhere.[8] Briefly, pRSETB plasmid was isolated from *E. coli* (JM 109) by small-scale plasmid preparations.[33] Each reaction contained test compound (4 μM, final DMSO concentration = 0.2%), DNA (2 μg), NADH (10 mM), and DT-diaphorase (0.48 μg) in a final volume of 100 μL of sodium phosphate buffer (100 mM, pH 7.4) containing 0.14% bovine serum albumin. Following incubation at 37 °C for 1 h, 25 μL of stop buffer (60% glycerol, 0.5% SDS, 5 mM EDTA, 0.01% bromophenol blue) was added to each sample prior to loading 50 μL into a 1% agarose gel. Following electrophoresis (100 V for 3−4 h), DNA was visualized by ethidium bromide staining and gels were photographed under UV transillumination. Selected compounds were evaluated in the presence and absence of the enzyme catalase (1200 units/reaction) according to the methodology described above.

Cell Culture and Chemosensitivity Assays. A549 (human NSCLC) and HT-29 (human colon carcinoma) were

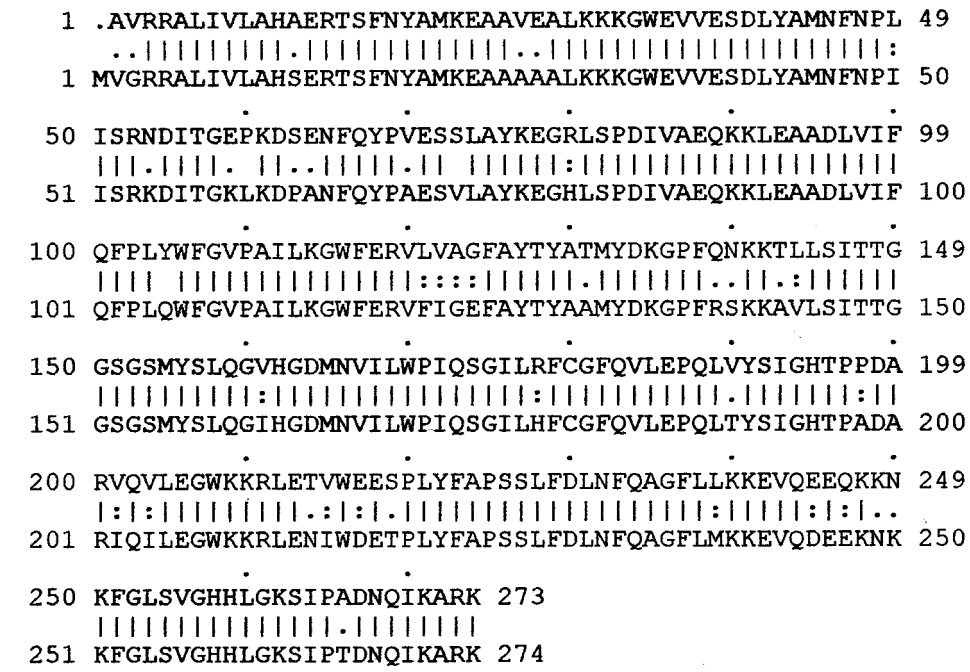

Figure 5. Sequence alignment of rat and human DT-diaphorase. The upper and lower sequences represent rat and human DT-diaphorase, respectively. There is 91.57% similarity and 85.34% identify between the rat and human DT-diaphorase sequences.

obtained from ATCC and routinely maintained as monolayer cultures in RPMI 1640 culture medium supplemented with 10% fetal calf serum, sodium pyruvate (1 mM), l-glutamine (2 mM), penicillin/streptomycin (50 IU/mL/50 μg/mL), and buffered by HEPES (25 mM). BE cells (human colon carcinoma) were a gift from Dr. T. Ward (Paterson Institute, Manchester, U.K.) and were routinely maintained as monolayer cultures as described above. Cells were plated into 96-well culture plates ((1−2) × 10^3 cells/well) and incubated overnight at 37 °C in an atmosphere containing 5% CO_2. Drugs were added to each well of the plate (8 wells/drug exposure) and cells exposed to the drug for 2 h at 37 °C. The final concentration of DMSO was 0.1% in all cases. Following drug exposure, cells were washed twice with HBSS prior to the addition of RPMI 1640 (200 μL/well). Cell survival was determined 5 days later using an MTT assay, details of which are described elsewhere.[34] DT-diaphorase activity was determined by measuring the dicumarol-sensitive reduction of DCPIP using NADH as the electron donor as described elsewhere.[32]

Modeling Human DT-Diaphorase. Human DT-diaphorase was modeled with homology to the available rat DT-diaphorase[29] structure (Brookhaven Protein Databank entry 1QRD) including tetramethyl-1,4-benzoquinone (duroquinone), Cibacron Blue (a potent inhibitor), and FAD. The physiological dimer was created from the PDB coordinates using appropriate transformations. The human and rat DT-diaphorase amino acid sequences had 85% identity and 91% similarity with a single amino acid insertion at the N-terminus (Figure 5). The additional amino acid was added to the structure and side chain mutations were performed. Particular attention was payed to the active-site residues, where it was noted that all the residues were identical in the rat and human DT-diaphorase sequences and hence retained the same coordinates in both the rat structure and the human model (Figure 5). The model was minimized with the substrate, inhibitor, and cofactor constrained to their spatial positions. Partial charges for the inhibitor, cofactor, and substrate atoms were determined from ab initio calculations at the STO-3G level. The complex was minimized using the CHARMM force field.[35] All calculations were performed on a Silicon Graphics workstation. The resulting structure was used as a starting model for docking simulations.

Modeling Enzyme−Substrate Complexes. Optimal drug binding orientations were identified using the program GRID.[36] Calculations were performed on the minimized human DT-diaphorase model following the removal of the duroquinone (DQN) substrate and Cibacron Blue (CBD) inhibitor from the model. GRID contours for the aromatic carbon probe indicated that the aromatic moieties of the substrates were likely to protrude in only one direction within the binding site. This direction led to the space previously occupied by the inhibitor. Additional GRID probes were also used in determining binding orientations. Using these GRID contours as a guide together with the superimposed position of DQN, substrates were docked into the enzyme at one of the active sites.

Each enzyme−substrate complex then underwent a minimization protocol of 500 steps steepest descent (SD) on hydrogens alone, 5 000 steps SD minimization on side chains, $FADH_2$, and substrate, and finally 15 000 steps adopted-basis Newton−Raphson (ABNR) minimization on side chains, $FADH_2$, and substrate. The final structures were visualized in order to identify any interactions which could explain the experimental results regarding sensitivity to substrate modifications.

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