Design, Synthesis, and Biological Evaluation of Indolequinone Phosphoramidate Prodrugs Targeted to DT-diaphorase

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A series of 2- and 3-substituted indolequinone phosphoramidate prodrugs targeted to DT-diaphorase (DTD) have been synthesized and evaluated. These compounds are designed to undergo activation via quinone reduction by DTD followed by expulsion of the phosphoramide mustard substituent from the hydroquinone. Chemical reduction of the phosphoramidate prodrugs led to rapid expulsion of the corresponding phosphoramidate anions in both series of compounds. Compounds substituted at the 2-position are excellent substrates for human DTD ($k_{cat}/K_M = (2-5) \times 10^6$ M^{-1} s^{-1}); however, compounds substituted at the 3-position are potent inhibitors of the target enzyme. Both series of compounds are toxic in HT-29 and BE human colon cancer cell lines in a clonogenic assay. There was a correlation found between cytotoxicity and DTD activity for the 2-series of phosphoramidates; however, there was no correlation between cytotoxicity and DTD activity in the 3-series of compounds. This finding suggests the presence of an alternative mechanism for the activation of these compounds.

Introduction

DT-diaphorase (E.C. 1.6.99.2, reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H):quinone acceptor oxidoreductase) is an obligate two electron quinone reductase. This flavoenzyme can utilize either NADH or NADPH to catalyze the reduction of various quinones. DT-diaphorase (DTD) is reported to play a protective role in the detoxification of various quinone-containing compounds.[1−3] Two electron reduction of quinones by this enzyme circumvents formation of semiquinone radicals and reactive oxygen species that can cause cellular damage.[1,3] DTD has also received a great deal of attention as a target for anticancer prodrug development and has been implicated in the bioreductive activation of mitomycin C and EO9, in addition to other quinone-containing compounds.[4,5] This enzyme is overexpressed in various types of cancers[6] and therefore offers the potential for selective prodrug activation and consequently selective tumor cell kill.[4]

We have previously exploited this enzyme for the selective activation of naphthoquinone- and benzimidazolequinone-containing phosphoramidate prodrugs.[7] In this approach, the phosphoramide esters themselves are inactive, and it was hypothesized that intracellular reduction of the parent compounds would result in release of a cytotoxic phosphoramide mustard capable of cross-linking DNA. Naphthoquinone phosphoramidates displayed low micromolar cytotoxicity when evaluated in vitro. These compounds were excellent substrates for the target enzyme ($k_{cat}/K_M = 2 \times 10^7$ to 3×10^8 M^{-1} s^{-1}); however, there was no correlation between DTD activity and cytotoxicity in vitro. The benzimidazolequinone phosphoramidates were also excellent substrates for DTD ($k_{cat}/K_M = 3-4.5 \times 10^7$ M^{-1} s^{-1}), but these compounds were 1−2 orders of magnitude less potent when evaluated in vitro. NMR experiments revealed that reduction of the benzimidazolequinone moiety did not result in release of the phosphoramidate anion as expected. The failure of these compounds to undergo activation is believed to be responsible for the observed lack of cytotoxicity.

The indolequinone nucleus has been studied extensively as a model for bioreductive prodrug development,[8−13] and structure−activity relationship (SAR) analysis of the indolequinone moiety and the molecular features relating to substrate specificity for human DTD have been examined.[14] Recently, it has been demonstrated that substitution with a potential leaving group at the 3-position of the indolequinone moiety is a viable means for bioreductive drug delivery.[15−17] In this approach, reductive elimination of the substituent at the 3-position results in activation of this substituent. Unfortunately, data from the SAR analysis led to the conclusion that substitution at the 3-position of the indolequinone nucleus with bulky leaving groups results in poor substrates, some of which inactivate the enzyme.[12,14,18] This is an undesirable feature for cytotoxic agents that are designed to be selectively activated by DTD. Another conclusion from this analysis was that modification of the 2-position of the indolequinone nucleus is favorable in terms of substrate specificity for the enzyme.[14] More recent studies have demonstrated that substitution at the 2-position with bulky substituents results in greater metabolism by the enzyme.[12] In light of this SAR data, it was believed that a prodrug strategy utilizing bioreductive drug delivery of a cytotoxin from the 2-position of the indolequinone would result in compounds having a high correlation between DTD activity and in vitro cytotoxicity. Bioreductive drug delivery from the 2-position of the indolequinone nucleus was also previously examined.[15] In contrast to the 3-regioisomers, compounds substituted at the 2-position failed to undergo activation following reduction (γ-

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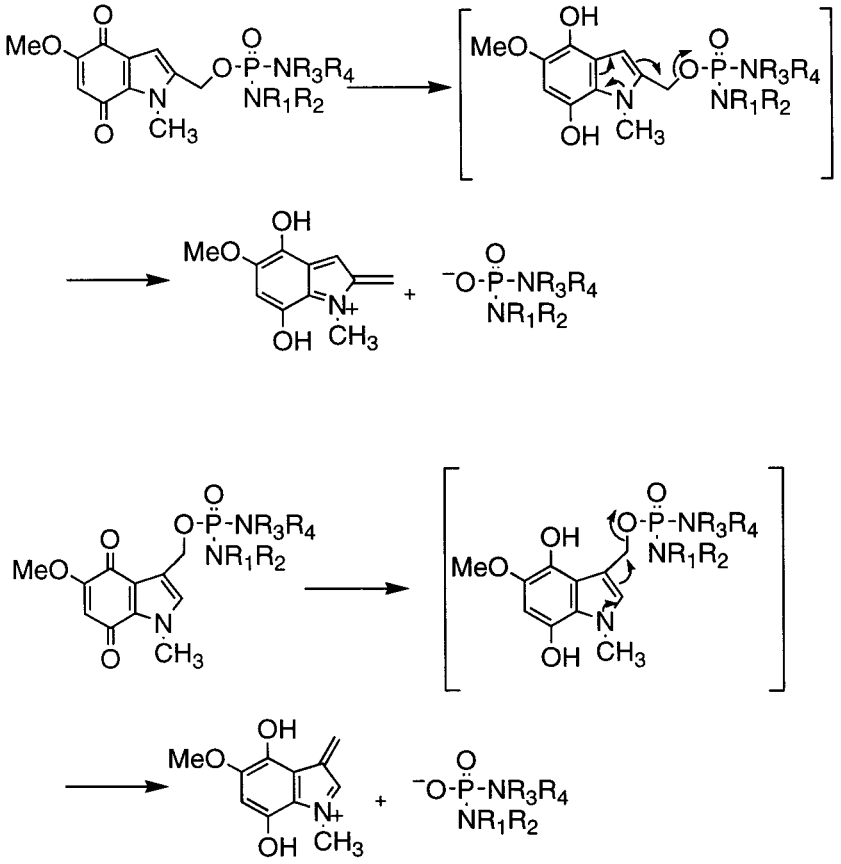
Scheme 1



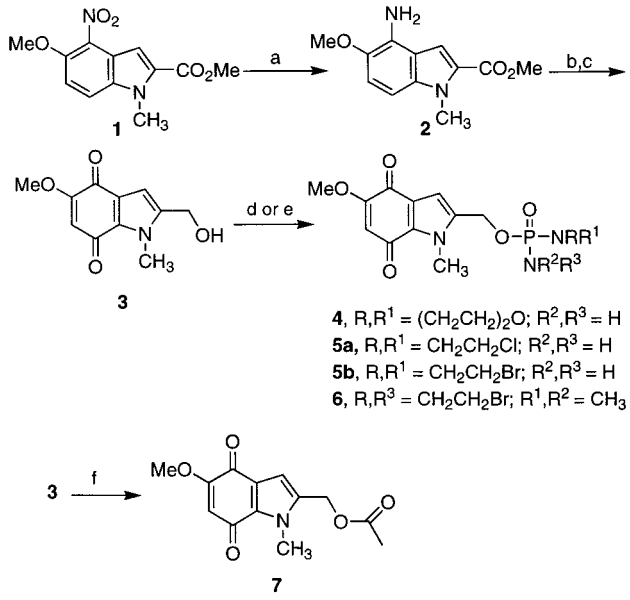
radiolysis). This led the authors to conclude that use of the indolequinone 2-position for drug delivery would not be a viable prodrug approach.

We have extended this indolequinone prodrug strategy to include the delivery of phosphoramide mustards from the 3-position. Because our desire was to prepare compounds that could be selectively activated by DTD, a series of 2-substituted indolequinone phosphoramidates were also prepared to evaluate the potential of this position for drug delivery. Herein, we report the synthesis, enzymatic activity, activation mechanism, and cytotoxicity of a novel series of indolequinone phosphoramidates.

Results and Discussion

Chemistry. The proposed mechanism for the activation of these prodrugs is shown in Scheme 1. Two electron reduction of the quinone moiety to the hydroquinone increases electron density on the indole nitrogen and leads to expulsion of a phosphoramidate anion. Expulsion from the parent quinone does not occur because the indole nitrogen is present as a vinylogous amide. The released phosphoramidate anion is capable of cross-linking DNA[19] and is hypothesized to be the active cytotoxic agent for this series of compounds. A series of 2- and 3-substituted indolequinone prodrugs containing an alkylating moiety were synthesized and evaluated. Additionally, the acetate (**7** and **11**) and morpholine phosphoramidate (**4** and **13**) compounds were prepared. These compounds do not contain an alkylating moiety and were designed to assess substituent effect on enzyme substrate activity, as well as to determine the contribution of the indolequinone moiety to the cytotoxicity of these compounds.

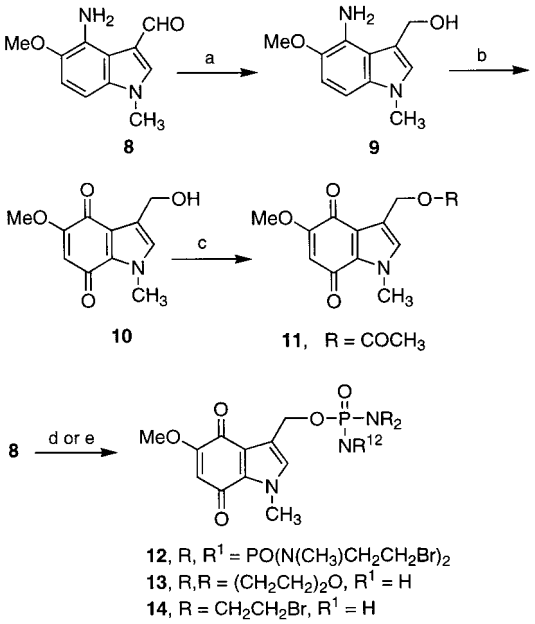
Scheme 2[a]



[a] Reagents: (a) Sn/HCl, EtOH. (b) LAH, THF. (c) Fremy's salt, $(CH_3)_2CO$, buffer. (d) (i) LHMDS; (ii) $POCl_2NRR^1$; (iii) NH_3. (e) (i) LHMDS; (ii) $POCl[NCH_3(CH_2CH_2Br)]_2$. (f) Ac_2O, $N(CH_2CH_3)_3$.

The synthesis of these compounds was carried out as depicted in Schemes 2 and 3. The 2-substituted indolequinone phosphoramidates were synthesized from **3**, which was prepared from 5-methoxyindole-2-carboxylic acid in five steps. First, 5-methoxyindole-2-carboxylic acid was methylated by treatment with sodium hydride

Scheme 3[a]



[a] Reagents: (a) LAH, THF. (b) Fremy's salt, buffer. (c) Ac_2O, $N(CH_2CH_3)_3$. (d) (i) LHMDS; (ii) $POCl_2NCH_3(CH_2CH_2Br)$; (iii) $HBrHNCH_3(CH_2CH_2Br$, *i*-Pr_2NEt). (e) (i) LHMDS; (ii) $POCl_2NRR$; (iii) NH_3.

followed by dimethyl sulfate.[20] Nitration of the methyl ester followed by reduction of the nitro substituent using either ammonium formate, Pd/C, or Sn/HCl yielded the corresponding amine intermediate **2**.[21,22] The ester moiety of this intermediate was then reduced using lithium aluminum hydride. The desired alcohol **3** was obtained by oxidation of the indole using Fremy's salt.[22] Phosphorylation of **3** to give **4** and **5a**,**b** was then carried out as previously described.[7] Briefly, the lithium alkoxide of **3** was formed and reacted with the corresponding phosphoryldichlorides.[7,23] Ammonia gas was then bubbled through the reaction mixture to yield **4** and **5a**,**b** after workup. The bis[*N*-methyl-*N*-(2-bromoethyl)]phosphoramidate **6** was prepared in a similar manner. The lithium alkoxide of **3** was formed and then reacted with bis[*N*-methyl-*N*-(2-bromoethyl)]phosphoramidic monochloride. Finally, the acetoxy compound **7** was prepared by reaction of **3** with acetyl chloride in the presence of triethylamine.

The 3-position regioisomers were prepared in a similar manner from indolequinone **10**. Intermediate **10** was initially synthesized from 5-methoxy-indole-3-carboxaldehyde as previously described.[22] Yields for the final step in this synthesis were low (40−50%); therefore, attempts were made to increase the yields for this sequence. It was found that overall yields for the preparation of **10** could be increased by reversing the order for the last two steps, such that aldehyde **8** is reduced to **9** prior to oxidation to the quinone. Once **10** was synthesized, compounds **11**, **13**, and **14** were prepared as described above for the corresponding 2-position regioisomers. The lithium alkoxide of **10** was much less reactive than the 2-regioisomer; therefore, **12** was prepared from the corresponding phosphoryldichloride. Briefly, the lithium alkoxide of **10** was reacted with [*N*-methyl-*N*-(2-bromoethyl)]phosphoramidic dichloride. Excess *N*-methyl-*N*-(2-bromoethyl)amine hydrobromide[23] and base were added to yield **12** after workup.

Table 1. Kinetic Analysis of Indolequinones[a]

compd	K_M (μM)	V_{max} (μM/s)	k_{cat} (1/s)	k_{cat}/K_M ($M^{-1}\,s^{-1} \times 10^{-6}$)
menadione[b]	1.5	0.85	960	63
3	12	0.69	77	6.7
4	2.8	0.045	4.9	2.0
5a	0.68	0.024	2.6	3.9
5b	0.29	0.014	1.6	5.3
6	0.55	0.01	1.28	2.3
7	8.2	0.27	30	3.7
10	4.1	1.25	136	33.3
11	0.74	0.035	4.0	5.4

[a] See Experimental Section for details of enzyme kinetics assays. [b] Enzyme assays with menadione were conducted using 1/10 of the enzyme concentration used for the other assays.

Enzyme Kinetics. The enzyme kinetic parameters for each compound were determined spectrophotometrically at 25 °C, using recombinant human DTD. The assay used was a standard coupled assay that monitors the absorbance change of cytochrome *c*.[24] Kinetic parameters were determined by Hanes analysis, using the initial linear reaction velocities. Hanes analysis was selected because some degree of substrate and product inhibition is observed at substrate concentrations near K_M as a consequence of the ping-pong mechanism of the enzyme. The results of these analyses are shown in Table 1. The 2- and 3-substituted indolequinones had very different results in this assay and therefore will be discussed separately. All of the 2-substituted indolequinones are excellent substrates for DTD (k_{cat}/K_M (2−5) $\times$ 10^6 M^{-1} s^{-1}) and do not significantly inhibit the enzyme at concentrations below K_M. The K_M values presented in Table 1 suggest that the affinity of these compounds for the enzyme increases as the size of the substituent at the 2-position increases. In contrast, turnover (k_{cat}) decreases with increased substituent size, perhaps as a result of the greater enzyme affinity and therefore a slower off rate. These data suggest that the enzyme tolerates various substituents at the 2-position, a finding consistent with data in the literature.[12,14]

The k_{cat}/K_M value for the 3-substituted acetoxy compound **11** is similar to the values obtained for compounds in the 2-series, and **11** does not significantly inhibit DTD at concentrations below K_M. Interestingly, indolequinone **10** was a superior substrate for DTD, having a much greater k_{cat}/K_M value than those seen for the other substrates. This is a consequence of the greater turnover by the enzyme for this compound, as the K_M is similar to those of the other compounds. In contrast, indolequinones substituted with phosphoramidates at the 3-position (**12**−**14**) are potent inhibitors of the enzyme (IC_{50} 670−820 nM, data not shown). From these data, it appears that the enzyme will tolerate small substituents at the 3-position; however, substitution with bulky leaving groups at this position leads to inactivation of the enzyme. We have demonstrated that this inhibition is NADH-dependent, suggesting that these compounds must be reduced by the enzyme prior to inhibition. Thus, it is likely that these compounds are acting as mechanism-based inactivators of DTD; however, additional experiments are required to determine the exact nature of this inhibition. This hypothesis is supported by recent data demonstrating that 5-meth-

Table 2. Indolequinone MTT 72 h Growth Inhibitory Activity[a]

compd	MDA231	PC-3	HT-29	PaCa-2	A498-2LM	A549	UMUC3
4	13	14	15	10	12	45	10
5a	0.87	0.26	0.21	0.031	0.98	0.022	0.010
5b	1.6	0.49	0.061	0.015	0.20	0.003	0.002
6	0.84	0.94	0.2	0.008	2.3	1.7	0.017
7	>38	>38	34	35	34	26	9.6
11	0.25	0.005	0.003	0.004	0.037	0.44	0.007
12	0.040	0.066	0.065	0.025	0.34	0.023	0.002
13	9.3	15	7.7	9.8	1.2	12	8.2
14	0.19	0.65	0.24	0.22	1.0	0.40	0.01
DTD activity (cyt c)[b]	4	500	1200	1400	1630	1700	200
DTD activity (DCPIP)[c]	0	50	200	160	240	980	30

[a] GI_{50} values expressed in micromolar; see Experimental Section for details of the MTT assay. [b] DTD activity expressed as nanomoles cytochrome *c* reduced/min/mg protein. [c] DTD activity expressed as nanomoles DCPIP reduced/min/mg protein.

oxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione is a mechanism-based inactivator of DTD.[25] These results imply that drug delivery from the 2-position may be a better prodrug strategy for targeting DTD.

In Vitro Growth Inhibition. The in vitro activity of these compounds against a panel of seven human solid tumor cell lines was determined using the MTT assay.[26] The results from these experiments are shown in Table 2. DTD activity for these cell lines was also determined using two different assay methods[24] (Table 2). It has been reported that use of the cytochrome *c*-coupled NADH:menadione oxidoreductase assay with cell lysates results in artifactually high levels of activity;[27] however, DTD activity obtained using this assay is included to facilitate comparison with other published values. Indolequinones that are substituted at either the 2- or 3-position with a phosphoramidate containing an alkylating group (**5a**,**b**, **6**, **12**, and **14**) were found to be broadly active inhibitors of tumor cell growth following 72 h of drug treatment. The greatest activity observed in the MTT assay was against the PaCa-2 and UMUC3 cell lines. On the other hand, phosphoramidates possessing a morpholine group (**4** and **13**) are 1−2 orders of magnitude less potent, suggesting that the alkylating moiety is essential for high potency. Similarly, acetoxy compound **7** did not significantly inhibit cell growth in this assay. These data imply that for the 2-series of compounds the alkylating moiety is required for significant growth inhibition and that the indolequinone portion of the molecule does not significantly contribute to growth inhibition in vitro. Acetoxy compound **11** is a potent inhibitor of cell growth in these cell lines. This result indicates that the indolequinone moiety may be the predominant growth inhibitor in the 3-series of compounds. The mechanism by which **11** exerts its growth inhibitory activity is not known at this time. It is interesting to note that growth inhibition has been seen for analogous acetoxy mitosenes.[20] It is important to point out that although some of these compounds are very potent inhibitors of cell growth, this in vitro activity cannot be directly correlated to DTD activity. This result suggests that there is an alternative mechanism of activation for these compounds. One possible mechanism might be reduction by NADPH:cytochrome P450 reductase and subsequent redox cycling.[28]

Compounds **5a**,**b**, **6**, **11**, and **12** were also evaluated in the NCI human tumor cell line in vitro screen. All of these compounds were found to be broadly active in this assay, with mean LC_{50} values between 3 and 29 μM. The compounds displayed some selectivity, with the greatest activity observed against the melanoma, renal, and central nervous system cell lines. Again, there was no direct correlation between in vitro activity and DTD activity.

Table 3. Clonogenic Survival of HT-29 and BE Cells[a]

compd	LC_{99}[b] HT-29 (μM)	LC_{99}[b] BE (μM)	selectivity BE/HT-29
4	>100	>100	N/A
5a	0.11	7.6	69
5b	0.077	4.2	55
6	0.35	2.0	6
7	>100	>100	N/A
11	0.54	5.3	10
12	0.07	0.14	2
13	>100	>100	N/A
14	0.24	0.47	2

[a] 2-h drug treatment; see Experimental Section for details of the clonogenic assay. [b] Concentration that reduces clonogenic survival by 2 log units (99%).

In Vitro Cytotoxicity. To assess the extent to which DTD contributes to the cytotoxicity of these compounds, clonogenic assays were conducted in HT-29 and BE human colon carcinoma cell lines. HT-29 cells have a high level of DTD activity, while the BE cell line has a C-to-T point mutation in the gene encoding for DTD that results in production of a mutant enzyme with no measurable activity.[29] Briefly, the cells were treated for 2 h in serum free media, washed, and plated. After 10 days, the viable cells were stained and cell colonies were counted. LC_{99} values were calculated from these data, and these results are shown in Table 3. Comparison of the data for **4** and **7** with **5a**,**b** and **6** indicates that an alkylating moiety is essential for cytotoxicity in this series of compounds. It also suggests that the indolequinone portion of the molecule does not significantly contribute to cytotoxicity. Examination of the results for **5a**,**b** in HT-29 and BE cells suggests that there is a correlation between in vitro cytotoxicity and DTD activity. These compounds are up to 69 times more potent in cells containing a high level of DTD activity; however, the demonstration of cytotoxicity in BE cells indicates that DTD is not a unique target for the activation of these compounds. Interestingly, compound **6** is only moderately selective for DTD in this assay. This unexpected result is a consequence of the decreased potency observed in HT-29 cells, as well as the increased potency in BE cells. Because compound **6** undergoes activation at a comparable rate and displays similar enzyme substrate activity to **5a**,**b**, one possible explanation for the decreased potency in HT-29 cells might be that the released phosphoramidate mustard is not as effective

a cross-linking agent as those released from **5a**,**b**. Results from the growth inhibition assays discussed above support the role of multiple enzymes involved in the activation of these prodrugs. Therefore, the relative increase in potency against BE cells may be a consequence of compound **6** being a better substrate than **5a** or **5b** for an alternative activating enzyme. Additional experiments are needed to test these hypotheses.

The alkylating moiety is also necessary for cytotoxicity of the phosphoramidates in the 3-series of compounds, as demonstrated by comparison of the data for **13** with **12** and **14**. It is interesting to note that **11** is also cytotoxic in this assay, although it is not as potent as the compounds containing an alkylating moiety. This perhaps suggests an additional role for the indolequinone in this series of compounds. In contrast to the 2-series of compounds, the correlation between DTD activity and cytotoxicity in the 3-series of compounds is less clear. Compounds that contain a phosphoramidate substituent at the 3-position are potent inhibitors of the enzyme and have no significant correlation between DTD activity and cytotoxicity. This finding again suggests an alternative target for the activation of these compounds. There is however a correlation between DTD activity and cytotoxicity for **11**, which interestingly is an excellent substrate for DTD.

Chemical Activation. It is hypothesized that the phosphoramidate anion is the active cytotoxic species for these prodrugs. The phosphoramidate anion must therefore be released rapidly following reduction so that the hydroquinone intermediate cannot diffuse out of the cell. Additionally, reoxidation of the hydroquinone moiety could occur if release is too slow. Either of these events would result in decreased selectivity and activity of these compounds. To ascertain if these prodrugs can liberate phosphoramidate anion rapidly following reduction of the quinone moiety, ^{31}P NMR experiments were carried out. Indolequinone phosphoramidates were chemically reduced with 3 equiv of sodium dithionite (1:1.5 CH_3CN/0.4 M cacodylate buffer, pH 7.4), and the reaction was monitored by ^{31}P NMR. Reactions were carried out at both room temperature and 37 °C for each of the compounds evaluated. Figure 1 is an example of a ^{31}P NMR stack plot showing the results from the dithionite reduction of **5b** at room temperature. The first spectrum, at 5 min, contains two resonances. The first resonance, at approximately −6 ppm, has the same chemical shift as the phosphoramidate **5b**. On the basis of the reductive environment of this experiment, this resonance is believed to be that of the corresponding hydroquinone of **5b**. The resonance for **5b** disappears with a half-life of 5 min at room temperature, with the concurrent appearance of the resonance at −12 ppm, which corresponds to the resonance for the phosphoramidate anion. These results confirm that the phosphoramidate is expelled from the 2-position following two electron reduction. The phosphoramidate anions for compounds containing haloethylamine moieties (**5a**,**b**, **6**, **12**, and **14**) are highly reactive species and undergo subsequent solvolysis.[7] Similar half-lives were found for compounds **4**, **5a**, and **6** at room temperature; however, activation of the 3-position regioisomers was more rapid. Activation was complete at the time the first spectrum was obtained (less than 5 min) for these analogues.

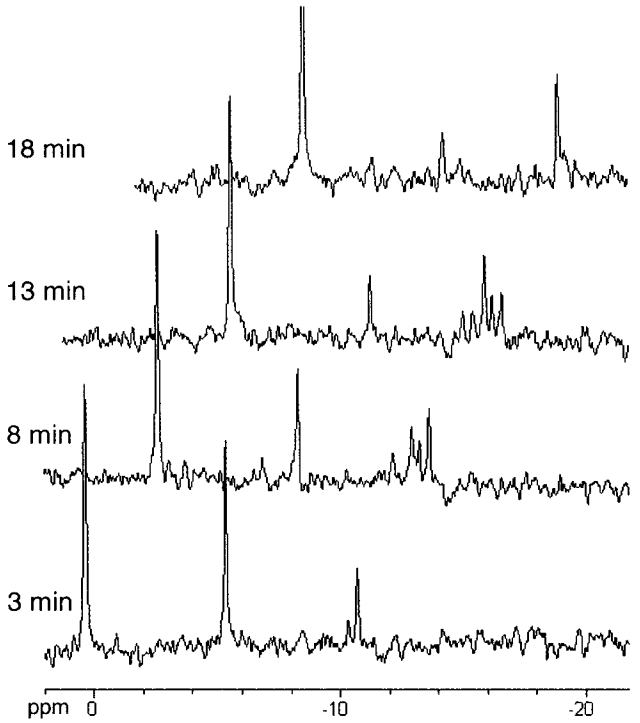


Figure 1. Reduction of indolequinone phosphoramidate **5b** with sodium dithionite (3 equiv, 1:1.5 CH_3CN:0.4 M cacodylate buffer, pH ∼7.4, room temperature). Chemical shifts are reported relative to the TPPO reference. See Results and Discussion for details.

Activation for all compounds was complete in less than 5 min for experiments carried out at 37 °C.

These results appear to be inconsistent with those previously reported. However, previous experiments investigating at the activation of 2-substituted prodrugs used a radiolytic one electron reduction, whereas a chemical two electron reduction was used for the activation experiments described here. It is therefore hypothesized that a one electron reduction is insufficient for activation of the 2-series of compounds and that a two electron reduction is required. Additional experiments to test this hypothesis are currently underway. However, it should be pointed out that these experiments utilized different types of leaving groups (carboxylate and propenyl); therefore, direct comparison may not be possible. In contrast, the 3-series of compounds undergoes activation following either a one or a two electron reduction. Because DTD is an obligate two electron reductase, selective activation of the 2-series of compounds following reduction by this enzyme should be feasible.

Conclusions

A series of indolequinone phosphoramidate prodrugs have been prepared as potential anticancer agents. Evaluation of these compounds in biological assays has led to the following conclusions. Both 2- and 3-substituted indolequinone phosphoramidates containing an alkylating moiety are highly potent inhibitors of cell growth with GI_{50} values in the low micromolar to nanomolar range. The potency of acetoxy compound **11** in this assay suggests that the indolequinone moiety may contribute to inhibition of cell proliferation for the 3-series of compounds; however, there is no data to

support a role for this moiety in the 2-series. Furthermore, the alkylating moiety is required for cytotoxicity in both series of compounds. In contrast to reports in the literature, bioreductive drug delivery from the 2-position of the indolequinone nucleus is a viable prodrug strategy. Activation and release of several phosphoramidate prodrugs from the 2-position following two electron reduction was demonstrated using ^{31}P NMR experiments. It is proposed that the difference between our results and literature reports may be attributable to the nature of the reducing agent. Finally, the contribution of DTD to the activation of these compounds is ambiguous. The 2-series of compounds are excellent substrates for this enzyme, but the 3-series of compounds are substituent-dependent. Compounds with small substituents are excellent substrates for the enzyme, while substitution with bulky leaving groups resulted in inhibition of the enzyme. Examination of the in vitro data reveals that the best substrates for DTD are not necessarily the most potent compounds (cytotoxicity, growth inhibition). Additionally, the 3-substituted indolequinone phosphoramidates, which are inhibitors of DTD, have similar in vitro potency to the 2-subsituted regioisomers, which are excellent substrates for DTD. These data imply that DTD is not a unique target for the activation of these prodrugs; therefore, alternative mechanisms of activation need to be investigated. Finally, a significant correlation between DTD activity and cytotoxicity was observed for the 2-series of compounds, suggesting that drug delivery from the 2-position is an attractive prodrug strategy for targeting DTD.

Experimental Section

General Methods. All ^1H NMR spectra were measured on a 250-MHz Bruker NMR system equipped with a multinuclear (^1H, ^{13}C, ^{19}F, and ^{31}P) 5 mm probe. The NMR data acquisition/processing program MacNMR was used with the Tecmag data acquisition system. ^1H chemical shifts are reported in parts per million from tetramethylsilane. All ^{31}P NMR spectra were obtained on the same instrument using broad band gated decoupling and a pulse width of 10 μs. Chemical shifts are reported in parts per million from a coaxial insert containing 1% triphenylphosphine oxide (TPPO) in benzene-d_6. All variable temperature experiments were conducted using a Bruker variable temperature unit. Chromatographic purifications were carried out by flash chromatography using silica gel grade 60. Melting points were determined on a MelTemp II apparatus and are uncorrected. Elemental analyses were performed by the Purdue University Microanalysis Lab, West Lafayette, IN. Mass spectral data were obtained from the Purdue University Mass Spectrometry Service, West Lafayette, IN. A glass calomel electrode on either a radiometer pH meter or an OrionPerpHect LogR meter, model 330, was used for acidity measurements. All anhydrous reactions were carried out in flame-dried flasks under argon. All organic solvents were distilled prior to use.

Methyl 5-Methoxy-1-methyl-4-nitroindole-2-carboxylate (1). A solution of concentrated nitric acid (12 mL) in acetic acid (43 mL) was added to a crude suspension of methyl 5-methoxy-1-methylindole-2-carboxylate[20] (5.73 g, 26.15 mmol) in acetic acid (260 mL) at 0 °C. Following the addition, the reaction mixture was warmed to room temperature and stirred for 2 h. The reaction mixture was poured over ice and filtered, and the precipitate was washed with H_2O to afford **1** (6.14 g, 89%) as a yellow solid; mp 185−186 °C (lit[22] 184−186 °C); R_f = 0.53 (50% EtOAc/hexanes). ^1H NMR ($CDCl_3$): δ 7.58 (1H, d, J = 8.7 Hz), 7.55 (1H, s), 7.18 (1H, d, J = 8.7 Hz), 4.11 (3H, s), 4.02 (3H, s), 3.94 (3H, s).

Methyl 4-Amino-5-methoxy-1-methylindole-2-carboxylate (2). Tin powder (5.0 g, 42.13 mmol), followed by HCl (4.0 M, 52 mL), was added to a solution of **1** (1.0 g, 3.78 mmol) in EtOH (200 mL). The reaction was stirred at room temperature for 2 h, neutralized with saturated $NaHCO_3$, and extracted with EtOAc to afford **2** (500 mg, 56%), which was carried on without further purification; R_f = 0.53 (50% EtOAc/hexanes). ^1H NMR ($CDCl_3$): δ 6.95 (1H, d, J = 8.7 Hz), 6.71 (1H, d, J = 8.7 Hz), 6.34 (1H, s), 4.77 (2H, s), 3.87 (3H, s), 3.74 (3H, s).

2-Hydroxymethyl-5-methoxy-1-methylindole-4,7-dione (3). Lithium aluminum hydride (1.77 mL, 1.77 mmol, 1.0 M solution in Et_2O) was added to a solution of **2** (180 mg, 0.77 mmol) in tetrahydrofuran (THF, 4 mL) under argon. The reaction was then heated at reflux for 15 min, quenched by careful addition of H_2O followed by 1 M NaOH, and then extracted with CH_2Cl_2 (3×). The combined organic layers were dried over Na_2SO_4 and evaporated to afford the alcohol intermediate as a brown oil, which was used without further purification. A solution of potassium nitrosodisulfonate $(KSO_3)_2NO$ (590 mg, 2.2 mmol) in sodium phosphate buffer (0.4 M, pH = 6, 13 mL) was added to a solution of the intermediate (130 mg, 0.63 mmol) in acetone (8 mL). The reaction mixture was stirred for 1 h at room temperature, ethyl acetate and water were added, and the aqueous layer was extracted (EtOAc, 3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (EtOAc) afforded **3** (122 mg, 72%) as an orange solid; mp 203−204 °C (lit[22] 205−206 °C); R_f = 0.62 (EtOAc). ^1H NMR ($CDCl_3$): δ 6.58 (1H, s), 5.67 (1H, s), 4.68 (2H, s), 4.03 (3H, s), 3.83 (3H, s).

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Morpholinophosphorodiamidate (4). LHMDS (0.12 mL, 1.0M in THF) was added dropwise to a solution of **3** (25 mg, 0.11 mmol) in THF (3 mL), and the solution was stirred for 10 min at −78 °C. A solution of morpholinophosphoramidic dichloride (25 mg, 0.12 mmol) in THF (2 mL) was added all at once to the alkoxide. The mixture was stirred at −78 °C for 1.5 h and warmed to −20 °C, and ammonia gas was bubbled through the reaction mixture for 8 min. The reaction was stirred for an additional 7 min and then added to CH_2Cl_2/H_2O and extracted (3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (5% MeOH/$CHCl_3$) afforded **4** (37 mg, 90%) as a yellow solid; mp 174−176 °C; R_f 0.42(10% MeOH/$CHCl_3$). ^1H NMR ($CDCl_3$): δ 6.67 (1H, s), 5.70 (1H, s), 4.97 (2H, dd), 4.03 (3H, s), 3.84 (3H, s), 3.59 (4H, m), 3.13 (4H, m), 2.63 (2H, bs). ^{31}P NMR ($CDCl_3$): δ −10.88. Anal. ($C_{15}H_{20}N_3O_6P\cdot0.5H_2O$) C, H; N: calcd, 11.11; found, 10.68.

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl *N*,*N*-Bis(2-chloroethyl)phosphorodiamidate (5a). LHMDS (0.25 mL, 1.0 M in THF) was added dropwise to a solution of **3** (50 mg, 0.23 mmol) in THF (10 mL). The solution was allowed to stir for 10 min at −78 °C, and a solution of bis(2-chloroethyl)-phosphoramidic dichloride (65 mg, 0.25 mmol) in THF (1 mL) was added all at once. The solution was stirred for 1.5 h at −78 °C and then warmed to −20 °C, and ammonia gas was bubbled through the reaction mixture for 7 min. The reaction was stirred for an additional 8 min and then added to CH_2Cl_2/H_2O and extracted (3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (20% EtOAc/Acetone) afforded **5a** (63 mg, 64%) as a golden solid; mp 112−113 °C; R_f = 0.47 (20% EtOAc/Acetone). ^1H NMR ($CDCl_3$): δ 6.68 (1H, s), 5.69 (1H, s), 5.01 (2H, m), 4.02 (3H, s), 3.83 (3H, s), 3.65 (4H, m), 3.47 (4H, m), 2.03 (2H, bs). ^{31}P NMR ($CDCl_3$): δ −9.35. Anal. ($C_{15}H_{20}Cl_2N_3O_5P\cdot1.5H_2O$): C, H, N.

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl *N*,*N*-Bis(2-bromoethyl)phosphorodiamidate (5b). Compound **5b** was prepared on a 0.11 mmol scale as described above for **5a**, except that bis(2-bromoethyl)phosphoramidic dichloride was used as the phosphorylating agent. Column chromatography of the crude product (30% EtOAc/Acetone) afforded **5b** (34 mg, 60%) as a yellow solid; mp 132−134 °C; R_f = 0.53 (30% EtOAc/Acetone). ^1H NMR ($CDCl_3$): δ 6.69 (1H, s), 5.70 (1H,

s), 5.05 (2H, d), 4.02 (3H, s), 3.83 (3H, s), 3.473 (8H, m), 2.90 (2H, bs). ^{31}P NMR ($CDCl_3$): δ −9.66. Anal. ($C_{15}H_{20}Br_2N_3O_5P$) C, H, N.

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Bis-[*N*-methyl-*N*-(2-bromoethyl)]phosphorodiamidate (6). LHMDS (1.36 mL, 1.0 M in THF) was added dropwise to a solution of **3** (300 mg, 1.36 mmol) in THF (30 mL). The solution was allowed to stir for 10 min at −78 °C, and a solution of bis[*N*-methyl-*N*-(2-bromoethyl)]phosphoramidic monochloride (500 mg, 1.40 mmol) in THF (3 mL) was added all at once. The solution was slowly warmed to room temperature and stirred overnight. The mixture was then quenched with saturated NH_4Cl and extracted with CH_2Cl_2 (3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (5% MeOH/$CHCl_3$) afforded **6** (480 mg, 65%) as a yellow solid; mp 98−100 °C; R_f = 0.33 (5% MeOH/EtOAc). ^{1}H NMR ($CDCl_3$): δ 6.69 (1H, s), 5.71 (1H, s), 5.03 (2H, d), 4.03 (3H, s), 3.84 (3H, s), 3.40 (8H, m), 2.70 (3H, s), 2.66 (3H, s). ^{31}P NMR ($CDCl_3$): δ −10.01. Anal. ($C_{17}H_{24}Br_2N_3O_5P$) C, H, N.

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Acetate (7). To a solution of **3** (100 mg, 0.45 mmol) in CH_2Cl_2 (10 mL) was added acetyl chloride (0.04 mL, 0.50 mmol), followed by triethylamine (0.07 mL, 0.50 mmol). The reaction was stirred at room temperature for 30 min and then added to EtOAc/brine and extracted (3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (50% EtOAc/Hexanes) afforded **7** (72 mg, 61%) as a orange solid; mp 178−180 °C; R_f 0.70 (EtOAc). ^{1}H NMR ($CDCl_3$): δ 6.68 (1H, s), 5.68 (1H, s), 5.29 (2H, s), 3.97 (3H, s), 3.82 (3H, s), 2.09 (3H, s). Anal. ($C_{13}H_{13}NO_5 \cdot 0.5H_2O$) H, N; C: calcd, 57.35; found, 56.33.

4-Amino-5-methoxy-3-hydroxymethyl-1-methylindole (9). Lithium aluminum hydride (9.8 mL, 9.8 mmol, 1.0 M solution in Et_2O) was added to a solution of **8** (1.0 g, 4.9 mmol) in THF (40 mL) under argon. The reaction was stirred at room temperature for 1 h. The reaction was quenched by the addition of water, the salts were filtered, and the filtrate was extracted with CH_2Cl_2 (3×). The combined organic layers were dried over Na_2SO_4 and evaporated to afford **9** (720 mg, 72%) as a brown solid, which was used without further purification; R_f = 0.65 (EtOAc). ^{1}H NMR ($CDCl_3$): δ 6.93 (1H, d, J = 8.6 Hz), 6.83 (1H, s), 6.62 (1H, d, J = 8.7 Hz), 4.83 (2H, s), 3.87 (3H, s), 3.65 (3H, s).

3-Hydroxymethyl-5-methoxy-1-methylindole-4,7-dione (10). A solution of potassium nitrosodisulfonate $(KSO_3)_2NO$ (4.60 g, 17.16 mmol) in sodium phosphate buffer (0.4 M, pH = 6, 70 mL) was added to a solution of **9** (1.18 g, 5.72 mmol) in acetone (50 mL). The reaction mixture was stirred for 10 min at room temperature, ethyl acetate and water were added, and the aqueous layer was extracted (EtOAc, 3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (EtOAc) afforded **10** (1.07 g, 84%) as an orange solid; mp 186−187 °C (lit[22] 185−186 °C); R_f = 0.53 (EtOAc). ^{1}H NMR ($CDCl_3$): δ 6.71 (1H, s), 5.69 (1H, s), 4.63 (2H, s), 3.93 (3H, s), 3.84 (3H, s).

3-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Acetate (11). To a solution of **10** (50 mg, 0.22 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added acetyl chloride (0.02 mL, 0.24 mmol), followed by triethylamine (0.04 mL, 0.24 mmol). The reaction was warmed to room temperature. After 3 h, the reaction mixture was then added to CH_2Cl_2/brine and extracted (3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (10% Hexanes/EtOAc) afforded **11** (46 mg, 79%) as a yellow solid; mp 154−155 °C; R_f 0.79 (EtOAc). ^{1}H NMR ($CDCl_3$): δ 6.82 (1H, s), 5.67 (1H, s), 5.26 (2H, s), 3.94 (3H, s), 3.82 (3H, s), 2.08 (3H, s). Anal. ($C_{13}H_{13}NO_5$) C, H; N: calcd, 5.32; found, 4.79.

3-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Bis-[*N*-methyl-*N*-(2-bromoethyl)]phosphorodiamidate (12). LHMDS (0.25 mL, 1.0 M in THF) was added dropwise to a solution of **10** (50 mg, 0.23 mmol) in THF (25 mL), and the solution was stirred for 15 min at −20 °C. The alkoxide was then cannulated into a solution of [*N*-methyl-*N*-(2-bromoethyl)]-phosphoramidic dichloride (319 mg, 1.25 mmol) in THF (10 mL) at −50 °C. After 1 h, the reaction was warmed to −40 °C and stirred for an additional 1 h. The reaction was warmed to −20 °C over 30 min and *N*-methyl-*N*-(2-bromoethyl)amine hydrobromide[7] (1.04 g, 4.74 mmol) followed by *i*-Pr_2NEt (1.65 mL, 9.48 mmol) was added. The reaction was slowly warmed to room temperature over 1 h. After 3 h and 45 min, CH_2Cl_2 was added and the reaction was quenched with saturated NH_4Cl. Following extraction with CH_2Cl_2 (3×), the combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (gradient EtOAc to 5% i-PrOH/EtOAc) afforded **12** (31 mg, 25%) as an amorphous solid; R_f = 0.48 (10:90 EtOH:ether). ^{1}H NMR ($CDCl_3$): δ 6.91 (1H, s), 5.68 (1H, s), 5.14 (2H, d, J = 7.5 Hz), 3.96 (3H, s), 3.83 (3H, s), 3.42 (8H, m), 2.72 (6H, d, J = 9.7 Hz). ^{31}P NMR ($CDCl_3$): δ −8.69. FAB MS [M + H]: calcd, 539.9900; found, 539.9901.

3-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Morpholinophosphorodiamidate (13). LHMDS (0.25 mL, 1.0 M in THF) was added dropwise to a solution of **10** (50 mg, 0.23 mmol) in THF (25 mL), and the solution was stirred for 10 min at −50 °C. The alkoxide was then cannulated into a solution of morpholinophosphoramidic dichloride (51 mg, 0.25 mmol) in THF (10 mL) at −50 °C. After 1 h, the reaction was warmed to −40 °C and stirred for an additional 1 h. The reaction was warmed to −20 °C over 30 min, and ammonia gas was bubbled through the reaction mixture for 5 min. The reaction was stirred for an additional 5 min, CH_2Cl_2 was added, and the reaction was quenched with saturated NH_4Cl. Following extraction with CH_2Cl_2 (3×), the combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (5%MeOH/$CHCl_3$) afforded **13** (56 mg, 67%) as a peach solid; mp 157−160 °C; R_f 0.58 (10% MeOH/$CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 6.67 (1H, s), 5.70 (1H, s), 4.97 (2H, dd), 4.03 (3H, s), 3.84 (3H, s), 3.59 (4H, m), 3.13 (4H, m), 2.63 (2H, bs). ^{31}P NMR ($CDCl_3$): δ −10.88. ESI MS [M + Na]: calcd, 392.0989; found, 392.0995.

3-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl *N*,*N*-Bis(2-bromoethyl)phosphorodiamidate (14). LHMDS (0.25 mL, 1.0 M in THF) was added dropwise to a solution of **10** (50 mg, 0.23 mmol) in THF (25 mL). The solution was stirred for 10 min at −20 °C. The alkoxide was then cannulated into a solution of bis(2-bromoethyl)phosphoramidic dichloride (393 mg, 1.13 mmol) in THF (10 mL) and stirred at −50 °C. After 1 h, the solution was warmed to −40 °C and stirred for an additional 1 h. The solution was warmed to −20 °C over 30 min, and ammonia gas was bubbled through the reaction mixture for 10 min. The mixture was stirred for an additional 5 min and then added to CH_2Cl_2/saturated NH_4Cl and extracted (CH_2Cl_2, 3×). The combined organic layers were dried over Na_2SO_4 and evaporated. Column chromatography of the crude product (5% MeOH/$CHCl_3$) afforded **14** (75 mg, 65%) as an amorphous orange solid; R_f = 0.25 (5% MeOH/$CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 6.88 (1H, s), 5.71 (1H, s), 5.04 (2H, m), 3.96 (3H, s), 3.84 (3H, s), 3.47 (8H, m), 3.22 (2H, bs). ^{31}P NMR ($CDCl_3$): δ −9.99. ESI MS [M + H]: calcd, 511.9585; found, 511.9597.

Enzyme Kinetics. The ability of these compounds to act as substrates for human DTD was determined. The assay used was a standard coupled assay that monitors the absorbance change of cytochrome *c* spectrophotometrically.[24] The reactions were monitored using Kinetics software by Varian, version 1.0, and a CARY 3 UV−visible spectrophotometer. Reaction mixtures, totaling 1 mL in volume, contained Tris buffer (0.05 M, pH 7.6) and dithiothreitol (0.01 mM), NADH (1 mM), cytochrome *c* (55 μM), purified recombinant human enzyme (4.8 × 10^{-4} mg), and various concentrations of the indolequinone in dimethyl sulfoxide (DMSO, 10 μL). The reactions were run in triplicate at 25 °C and started by the addition of the enzyme. Reactions were monitored by an increase in absorbance at 550 nm due to reduction of cytochrome *c*, and appropriate controls were performed without the addition of enzyme to determine the background rates for the reactions. Rates of the reduction

were measured from the initial linear portion of the reaction curve, and the kinetic parameters were determined using a Hanes plot. An extinction coefficient of 1.85×10^4 M^{-1} cm^{-1} for cytochrome *c* was used in the calculations. For enzyme inhibition data, IC_{50} values reported represent the concentration of indolequinone that reduces the rate of menadione (10 μM) to one-half its uninhibited value. All assays were performed in triplicate, and the results reported are the average of at least two experiments.

Measurement of DTD Activity. S9 supernatants were prepared from the cells using a procedure outlined by Fitzsimmons,[30] except that the cell pellets were resuspended immediately. Protein concentration was determined using a standard Bradford assay.[31] All assays were carried out at 37 °C. The NADH:menadione oxidoreductase assay is the cytochrome *c* coupled assay described above. The same reaction mixtures were used, except that menadione (2 μM) was used as the substrate and the reactions were initiated by the addition of NADH. The activity inhibited by dicumarol (10 μM) is attributed to DTD and is expressed as nanomoles cytochrome *c* reduced/min/mg protein. The NADPH:DCPIP reductase assay was carried out as described by Hodnick.[27] Reaction mixtures, totaling 1 mL in volume, contained potassium phosphate buffer (0.05 M, pH 7.5), NADPH (0.3 mM), 2,6-dichlorophenolindophenol (DCPIP) (0.04 mM), BSA (0.07%), and cell lysate (varied). The reactions were run in triplicate at 37 °C and started by the addition of NADPH. Reactions were monitored by a decrease in absorbance at 600 nm due to oxidation of DCPIP, and appropriate controls were performed without the addition of enzyme to determine the background rates for the reactions. Rates of the reduction were measured from the initial linear portion of the reaction curve. An extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} for DCPIP was used in the calculations. The activity inhibited by dicumarol (10 μM) is attributed to DTD and is expressed as nanomoles DCPIP reduced/min/mg protein. All assays were performed in duplicate, and the results reported are the average of at least two experiments.

Growth Inhibition In Vitro. Growth inhibition was determined using the MTT colorimetric assay.[26] Cells were plated in 96 well plates at a density of $(1-2) \times 10^4$ and allowed to attach for 24 h. Indolequinones were dissolved in DMSO, and serial dilutions were carried out in media, such that the final concentration of DMSO was 1% or less. The indolequinone solutions were applied, and the cells were incubated for 72 h. MTT was added to each well, and the cells were incubated. After an additional 4−5 h, the medium/MTT was removed and 100 μL of DMSO was added to each well to dissolve the MTT formazan crystals. The optical absorbance at 570 nm was then determined on a plate reader. GI_{50} values were determined from plots of the percent of the control vs concentration. The compounds were all assayed twice, and the average values are reported.

Cytotoxicity Studies In Vitro: HT-29 and BE Cells. Clonogenic survival of cells was determined using a modified procedure by Mirabelli et al.[32] HT-29 and BE cells in exponential growth were suspended in unsupplemented Eagles MEM medium (9.9 mL) at a final density of $1-1.2 \times 10^5$ cells/mL. Unsupplemented medium contains MEM (Gibco) and HEPES (0.02 M). The drug stock solutions were prepared using ethanol as the solvent. The final amount of ethanol used in the drug treatments was 1% of the final volume (100 μL). Appropriate volumes of the drug stock solution and ethanol (final volume 100 μL) were added to five vials of the cell suspensions, to give five different final drug concentrations, and 100 μL of ethanol was added to a sixth vial for a control. The treated cells were incubated for 2 h (37 °C, 5% CO_2). The cells were spun down and rinsed three times with supplemented medium (3 mL) and then diluted in 5 mL of supplemented medium. Supplemented medium was prepared by adding fetal bovine serum (10%), gentamicin (0.05 mg/mL), nonessential amino acids, L-glutamine (0.03 mg/mL), and sodium pyruvate (0.1 mM) to unsupplemented medium. The cells were counted using a Coulter Counter, plated at 2−3 different densities for each drug concentration (in replicates of five), and incubated for 10 days. The colonies were stained with 0.5% crystal violet in 95% ethanol, and those colonies comprised of 50 or more cells were counted using a dissecting microscope and pen-style counter. The LC_{99} of each compound was determined by plotting the log surviving fraction vs drug concentration. All experiments were carried out at least twice, and the average values are reported.

^{31}P NMR Kinetics. The indolequinone prodrug was dissolved in CH_3CN (0.2 mL) and cacodylate buffer (0.1 mL, 0.4 M, pH 7.4), and sodium dithionite was dissolved in cacodylate buffer (0.2 mL, 0.4 M, pH 7.4). The solution containing sodium dithionite was added to the indolequinone solution, and the pH of the mixture was adjusted to ∼7.4. The reaction mixture was transferred to a 5 mm NMR tube, and the data acquisition was started (pulse delay 30 μs). Spectra were taken every 5 min for 1 h and then every 10 min for 1 h, and time points for each spectrum were assigned from the initiation of the reaction. Chemical shifts are reported relative to the TPPO reference. The temperature of the probe was maintained at 37 °C, if necessary, using the Bruker variable temperature unit. The relative concentrations of the intermediates were determined by measuring the peak areas.

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