Studies on the Mechanisms of Activation of Indolequinone Phosphoramidate Prodrugs

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Previously a series of 2- and 3-substituted indolequinone phosphoramidate prodrugs was synthesized, and the compounds were shown to be nanomolar inhibitors of cell proliferation. The activation of these compounds following both one- and two-electron reduction has been investigated. ^{31}P NMR experiments demonstrated that both series of compounds undergo rapid activation following two-electron reduction. Additionally, the 3-series of compounds undergo rapid activation following one-electron reduction, while activation of the 2-series of compounds via this mechanism is very slow. The activation of these prodrugs by direct displacement using sulfur nucleophiles such as glutathione has been examined. Activation via this route is rapid for the 3-regioisomers, but is considerably slower for the 2-substituted analogues under similar conditions. Together these findings suggest that drug delivery via two-electron reduction from the 2-position is the more selective prodrug strategy.

Introduction

The quinone moiety is common among bioreductive anticancer agents. In this context, prodrugs in the quinone form are inactive species; however, these compounds are easily converted physiologically to their hydroquinone counterparts through the action of various reductases. It is this conversion to the hydroquinone that consequently results in the formation of a biologically active species. The advantage of such prodrug strategies is the potential for selective activation and delivery of cytotoxins to tumor cells. Various types of quinones have been utilized in bioreductive prodrug strategies, including indolequinones.[1−6]

There are two main strategies used in the design of bioreductive agents; one targets the enzyme NAD(P)H: quinone oxidoreductase (NQO1) for activation, and the other relies on the hypoxic environment of tumor cells for activation. In the first approach, NQO1 is an obligate two-electron quinone reductase that is overexpressed in various cancer cell lines.[7] This finding, coupled with the otherwise low abundance of two-electron reductases, makes NQO1 an obvious target for the development of selective antitumor agents.[8−10] It has been demonstrated that NQO1 is important for the activation of quinone containing prodrugs including mitomycin C.[11] In light of the interest in mitomycin C, additional indolequinone prodrug strategies targeting NQO1 have been developed.[1−6] In one approach, the indolequinone nucleus itself is converted to a reactive and cytotoxic species following reduction to the hydroquinone. Alternatively, the indolequinone nucleus has been used for the delivery of other agents. In this strategy a substituent appended to the indolequinone is converted to a reactive species following reductive expulsion. Reductive elimination of substituents from indolequinones and related analogues has been demonstrated following two-electron reduction.[4,12−14] These findings support a role for two-electron reductases, including NQO1, in the activation of indolequinone prodrugs in vivo.

We have previously reported the synthesis and in vitro activity of a series of indolequinone phosphoramidate prodrugs.[14] These compounds were designed to be activated selectively by NQO1 to release a cytotoxic phosphoramidate anion upon reduction. Although a number of the indolequinone phosphoramidates displayed potent in vitro activity, there was no correlation between growth inhibition and NQO1 activity. This finding led us to investigate alternative activation pathways for these compounds. Herein we report our findings on the mechanisms of activation of 2- and 3-substituted indolequinone phosphoramidate prodrugs.

Results and Discussion

The indolequinone phosphoramidate prodrugs are designed to undergo bioreductive activation as shown in Scheme 1. Two-electron reduction of the quinone moiety to the hydroquinone increases the electron density on the indole nitrogen, resulting in expulsion of a phosphoramidate anion. The indole nitrogen in the parent indolequinone is present as a vinylogous amide, and therefore expulsion of the phosphoramidate does not occur. The liberated phosphoramidate anion is capable of cross-linking DNA[15] and is hypothesized to be the cytotoxic species for this series of compounds. This paper explores the possible mechanisms by which this activation can occur. The model experiments described are designed to determine whether the anticipated reactions under physiological conditions are likely to result in the desired bond cleavage and ultimately the activation of these compounds.

Reductive Activation. The mechanisms of activation of the prototypical quinone antitumor agent mitomycin C (MMC) have been widely examined. The one-electron reductases NADH:cytochrome P450 reductase, NADPH:cytochrome b5 reductase, and xanthine oxidase

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Scheme 1

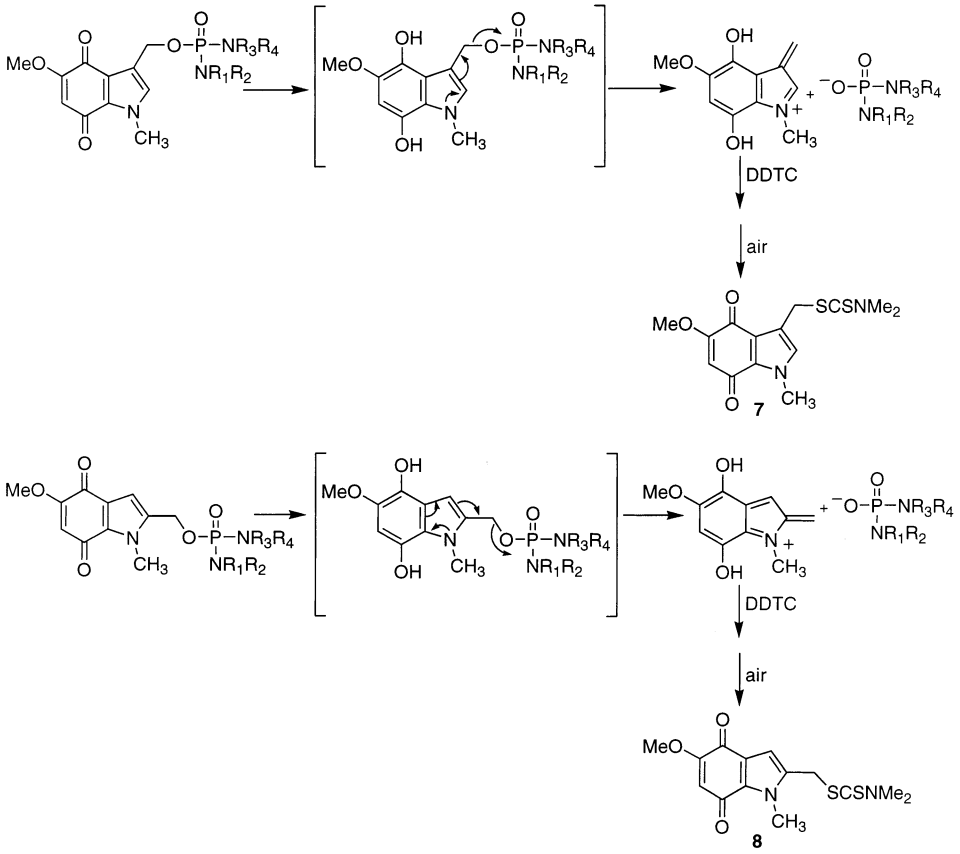
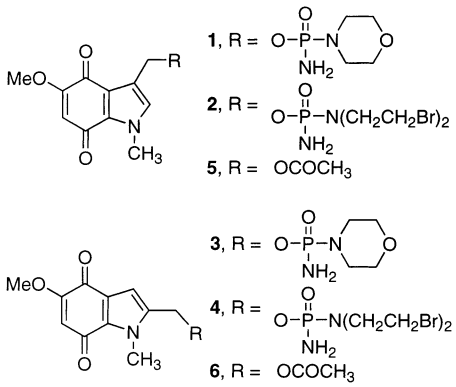


Chart 1



have been implicated in the activation of MMC, as have the two-electron reductases NQO1 and xanthine dehydrogenase.[16−22] On the basis of the structural similarities of the indolequinone compounds in Chart 1 to MMC, it was hypothesized that activation of these compounds could occur via one- and two-electron reduction.

Activation following two-electron reduction was previously demonstrated for indolequinone phosphoramidates **1**−**4** using ^{31}P NMR.[14] Expulsion of phosphoramidate anion from both the 2- and 3-positions was complete in less than 5 min at 37 °C. These data support a role for the activation of these compounds by two-electron reductases. The 2-regioisomers were also demonstrated to be excellent substrates for NQO1, and together these data support that this enzyme is likely to participate in the activation of this series of compounds in vivo. In contrast, the indolequinones containing phosphoramidates at the 3-position were potent inhibitors of NQO1, making it difficult to assess whether this enzyme could contribute to the activation of these prodrugs in vivo.

Activation of the indolequinone phosphoramidates via the mechanism shown in Scheme 1 would result in the generation of an iminium ion. To confirm that this mechanism is operative, the indolequinones were reduced chemically in the presence of a sulfur nucleophile to trap the intermediate. This approach is outlined in Scheme 1 and was previously used to demonstrate reductive elimination of substituents from mitosenes and indolequinones.[4,13] Excess sodium dithionite in buffer was added to a solution of indolequinone **5** in THF which contained the highly reactive sulfur nucleophile sodium dimethyldithiocarbamate (DDTC) for trapping. Rapid reduction of the indolequinones was demonstrated, because the solutions became colorless upon the addition of sodium dithionite. The indolequinones were then reoxidized by stirring under an air atmosphere. As anticipated, **7** was the product identified following the reaction of **5**. Control experiments in the absence of sodium dithionite confirmed that, under these conditions, the reaction proceeded via reductive elimination with subsequent nucleophilic trapping. Reductive elimination of phosphoramidate anion from **3**, followed by nucleophilic trapping, yielded **8** under similar conditions (10 equiv $Na_2S_2O_4$, 5 equiv of DDTC, 1:1.5 CH_3CN/0.4 M cacodylate buffer, pH 7.4). These results confirm the generation of the reactive iminium ion intermediate following expulsion of the phosphoramidate anion.

One-electron reductases also contribute significantly to the activation of quinone-containing antitumor agents. Therefore the ability of the indolequinone phosphora-

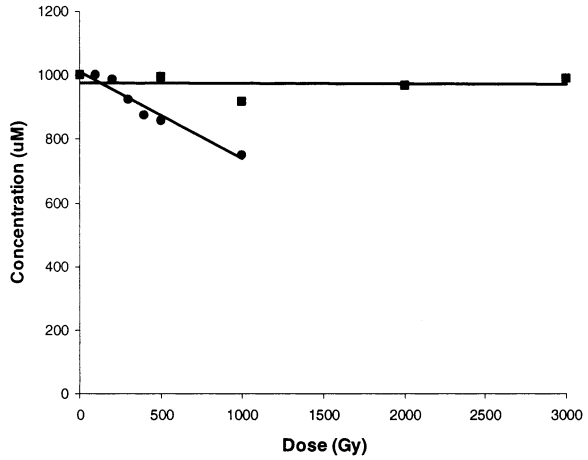


Figure 1. Dose dependent activation of **1** (●) and **3** (■) following irradiation with a ^{60}Co source. See Experimental Section for complete details.

midates to undergo activation following one-electron reduction was evaluated. Radiolytic reduction is a technique commonly used for one-electron reduction in the evaluation of hypoxia-selective agents.[23−25] Consequently the liberation of phosphoramidate anion from the model compounds **1** and **3** following γ-radiolysis was examined. Solutions of indolequinones **1** and **3** in 50/50 i-PrOH/0.4 M cacodylate buffer (pH 7.4) were degassed and irradiated with a ^{60}Co source. To prevent reactions with oxygen, nitrogen bubbling was continued throughout the course of the experiments. Aliquots were removed after different radiation doses and the extent of activation was determined using ^{31}P NMR. The concentration of remaining starting material was plotted against radiation dose and the *G* values were calculated from the linear slopes of these plots. Indolequinone **1** underwent dose-dependent activation with a $G(-Q)$ value of 0.32 μmol/J (Figure 1). This value is somewhat smaller than those reported for other 3-substituted indolequinones.[4,26,27] Nonetheless, one-electron reduction may represent a viable mechanism of activation for this 3-series of compounds.

Elimination of phosphoramidate anion from **3** was at least 150-fold slower than the activation of **1**, with a $G(-Q)$ value of $\leq$0.0025 μmol/J (Figure 1). This finding is consistent with previous reports where reductive elimination of leaving groups from the 2-position did not occur following γ-radiolysis.[28] The mechanisms for reductive elimination of substituents from indolequinones following one-electron reduction have been studied, and the results are summarized in Scheme 2.[4,26,27,29] It was found that elimination could occur either directly from the semiquinone via a first-order process, or alternatively the semiquinone could undergo a disproportionation reaction and subsequently elimination could occur from the hydroquinone. To confirm that the slow expulsion was not related to the initial reduction, pulse radiolysis experiments were carried out on **3**. The semiquinone was formed with a half-time of 47 ns ($k_1 = 1.5 \times 10^7$ s^{-1}) and subsequently disappeared with an instantaneous rate of disappearance that was approximately 5 orders of magnitude slower ($t_{1/2} = 265$ ms, second-order rate constant $k_2 = 8.7 \times 10^6$ M^{-1} s^{-1}). These rate constants are comparable to the rates reported for the 3-substituted compounds.[26] Formation

Scheme 2

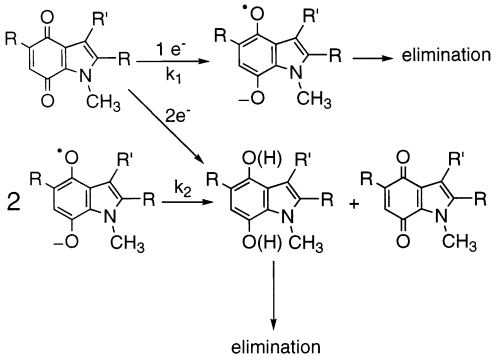


Table 1. Reduction Potentials of Indolequinones[a]

compound	E (Q/Q$^{\bullet-}$), V	E (Q$^{\bullet-}$/QH$_2$), V
1	−0.84	not determined
3	−0.87	−1.39
5	−0.87	−1.41
6	−0.87	−1.41

[a] Reduction potentials reported against Ag/AgCl reference. See Experimental Section for complete details.

of the semiquinone does occur rapidly, and therefore the slow activation of **3** is not a result of slow reduction. The slow rate of activation for this compound suggests that one-electron reduction is unlikely to be a significant mechanism of activation for the 2-series of analogues.

Reduction Potential. In the experiments described above, elimination of phosphoramidate anion from **1** was approximately 150-fold faster than elimination from **3** following radiolytic reduction. The leaving phosphoramidate anion is identical for the two regioisomers, and therefore leaving group ability cannot account for the observed rate difference. As mentioned previously, elimination could occur from either the semiquinone or hydroquinone and therefore it is possible that the differences in the rates of activation of **1** and **3** following radiolysis are attributable to differences in either the one- or two-electron reduction potentials of these compounds. Cyclic voltammetry has been used for the determination of reduction potentials of quinone containing compounds, including mitomycin analogues,[30−32] and therefore this method was used to determine the one- and two-electron reduction potentials for select indolequinones in Chart 1.

Reduction potentials of indolequinones were determined in acetonitrile containing tetrabutylammonium hexafluorophosphate as the supporting electrolyte with a glassy carbon electrode, and these results are shown in Table 1. Voltammograms were recorded in the potential range of 0.0 to −1.7 V using a Ag/AgCl reference electrode. The voltammograms for **1** and **3** are shown in Figure 2. Comparison of these voltammograms shows the one-electron reduction potentials for **1** and **3** are similar (−0.84 V and −0.87 V, respectively); however, the two-electron reduction potentials cannot be compared because of the complex nature of the voltammogram in Figure 2A. Similar results were obtained for mitosenes that contained good leaving groups and were attributed to the formation of compounds with different reduction characteristics.[31] The reduction potentials for **5** and **6** were then examined as model compounds in order to compare the two-electron reduction potentials of the 2- and 3-regioisomers. Both the

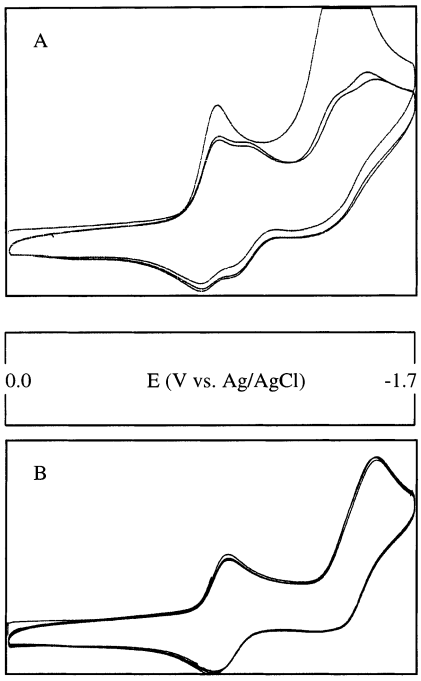


Figure 2. Cyclic voltammograms of (A) 1 mM **1** and (B) 1 mM **3** in CH_3CN. The potentials were recorded over the range of 0 to −1.7 V vs Ag/AgCl at a scan rate of 50 mV/s.

one- and two-electron reduction potentials for **5** and **6** are identical (−0.87 and −1.41 V), suggesting that differences in reduction potential cannot account for the rate differences in activation found in the radiolysis experiments. This finding may suggest that the differences in the activation of the two series of compounds are attributable to differences in the rates of elimination following radiolytic reduction and not the reduction potentials of the compounds.

Nucleophilic Activation. Nucleophilic prodrug activation by glutathione has been reported for agents designed to undergo bioreductive activation.[33] Because glutathione (GSH) and glutathione S-transferase (GST) have increased levels and/or activity in certain tumors,[34−36] prodrug activation via this pathway could be significant. Nucleophilic activation of the indolequinone phosphoramidate is undesirable because it could decrease the selectivity of these agents, so the reaction of indolequinone phosphoramidates with sulfur nucleophiles was evaluated. GSH and DDTC were utilized as the nucleophiles in these experiments, and the conversion of the prodrugs to phosphoramidate anion was monitored using ^{31}P NMR. GSH is the physiologically relevant sulfur nucleophile, and DDTC was used as a model for the GST reaction because it is present as the thiolate at neutral pH. The indolequinone phosphoramidates were reacted with DDTC or GSH under pseudo first-order conditions (10 equiv, 1:1.5 CH_3CN/0.4 M cacodylate buffer, pH 7.4, 37°C) and the reactions were monitored by ^{31}P NMR. The results for these experiments are summarized in Table 2. In general the 3-regioisomers were more reactive than the corresponding 2-regioisomers under similar reaction conditions. Figure 3 is a typical ^{31}P NMR stack plot showing the results of the reaction of **1** with DDTC. The first spectrum, at 3 min, contains one resonance at −7.28 ppm corresponding to **1**. This resonance disappears with a half-life of 34 min, giving rise to the resonance of the morpholine phosphoramidate anion at −12.28 ppm. These results confirm that the phosphoramidate anion is displaced from the 3-position following reaction with DDTC. Comparison of the reaction rates of **1** and **3** with DDTC shows that **1** is converted to phosphoramidate anion approximately 20-fold faster than **3**; the starting indolequinone phosphoramidates have half-lives of 34 and 660 min, respectively. When glutathione is used as the nucleophile, reaction of **1** and **3** occur with half-lives of 83 and 720 min for the respective indolequinone phosphoramidates. The conversion of **1** to the morpholine phosphoramidate anion occurs approximately 2.5-fold slower when the nucleophile is GSH compared to DDTC, presumably because DDTC is a better nucleophile than GSH at physiologic pH. The rate of the reaction with glutathione, however, is rapid enough to support nucleophilic activation as a potentially significant mechanism of activation for the 3-series of compounds. In contrast, the rates of activation of **3** with GSH and DDTC are considerably slower, suggesting that nucleophilic activation of the 2-series of compounds does not contribute significantly to drug activation. However, it should be pointed out that the rates of activation might be accelerated in vivo by GST. The rates of nucleophilic activation in the above experiments were linearly dependent on the concentration of nucleophile (data not shown), confirming that activation of the indolequinone phosphoramidates proceeds via an S_N2 direct displacement mechanism (Scheme 3, path a).

Interestingly, in the reactions of **2** and **4** with DDTC direct displacement of bromide from the parent compounds was also observed (Scheme 3, path b). Figure 4 is a ^{31}P NMR stack plot showing the reaction of **2** with DDTC. In the first spectrum, there is one resonance at −6.54 ppm that corresponds to **2**. In the spectra that follow, there is the appearance of two resonances

Table 2. Half-lives for Disappearance of Indolequinone Phosphoramidates in Reactions with Sulfur Nucleophiles[a]

compound	DDTC[b] $t_{1/2}$ (min)	GSH[c] $t_{1/2}$ (min)
1	34 ± 4	83 ± 7
2	14 ± 1	35 ± 6
3	659 ± 21	716 ± 59
4	26 ± 3	653 ± 71

[a] Data represent the mean ± SEM of at least three separate experiments. [b] Reaction of indolequinones with 10 equiv sodium dimethyldithiocarbamate, pH ∼ 7.4 at 37 °C. Half-lives were determined using ^{31}P NMR. See Experimental Section for complete details. [c] Reaction of indolequinones with 10 equiv of glutathione, pH ∼ 7.4 at 37 °C. Half-lives were determined using ^{31}P NMR. See Experimental Section for complete details.

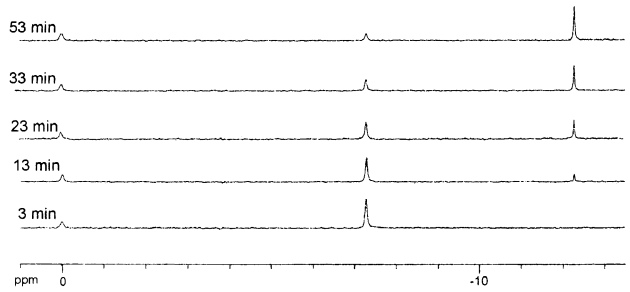


Figure 3. Reaction of indolequinone phosphoramidate **1** with DDTC (10 equiv, 1:1.5 CH_3CN: 0.4 M cacodylate buffer, pH ∼ 7.4, 37 °C). Chemical shifts are reported relative to the TPPO reference. See Results and Discussion for details.

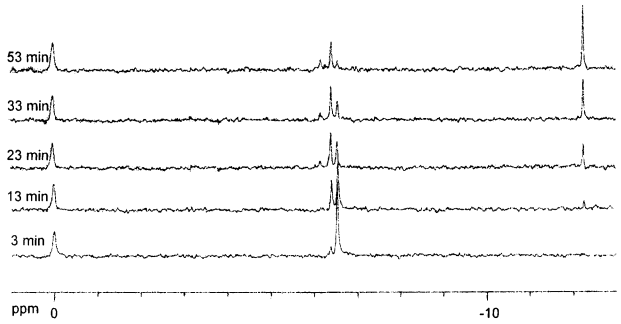
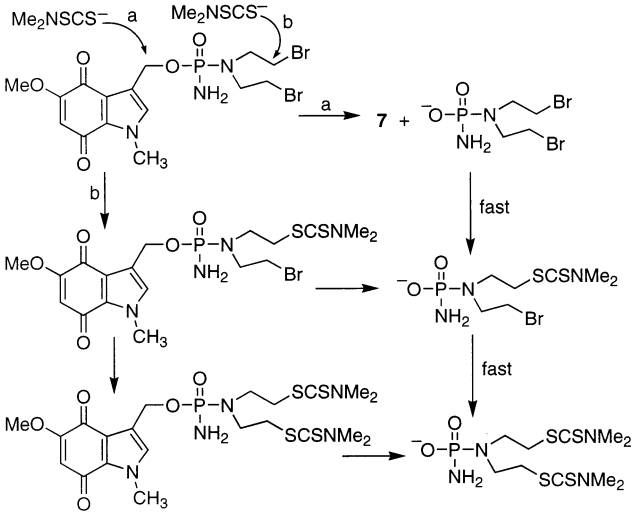


Figure 4. Reaction of indolequinone phosphoramidate **2** with DDTC (10 equiv, 1:1.5 CH_3CN: 0.4 M cacodylate buffer, pH ~ 7.4, 37 °C). Chemical shifts are reported relative to the TPPO reference. See Results and Discussion for details.

Scheme 3



downfield at −6.39 ppm and −6.17 ppm. This pattern in the ^{31}P NMR spectra is characteristic of halogen displacement from phosphoramidates containing haloethylamine moieties.[37−39] Liberation of the phosphoramidate anion (−12.25 ppm) is also shown in Figure 3 and is presumed to occur via a direct displacement mechanism (Scheme 3, path a). It is interesting to note that direct displacement of chloride from the bis(2-chloroethyl)amine analogue of **4** by DDTC was not observed. This finding suggests that utilization of chloro analogues might be the superior prodrug strategy.

Conclusions

Mechanisms for the activation of indolequinone phosphoramidate prodrugs substituted at both the 2- and 3-positions have been evaluated. Since these compounds were designed to undergo bioreductive activation, the activation of these compounds following both one- and two-electron reduction was examined. Previously it was demonstrated that both series of compounds underwent activation rapidly following two-electron reduction, a finding that supports a role for two-electron reduction as a mechanism of activation in vivo. Activation of the 3-subsitituted indolequinone **1** also occurred readily following radiolytic reduction. Consistent with the literature, these results suggest that activation via one-electron reduction may represent a physiologically relevant mechanism of activation for 3-substituted indolequinones. In contrast the activation of **3** following one-electron reduction was significantly slower and consequently is not likely to represent a physiologically relevant activation pathway.

Reduction potentials for 2- and 3-substituted indolequinones were determined. Both the one- and two-electron potentials were similar for the analogues examined and therefore it is unlikely that differences in reduction potential can account for the differences in the rates of activation. This suggests that the differences in the rates of activation may be a due to differences in the rate of elimination following reduction of the indolequinone.

The ability the indolequinone phosphoramidate prodrugs to undergo nucleophilic activation has been explored using the sulfur nucleophiles GSH and DDTC. In these experiments phosphoramidate anion was expelled rapidly from indolequinone **1**, suggesting that nucleophilic activation might be a significant mechanism of activation for the 3-series of compounds. The data also suggest that this activation occurs via direct displacement of the phosphoramidate anion from the indolequinone. In contrast, the rate of activation of the 2-series of compounds by this mechanism was extremely slow, and therefore, unlikely to be of physiologic significance.

The above results demonstrate that the 3-series of compounds are capable of undergoing significant activation via several pathways, which will likely lead to decreased selectivity for this series of analogues. In contrast, the 2-series of compounds undergo preferential activation via two-electron reduction. As a consequence, drug delivery from the 2-position appears to be the more selective prodrug strategy.

Experimental Section

General Methods. All ^1H NMR spectra were measured on a 250-MHz Bruker NMR system equipped with a multinuclear 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 broadband 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. A glass calomel electrode on either a radiometer pH meter or an Orion PerpHect LogR meter, model 330, was used for acidity measurements.

Synthesis. Indolequinones **1**−**6** were prepared as previously reported.[14]

Reductive Activation. To a solution of indolequinone **3** or **5** (1.6 mg, 6×10^{-3} mmol) and sodium dimethyldithiocarbamate (10.5 mg, 60×10^{-3} mmol) in THF (2 mL)/0.4 M cacodylate buffer (0.05 mL) or CH_3CN (0.4 mL)/0.4 M cacodylate buffer (0.6 mL) was added sodium dithionite (4.3 mg, 30×10^{-3} mmol) in cacodylate buffer (0.1 mL). The pH was adjusted to ~7.4, and the solution was capped and stirred at room temperature. Reduction of the indolequinone was presumed to be instantaneous, because the solution became colorless immediately upon addition of sodium dithionite. After 1 h the reaction was uncapped and stirred under an aerobic atmosphere for 15 min. Reoxidation of the quinone occurred and the gold color returned. The reaction was quenched with saturated NH_4Cl, extracted with CH_2Cl_2, and dried over Na_2SO_4 to yield the trapped indolequinone **7** or **8**.

3-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Dimethyldithiocarbamate (7). ^1H NMR ($CDCl_3$): δ 7.04 (1H,

s), 5.65 (1H, s), 4.72 (2H, s), 3.92 (3H, s), 3.82 (3H, s), 3.54 (3H, s), 3.33 (3H, s).

2-(5-Methoxy-1-methyl-4,7-indolequinonyl)methyl Dimethyldithiocarbamate (8). ^{1}H NMR ($CDCl_3$): δ 6.65 (1H, s), 5.65 (1H, s), 4.60 (2H, s), 3.98 (3H, s), 3.82 (3H, s), 3.57 (3H, s), 3.37 (3H, s).

^{31}P NMR Kinetics. The indolequinone prodrug (2−3.9 mg, final concentration of indolequinone 9.2−14.4 mM) was dissolved in CH_3CN (0.2 mL), and sodium dimethyldithiocarbamate or glutathione (10 equiv) was dissolved in cacodylate buffer (0.3 mL, 0.4 M, pH 7.4). The solutions were combined, 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). For 2 and 3 h experiments, spectra were taken every 2.5 min for 1 h, and then every 10 min for an additional 1 or 2 h. For 12 h experiments, spectra were taken every 5 min for 1 h, every 10 min for 1 h, every 20 min for 1 h, every 30 min for 3 h, and every 1 h for 6 h. 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 using the Bruker variable temperature unit. The relative concentrations of the intermediates were determined by measuring the peak areas.

Steady-State Radiolysis. Indolequinone solutions (1 mM) were prepared in 2-propanol/0.4 M cacodylate buffer (50% v/v), the pH adjusted to ∼7.4 and the solutions degassed with N_2 bubbling. The solutions were irradiated with a ^{60}Co source at a dose rate of 16.2−18.4 Gy/min as determined by dosimetry. Nitrogen bubbling was continued throughout the entire course of the experiments to prevent reoxidation of the quinone. Aliquots were removed from the sample after various doses of radiation, and the extent of activation was determined using ^{31}P NMR. The slopes from plots of starting material concentration (based on ^{31}P NMR peak areas) vs radiation dose were used to determine the *G* values reported in μM/J.

Pulse Radiolysis. Solutions of indolequinone **3** (1 mM) were prepared in 2-propanol/0.4 M cacodylate buffer (50% v/v), the pH adjusted to ∼7.4 and the solutions degassed with N_2 bubbling. An 8 MeV linear accelerator at the Notre Dame Radiation Laboratory was used to generate the electron pulse (∼5 ns) in these experiments. The thiocyanate dosimeter[40] was used to determine the radiation dose (3−5 μM/pulse). Nitrogen bubbling was continued throughout the entire course of the experiments to prevent reoxidation of the quinone. The rate of semiquinone formation and disappearance was determined by analyzing the change in absorbance at 370 nm.

Cyclic Voltammetry. Reduction potentials were determined at room temperature using the cyclic voltammograph Model CV-27 from Bioanalytical Systems, Inc. The electrochemical cell consisted of a glassy carbon working electrode, Ag/AgCl (saturated KCl) reference electrode, and a platinum wire auxiliary electrode. Indolequinone solutions (1 mM) were prepared in CH_3CN containing 0.1 M tetrabutylammonium hexafluorophosphate as the supporting electrolyte and degassed with N_2 bubbling for 15−20 min. The solutions were kept under a nitrogen atmosphere throughout the course of the experiments. Voltammograms were recorded in the potential range of 0 to −1.7 V vs Ag/AgCl at a scan rate of 50 mV/s.

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