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Efficacious Cyclic *N*-Acyl *O*-Amino Phenol Duocarmycin Prodrugs

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Abstract

Two novel cyclic *N*-acyl *O*-amino phenol prodrugs are reported as new members of a unique class of reductively cleaved prodrugs of the duocarmycin family of natural products. These prodrugs were explored with the expectation that they may be cleaved selectively within hypoxic tumor environments that have intrinsically higher concentrations of reducing nucleophiles and were designed to liberate the free drug without the release of an extraneous group. In vivo evaluation of the prodrug **6** showed that it exhibits extraordinary efficacy (*T/C* > 1500, L1210; 6/10 one year survivors) substantially exceeding that of the free drug, that its therapeutic window of activity is much larger permitting a dosing 40-fold higher than the free drug, and yet that it displays a potency in vivo that approaches the free drug (within 3-fold). Clearly, the prodrug **6** benefits from either its controlled slow release of the free drug or its preferential intracellular reductive cleavage.

Introduction

Duocarmycin SA (**1**)¹ and CC-1065 (**2**)² are the two most widely recognized members of a class of exceptionally potent naturally occurring antitumor agents that also include duocarmycin A³ and yatakemycin⁴ (Figure 1). This unique class of natural products derives its antitumor properties from their ability to alkylate DNA in a sequence selective manner.^{5,6} In depth studies of the natural products, their synthetic unnatural enantiomers,⁷ and key analogues have defined many of the fundamental features that control their DNA alkylation selectivity, efficiency, and catalysis, resulting in a detailed understanding of the relationships between structure, reactivity, and biological activity.^{6,7,8}

1,2,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI, Figure 1) is one of the most extensively studied synthetic analogues of the duocarmycin family since we first introduced it in 1989.⁹ The CBI alkylation subunit is not only synthetically more accessible and possesses DNA alkylation properties identical to those of the natural products,¹⁰ but it is also four times more stable and four times more potent than the naturally occurring

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Associated Content

Supporting Information. Figures illustrating the DNA alkylation of **6** without reductive activation, the DNA alkylation reaction of **19**, the synthesis and characterization of *N*-Boc-MACBI (**21**), and reactivity (solvolysis) data of **21**.

alkylation subunit of CC-1065 (**2**), approaching the stability and potency of the duocarmycin SA (**1**) alkylation subunit. Because the CBI-based analogues have also been established to exhibit efficacious in vivo antitumor activity in animal models, it has become an excellent synthetic replacement on which to examine the structure-function features of the natural products, including new prodrug designs.¹¹

In the course of the early total syntheses⁷ of the natural products and related analogues including CBI-indole₂ (**5**),¹¹ synthetic phenol precursors such as **4**, which have yet to undergo the Winstein Ar-3' spirocyclization, were found to be equipotent to and indistinguishable from their cyclized cyclopropane containing counterparts in cell growth inhibition assays, DNA alkylation studies, and in vivo antitumor models. Due to this indistinguishable behavior, protection of the phenol provides an especially effective site on which to prepare inactive prodrugs that can be cleaved in vivo releasing the active drug.¹² Prodrugs that use this protection and release strategy have been developed where the phenol release in vivo is coupled to features that might allow selective tumor cell delivery or cleavage, but surprisingly few prodrug classes have been studied to date.^{13,14,15}

There are largely two main prodrug strategies that have been examined for the duocarmycins. The most widely examined strategy utilizes an acylation of the phenol that can be cleaved hydrolytically or enzymatically. Many groups have examined such ester and carbamate prodrugs¹² and two such derivatives, a duocarmycin derivative (KW-2189)^{12c} and carzelesin^{12a}, entered clinical trials. With the exception of a notable recent example,^{12f} these have been traditionally designed to permit rapid free drug release upon in vivo administration and typically incorporate functionality to improve the drug physical properties (e.g., solubility). The second but less extensively explored approach involves the development of functionality that can be reductively activated.¹⁶ Past examples include Denny's nitro precursors to aryl amine variants of the phenol drugs,^{14a} Lee's use of an ester that is subject to cleavage upon a tethered quinone reduction,^{14d} and our report of mitomycin-like quinone precursors which undergo an analogous *o*-spirocyclization upon reductive activation.^{14e} More recently, we introduced a set of *N*-acyl *O*-amino phenol prodrugs that were designed to potentially take advantage of the hypoxic tumor environment and its increased concentration of reducing nucleophiles such as glutathione that can irreversibly cleave an activated N-O bond (Figure 2).¹⁷ Just as significantly, intracellular concentrations of such reducing thiols are as much as 100-fold higher than plasma concentrations,¹⁸ suggesting that, unlike the more traditional carbamates, such prodrugs may be subject to preferential intracellular release. Precedent for the behavior of such prodrugs may be found in the natural product FR900482 and its related congeners¹⁹ that contain hydroxylamine hemiketals and are irreversibly activated by reductive cleavage of an N-O bond. In our initial studies, a remarkable range of prodrug stabilities and propensity for N-O bond cleavage was observed even with subtle variations in the electronic and steric environment around the weak N-O bond. Significantly, the in vivo evaluation of several such prodrugs demonstrated that those that exhibit a balanced N-O stability/reactivity approach the potency and substantially exceed the efficacy of the free drug itself, suggesting that they may afford advantages related to their controlled or targeted release. Based on this demonstrated capability to structurally tune the free drug release, we have now examined a unique cyclic variant of the *N*-acyl *O*-amino phenol motif. Herein, we report the synthesis and biological evaluation of two cyclic oxazinone N-O CBI prodrugs that can be reductively cleaved to provide the active compound without the accompanying release of any extraneous material (Figure 3).

Chemistry

Synthesis

Prodrugs **6** and **7** were synthesized from a common intermediate, carboxylic acid **9** (Scheme 1). Carboxylic acid **9** was generated by phenyl lithium-mediated metal-halogen exchange of the aryl bromide **8**^{12h} and subsequent trap of the organolithium intermediate with CO₂ (g), providing **9** in excellent yield. The *N*-methyl oxazinone **11** was produced from **9** in a three-step sequence. First, the acid chloride was formed in situ using oxalyl chloride and catalytic DMF and subsequently condensed with *N*-methyl hydroxylamine producing the hydroxamic acid **10** in superb yield. The benzyl ether protecting group was removed by palladium-catalyzed hydrogenation, yielding the phenol **11** in a reaction that was run for only 60 min to avoid competitive formation of the N-O cleavage byproduct **18** observed at longer reaction times. The key oxazinone was closed upon exposure of **11** to Swern oxidation conditions yielding **12** in modest yield. This represents a new and unique method for “oxidative” formation of an N-O bond and presumably entails activation of the hydroxamic acid alcohol as its dimethyl sulfoxonium cation for subsequent intramolecular phenol displacement. In contrast and interestingly, attempts to prepare such oxazinones through a Mitsunobu type reaction of the hydroxamic acid **13** using triphenylphosphine (Ph₃P) and diethyl azidocarboxylate (DEAD) yielded only the carbamate product **14**, obtained from a Lossen rearrangement-derived isocyanate (Scheme 2). The racemic *N*-methyl oxazinone **12** was resolved into its two enantiomers by chiral phase (ChiraCel OD) HPLC with 5% *i*PrOH/hexanes as the eluent, and each enantiomer was coupled with **16** using EDCI following an acid-catalyzed deprotection of the Boc group providing (+)- and *ent*-(-)-**6**.

The oxazinone **7** was prepared in an analogous manner with minor changes to the overall synthetic sequence (Scheme 3). An activated triazine ester of **9** was generated upon reaction with 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and *N*-methyl morpholine (NMM) and trapped in situ with hydroxylamine to give the corresponding hydroxamic acid. Hydrogenolysis of the benzyl ether to provide **13** and oxazinone closure upon exposure of **13** to Swern oxidation conditions provided **15**, but competitive N-O cleavage during the hydrogenation was more facile with the secondary hydroxamic acid and the reaction was more sensitive to the reaction time. Compound **15** was resolved by chiral phase (ChiraCel OD) HPLC with a 15% *i*PrOH/hexanes eluent. Boc deprotection of each enantiomer of **15** followed by coupling with **16** using EDCI did not provide **7**, presumably due to competitive reaction of the acidic oxazinone. Therefore, the carboxylic acid **16** was first activated as its pentafluorophenyl ester **17** and used in the coupling reaction yielding (+)- and *ent*-(-)-**7**.

In addition to the key prodrugs **6** and **7**, the parent *N*-methyl amide **18** was prepared by extending the hydrogenation time of **10**, resulting in removal of the benzyl ether and cleavage of the hydroxamic acid. Acid-catalyzed Boc deprotection of **18** and coupling the released free amine with **16** using EDCI produced the parent free drug **19** as a racemate (Scheme 4).

Stability and Reactivity of the N-O Prodrugs

Since little is known about the chemical reactivity of such oxazinones, the stability of each Boc-protected prodrug (**12** and **15**) and their propensity to release the free drug were examined under representative acidic, basic, and nucleophilic conditions (Figure 4 and Supporting Information Table S1). As expected based on observations made throughout their synthesis, both **12** and **15** are remarkably stable and **15** proved to be more reactive and less stable than **12** under all conditions examined. In acidic media (4 N HCl/EtOAc, 23 °C), **12** and **15** exhibited half-lives (*t*_{1/2}) of 24 h and 2 h, respectively (after initial Boc deprotection). The full prodrug **6** was even more stable, exhibiting half-lives of >72 h and 30

h in 4 N HCl/EtOAc and 1:1 TFA/CH₂Cl₂ at 23 °C, respectively. Compound **12** was found to be stable upon exposure to basic conditions, being unreactive toward NaHCO₃ in a variety of solvents (THF, MeOH, DMF/H₂O, 23 °C) for more than 5 days. Notably, not only is the cyclic *N*-methyl oxazinone stable, but even the primary chloride is unreactive toward displacement in the protic solvents under these basic conditions. Comparatively, the unsubstituted oxazinone **15** was stable to NaHCO₃ in THF/H₂O at 23 °C, but was slowly consumed in MeOH under the same conditions (*t*_{1/2} = 70 h). Both oxazinones were found to be remarkably stable toward model nucleophiles with **15** exhibiting a more rapid cleavage relative to the more stable **12** and with thiols expectedly reacting much more effectively than amines (Figure 4). The greater stability of **12** versus **15** toward nucleophilic cleavage of the N-O bond can be attributed to the increased steric hindrance of the tertiary versus secondary nitrogen consistent with prior observations.^{17b} Finally, even the more stable of the two prodrugs (**6**) was rapidly cleaved under reductive conditions (H₂, Pd/C, MeOH, 23 °C, 1 h or Mo(CO)₆, MeCN/H₂O, reflux, 3 h).

The stability of the prodrug **6** was examined in pH 7.0 phosphate buffer (*t*_{1/2} > 4 weeks), human plasma (*t*_{1/2} > 1 week, <5% free drug), and mouse plasma (*t*_{1/2} > 48 h), demonstrating that its N-O bond is remarkably stable under such conditions. In contrast, the more reactive prodrug **7** was found to be slowly reactive in pH 7.0 phosphate buffer (*t*_{1/2} = 1 week), and slowly cleaved in human plasma (*t*_{1/2} = 4 days) and mouse plasma (*t*_{1/2} > 48 h, <5% free drug).

Biological Properties

DNA Alkylation Properties

Consistent with expectations and in the absence of a source of reductive activation, the prodrug (+)-**6** was found to be incapable of alkylating DNA in cell-free systems²⁰ at concentrations as much as 1000-fold higher than those where the free drug (+)-**4** is effective (Supporting Information Figures S1 and S2). Similarly, the prodrug (+)-**7** failed to alkylate DNA in a cell free assay without deliberate activation, whereas addition of L-glutathione promotes an effective, time-dependent release of the free drug and a slow DNA alkylation reaction that proceeds with a selectivity identical to the parent free drug (Figure 5).

This DNA alkylation behavior of (+)-**7** proved to be analogous to that of (+)-**3c** that also fails to effectively alkylate DNA in the cell free assay, but does so effectively upon activation by addition of L-glutathione (Figure 6).

Finally, the free drug **19**, released upon reductive cleavage of (+)-**6**, was found to alkylate DNA in a manner analogous to (+)-**4** indicating that the C5-carboxamide does not significantly alter the DNA alkylation properties and capabilities of the parent drug **4** (Supporting Information Figure S3).

These studies indicate that the in vitro cytotoxic activity and in vivo antitumor activity of the N-O prodrugs are not likely derived from the prodrugs **3**, **6**, or **7** themselves, but are the result of their reductive cleavage to release the free phenols that in turn are effective DNA alkylating agents and potent cytotoxic compounds. Moreover, the studies indicate that thiols alone are capable of providing this reductive activation through nucleophilic cleavage of the N-O bond, although they do not preclude other reductive mechanisms being operative.

In Vitro Cytotoxic Activity

Both enantiomers of **6** and **7** as well as their precursors **12** and **15** were tested for cell growth inhibition in a cytotoxic assay with L1210 murine leukemia cells alongside the free drug **19** and related control compounds (Figure 7). Prodrug (+)-**6** was found to exhibit an IC₅₀ of

0.99 nM, whereas the more reactive prodrug (+)-**7** was found to have an IC₅₀ of 0.30 nM. By comparison, *seco*-CBI-indole₂ derivatives that lack a C5 substituent needed to support a spirocyclization (e.g., H or OMe) exhibit IC₅₀ values at least 100-fold higher than (+)-**6** and 350-fold higher than (+)-**7** (IC₅₀ >100 nM, L1210).¹⁷ The 3-fold difference in potency between (+)-**6** and (+)-**7** reflects their relative reactivity toward N-O cleavage with the more reactive prodrug providing a more facile release of the free drug under the conditions on the assay. Both prodrug unnatural enantiomers, (–)-**6** and (–)-**7**, were found to be >200 fold less potent than the natural enantiomers. The parent free drug **19**, tested as a racemic mixture, was found to be 2.5 times less potent than *seco*-(+)-CBI-indole₂, indicating that its natural enantiomer closely approximates the activity of (+)-CBI-indole₂. Combined, these studies indicate that the prodrugs **6** and **7** are effectively, but not rapidly or completely cleaved to provide the free drug under the conditions of the cellular assay since their IC₅₀ values are between 8 and 25 fold higher than the free drug **19** itself. Despite this lower in vitro potency relative to the free drug, it is notable that both prodrugs still display potent sub nanomolar cellular activity, being more potent than most clinically used antitumor agents. Moreover, their inherent chemical stability suggest they, like **3c**, possess an appropriate balance between chemical stability and reductive cleavage propensity to display more efficacious in vivo antitumor activity than the free drug itself.

In Vivo Antitumor Activity

Although the results of the functional cellular assays show that (+)-**6** is less potent than its parent drug **19** and less potent than (+)-**7**, the slow release of the free drug could prove advantageous in vivo due to the parent compound's remarkable potency and inherent toxicity.^{12h} Therefore, the preliminary evaluation of the in vivo antitumor activity of the cyclic N-O prodrugs was assessed with (+)-**6** and was conducted alongside *seco*-CBI-indole₂ (**4**) in an antitumor model consisting of L1210 murine leukemia cells implanted i.p. in B6D2F1 mice traditionally used to initially compare new duocarmycin derivatives (Figure 8). A dose range of 60 to 2500 µg/kg for prodrug (+)-**6** (scaled to its determined IC₅₀) and 60 to 500 µg/kg for *seco*-CBI-indole₂ (**4**), and a dosing schedule (administered ip three times on days 1, 5, and 9) traditionally used for this class of compounds were employed. A subtle, but important additional empirical observation made in the studies was that the prodrug administration was tolerated at the injection sites of the animals much better than the free drug.

seco-CBI-indole₂ was administered at a range (60 to 500 µg/kg) beyond its optimal dosing (60 or 100 µg/kg)^{11,17} to highlight its small therapeutic window in comparison to the potential efficacy of prodrug (+)-**6**. In line with previous observations, administration of (+)-**4** at 60 µg/kg resulted in only 1/10 long term survivor (*T/C* > 284) at the termination of the study (366 days). At all higher doses (100–500 µg/kg), (+)-**4** proved toxic leading to premature death of the animals due to drug toxicity. Remarkably, administration of (+)-**6** produced 6/10 long term survivors at 2500 µg/kg (*T/C* > 1500) and 4/10 long term survivors at the next two lower doses of 1000 µg/kg (*T/C* > 1130) and 500 µg/kg (*T/C* > 1142). Because the response to the administration of (+)-**6** was so remarkable, the length of the study was extended to 366 days (1 year) where the live animals represent long term survivors with no evidence of a delayed toxicity. Even at a dose of (+)-**6** (200 µg/kg) only 3-fold higher than the optimal dose of (+)-**4** (60 µg/kg), the prodrug outperformed the free drug (*T/C* 386 vs 284). This data indicates that (+)-**6** does not suffer from the characteristic toxicity of (+)-**4** even at 40-fold higher doses, that it possesses a much larger therapeutic window (200 to > 2500 µg/kg) compared to the narrow 30 to 100 µg/kg dose range of (+)-**4**,^{11,17} and that it is much more and extraordinarily efficacious in vivo. It is possible that the prodrug (+)-**6** could benefit from an even higher dosing level or alternative dose schedule beyond our initial exploration, and that such higher doses may be even more

efficacious. These studies indirectly establish that the free drug is released in vivo, but they do not define the site or mechanism of free drug release. In addition, the studies indicate that the in vivo behavior of the cyclic *N*-acyl *O*-amino phenol prodrug **6** relative to the free drug **4** itself benefits from either its controlled slow release of the free drug or its targeted intracellular reductive cleavage.

Hypoxia-Selective Cytotoxic Activity

In a preliminary effort to address whether the remarkable in vivo efficacy of (+)-**6** might be related to its mechanism of reductive activation rather than its controlled rate of release, its relative cytotoxic activity was examined against two different human tumor cell lines, a human lung cancer cell line (A549) and a human prostate cancer cell line (PC3/MM2), under hypoxic versus normal aerobic conditions (Figure 9). For comparison, the efficacious, but hydrolytically released, cyclic carbamate prodrug **20**^{12h} of (+)-**4** was used as a control. Unlike **20** (HCR = 1–2), which would not be expected to display hypoxia selective cytotoxic activity, the prodrug (+)-**6** exhibited increased cytotoxic potency under the conditions of hypoxia, providing a 5-fold improvement in activity against A549 (HCR = 5) and an 10-fold increase against PC3/MM2 (HCR = 10) relative to its activity under normal aerobic conditions. This indicates that the prodrug (+)-**6** more effectively releases the free drug under hypoxic conditions, consistent with a reductive release and potentially contributing to its improved in vivo efficacy. However, it does not define the mechanism of reductive release (e.g., thiol addition or enzymatic cleavage), nor does it rule out the important additional roles that the site of release (intracellular vs extracellular) or the controlled slow release of free drug may play in its improved in vivo efficacy. These and related questions are the subject of ongoing studies.

Conclusions

Two novel cyclic *N*-acyl *O*-amino phenol duocarmycin prodrugs, (+)-**6** and (+)-**7**, were synthesized and analyzed for their antitumor activity in vitro and in vivo. The prodrugs were designed to be subject to reductive activation, perhaps being capable of selective cleavage with release of the free drug within a hypoxic tumor cell environment due to the increased presence of reducing nucleophiles that can cleave the weak N-O bond. Of the two candidate prodrugs, *N*-methyl oxazinone (+)-**6** proved more stable than (+)-**7**, but both proved remarkably stable toward acidic and basic conditions as well as reaction with model nucleophiles. Neither prodrug was found to be effective at alkylating DNA in cell free assays, but they were found to be activated for DNA alkylation by thiols (L-glutathione) like the N-O prodrug **3c**. This reactivity toward cleavage of the protective N-O bond was reflected in their in vitro cytotoxic activity with the more stable prodrug (+)-**6** displaying a less potent IC₅₀ (0.99 nM) than the more reactive prodrug (+)-**7** (IC₅₀ = 0.30 nM). Prodrug (+)-**6**, which was also found to be stable in pH 7.0 phosphate buffer, mouse plasma, and human plasma, was examined in vivo in a leukemia mouse model conducted for an extended duration (366 days) and found to exhibit extraordinary efficacy (*T/C* > 1500, L1210; 6/10 one year survivors) substantially exceeding the efficacy of the free drug. Prodrug (+)-**6** displayed a therapeutic window of efficacy that is much larger than the free drug permitting dosing 40-fold over that of the free drug and yet it displays an in vivo potency that is within 3-fold of that of the free drug. Clearly and on top of the intrinsic selective activity displayed by this class of antitumor drugs, the in vivo behavior of (+)-**6** additionally benefits from a controlled release of the active free drug, its site (intracellular) of free drug release, or its mechanism of reductive cleavage. Consistent with a role for the latter, (+)-**6** exhibited improved in vitro cytotoxic activity under hypoxic versus aerobic conditions. These and related aspects of the in vivo behavior of **6** are under investigation.

Experimental Section

General

Reagents and solvents were purchased reagent-grade and used without further purification. Pooled human plasma, with sodium citrate as an anticoagulant, was purchased from Innovative Research and stored at -20°C . THF was freshly distilled from sodium benzophenone ketyl. All reactions were performed in oven-dried glassware under an Ar atmosphere. Evaporation and concentration in vacuo was performed at 20°C . TLC was conducted using precoated SiO_2 60 F254 glass plates from EMD with visualization by UV light (254 or 366 nm). Chiral phase HPLC was performed using a Shimadzu HPLC on a semi-preparative Diacel ChiralCel OD column ($0.46\text{ cm} \times 25\text{ cm}$) with a flow rate of 7 mL/min and with UV detection at $\lambda = 254\text{ nm}$. Optical rotations were determined on a Rudolph Research Analytical Autopol III Automatic Polarimeter ($\lambda = 589\text{ nm}$, 25°C). NMR (^1H or ^{13}C) were recorded on Bruker DRX-500 and DRX-600 NMP spectrophotometers at 298K. Residual solvent peaks were used as an internal reference. Coupling constants (J) (H,H) are given in Hz. Coupling patterns are designated as singlet (s), doublet (d), triplet (t), quadruplet (q), multiplet (m), or broad singlet (br). IR spectra were recorded on a Thermo Scientific Nicolet 380 FT-IR spectrophotometer and measured neat. High resolution mass spectral data were acquired on an Agilent Technologies high resolution LC/MSD-TOF, and the detected masses are given as m/z with m representing the molecular ion. The purity of each tested compound ($>95\%$) was determined by HPLC on an Agilent 1100 LC/MS instrument using a ZORBAX SB-C18 column (3.5 mm , $4.6\text{ mm} \times 50\text{ mm}$, with a flow rate of 0.75 mL/min and detection at 220 and 254 nm) with a 10–98% acetonitrile/water/0.1% formic acid gradient.

***tert*-Butyl 1,2-Dihydro-5-(benzyloxy)-1-(chloromethyl)-6-(carboxylic acid) -benzo[e]indole-3-carboxylate (9)**

Compound **8** (206 mg, 0.409 mmol) was dissolved in freshly distilled anhydrous THF (4.1 mL) and cooled to -78°C . Phenyl lithium (0.23 mL, 2.0 M solution in THF) was added dropwise. After 45 min, CO_2 (g) was bubbled into the reaction mixture for 10 min after which it was allowed to slowly warm to 0°C . The reaction mixture was quenched with the addition 2 N aqueous HCl. The slurry was extracted with Et_2O (3 $\times$). The organic extracts were combined, washed with H_2O (2 $\times$), saturated aqueous NaCl, dried over Na_2SO_4 , and concentrated under reduced pressure. Flash chromatography (SiO_2 , 20–100% EtOAc/hexanes gradient elution) provided **9** (180 mg, 94%) as a tan solid. ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) δ 7.72 (br, 2H), 7.52 (d, $J = 7\text{ Hz}$, 1H), 7.38–7.32 (m, 3H), 7.25 (t, $J = 7.5\text{ Hz}$, 1H), 6.99 (d, $J = 7.0\text{ Hz}$, 1H), 4.11–4.01 (m, 3H), 3.94 (d, $J = 8.0\text{ Hz}$, 1H), 3.75 (dd, $J = 7.5$, 11 Hz, 1H) 1.51 (s, 9H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz) δ 154.9, 151.4, 136.7, 136.1, 130.2, 129.1, 128.4, 128.2, 128.1, 127.5, 127.2, 126.7, 125.7, 121.4, 117.1, 114.7, 97.2, 79.8, 69.9, 52.4, 47.6, 27.9. IR (film) ν_{max} 2980, 2903, 1693, 1415, 1141 cm^{-1} . ESI-TOF HRMS m/z 468.1569 ($\text{M}+\text{H}^+$, $\text{C}_{26}\text{H}_{26}\text{ClNO}_5$ requires 468.1572).

***tert*-Butyl 10-(Chloromethyl)-5-methyl-4-oxo-9,10-dihydro-4H-pyrrolo[3',2':5,6]naphtho[1,8-de][1,2]oxazine-8(5H)-carboxylate (12)**

The carboxylic acid **9** (199 mg, 0.420 mmol) in anhydrous CH_2Cl_2 (8.5 mL) at 0°C was treated with $(\text{COCl})_2$ (42 μL , 0.510 mmol). A drop of DMF was added and the reaction mixture was stirred for 1 h. After 1 h, a premixed solution of $\text{MeNHOH}\cdot\text{HCl}$ (177 mg, 2.13 mmol), Et_3N (0.29 mL, 2.13 mmol), 3 drops of H_2O , and THF (21.3 mL) was added by cannula. After the addition was complete, the reaction mixture was allowed to warm to room temperature over 1 h. The reaction mixture was concentrated under reduced pressure and then diluted with 2 N aqueous HCl. The aqueous phase was extracted with EtOAc (3 $\times$). The organic extracts were combined, washed with H_2O and saturated aqueous NaCl, dried over

Na₂SO₄, and concentrated under reduced pressure. The crude solid (209 mg, 99%) was carried forward without further purification. ESI-TOF HRMS m/z 497.1854 (M+H⁺, C₂₇H₂₉ClN₂O₅ requires 497.1838). The *N*-methyl hydroxamic acid **10** (209 mg, 0.420 mmol) was dissolved in anhydrous CH₃OH (8.4 mL) under Ar. 10% Pd/C (44 mg, 0.0042 mmol) was added and the atmosphere was exchanged with H₂. The reaction mixture was allowed to stir at 25 °C for 1 h. The reaction mixture was diluted with Et₂O, filtered through Celite, and concentrated under reduced pressure. The residue was purified by PTLC (SiO₂, EtOAc elution) providing **11** (78 mg, 45%) as an orange foam. ESI-TOF HRMS m/z 407.1367 (M+H⁺, C₂₀H₂₃ClN₂O₅ requires 407.1368). A stirred solution of (COCl)₂ (24 μL, 0.280 mmol) in anhydrous CH₂Cl₂ (4 mL) at -78 °C was treated with Me₂SO (44 μL, 0.570 mmol) in 3 mL anhydrous CH₂Cl₂ dropwise. After 10 min, **11** (78 mg, 0.190 mmol) in 12 mL of anhydrous CH₂Cl₂ was added dropwise. After the addition, the reaction mixture was allowed to warm slowly to -10 °C over 2 h. The reaction was quenched with the addition of saturated aqueous NH₄Cl and extracted with EtOAc. The organic layer was washed with saturated aqueous NaCl, dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by PTLC (SiO₂, 50% EtOAc/hexanes elution) providing **12** (12.7 mg, 17%) as a bright yellow solid. ¹H NMR (acetone-*d*₆, 600 MHz) δ 7.99 (d, *J* = 8.4 Hz, 1H), 7.82 (d, *J* = 7.2 Hz, 1H), 7.77 (br, 1H), 6.63 (t, *J* = 7.8 Hz, 1H), 4.21 (m, 2H), 4.14 (m, 1H), 4.00 (dd, *J* = 11.1, 3.6 Hz, 1H), 3.79 (dd, *J* = 8.4, 11.4 Hz, 1H), 3.56 (s, 3H), 1.58 (s, 9H). ¹³C NMR (acetone-*d*₆, 150 MHz) δ 161.8, 153.7, 153.3, 144.8, 131.2, 130.0, 127.9, 123.9, 121.6, 118.5, 118.1, 99.2, 82.7, 54.7, 48.7, 45.5, 36.2, 29.5. IR (film) ν_{max} 2978, 1702, 1404, 1141 cm⁻¹. ESI-TOF HRMS m/z 389.1268 (M+H⁺, C₂₀H₂₁ClN₂O₄ requires 389.1263).

The enantiomers were resolved on a semi-preparative Diacel ChiralCel OD column (0.46 cm × 25 cm) with 5% *i*-PrOH/hexanes elution. α = 1.14

(1*S*)-**12**: [α]_D²³ -35 (*c* 1.22, THF), natural enantiomer.

(1*R*)-**12**: [α]_D²³ +40 (*c* 0.42, THF), unnatural enantiomer.

***N*-(2-(10-(Chloromethyl)-5-methyl-4-oxo-5,8,9,10-tetrahydro-4*H*-pyrrolo[3',2':5,6]naphtho[1,8-*de*][1,2]oxazine-8-carbonyl)-1*H*-indol-5-yl)-1*H*-indole-2-carboxamide (**6**)**

N-Methyl oxazinone **12** (6.8 mg, 0.017 mmol) was dissolved in 4 N HCl in EtOAc (0.5 mL) and the mixture was allowed to stir at room temperature for 15 min. The solvent was removed under a stream of nitrogen and the residue was redissolved in anhydrous DMF (0.34 mL). EDCI (10.0 mg, 0.052 mmol) and **16** (6.1 mg, 0.019 mmol) were added and the reaction mixture was allowed to stir at 25 °C for 20 h. The reaction mixture was quenched with the addition H₂O and diluted with EtOAc. The organic phase was washed with 2 N aqueous HCl (3×), saturated aqueous NaHCO₃ (5×) and saturated aqueous NaCl. The organic extract was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by PTLC (SiO₂, 40% THF/toluene) providing **6** (5.7 mg, 56%) as a yellow solid. ¹H NMR (DMSO-*d*₆, 600 MHz) δ 11.84 (s, 1H), 11.73 (s, 1H), 10.18 (s, 1H), 8.25 (s, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.09 (br, 1H), 7.90 (d, *J* = 7.2 Hz, 1H), 7.74 (t, *J* = 8.4 Hz, 1H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.59 (d, *J* = 9.0 Hz, 1H), 7.50–7.47 (m, 2H), 7.43 (s, 1H), 7.29 (s, 1H), 7.21 (t, *J* = 8.4 Hz, 1H), 7.07 (t, *J* = 7.2 Hz, 1H), 4.91 (t, *J* = 9.6 Hz, 1H), 4.65 (dd, *J* = 11.4, 2.4 Hz, 1H), 4.39 (m, 1H), 4.09–4.04 (m, 1H), 4.03–4.01 (m, 1H), 3.58 (s, 3H). ¹³C NMR (DMSO-*d*₆, 150 MHz) δ 160.3, 159.5, 159.4, 150.1, 142.9, 136.6, 133.4, 131.77, 131.70, 130.6, 128.5, 128.4, 127.04, 127.02, 126.9, 123.4, 121.5, 121.3, 120.5, 119.7, 119.5, 118.2, 117.0, 112.8, 112.27, 112.24, 106.2, 103.3, 98.9, 54.9, 47.5, 40.7, 34.9. ESI-TOF HRMS m/z 590.1592 (M+H⁺, C₃₃H₂₄ClN₅O₄ requires 590.1589).

(1*S*)-**6**: [α]_D²³ +54 (*c* 0.38, THF), natural enantiomer.

(1*R*)-**6**: $[\alpha]^{23}_{\text{D}} -57$ (c 0.24, THF), unnatural enantiomer.

***tert*-Butyl 10-(Chloromethyl)-4-oxo-9,10-dihydro-4*H*-pyrrolo[3',2':5,6]naphtho[1,8-*de*][1,2]oxazine-8(5*H*)-carboxylate (15)**

The carboxylic acid **9** (191 mg, 0.41 mmol) in anhydrous THF (2.0 mL) at 25 °C was treated with CDMT (78.0 mg, 0.45 mmol) and NMM (134 μ L, 1.22 mmol). After 2 h, $\text{NH}_2\text{OH}\cdot\text{HCl}$ (31 mg, 0.45 mmol) in 2.0 mL of DMF was added and the reaction mixture was stirred for 6 h. After 6 h, the reaction mixture was quenched with the addition of H_2O and extracted with EtOAc (3 $\times$). The organic layers were combined, washed with 2 N aqueous HCl, saturated aqueous NaCl, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by PTLC (SiO_2 , 50% EtOAc/hexanes elution) providing the hydroxamic acid (130 mg, 93% BRSM) as a light brown foam. ESI-TOF HRMS m/z 483.1677 ($\text{M}+\text{H}^+$, $\text{C}_{26}\text{H}_{27}\text{ClN}_2\text{O}_5$ requires 483.1681). The hydroxamic acid (130 mg) was dissolved in anhydrous CH_3OH (3.36 mL) under Ar. 10% Pd/C (28.6 mg, 0.026 mmol) was added and the atmosphere was exchanged with H_2 . The reaction mixture was allowed to stir at 25 °C for 50 min. The reaction mixture was diluted with Et_2O , filtered through Celite, and concentrated under reduced pressure. The residue was purified by PTLC (SiO_2 , EtOAc elution) providing **13** (46 mg, 56% BRSM) as an orange foam. ESI-TOF HRMS m/z 393.1208 ($\text{M}+\text{H}^+$, $\text{C}_{19}\text{H}_{21}\text{ClN}_2\text{O}_5$ requires 393.1212). A stirred solution of $(\text{COCl})_2$ (14.7 μ L, 0.175 mmol) in anhydrous CH_2Cl_2 (3 mL) at -78 °C was treated with Me_2SO (27.3 μ L, 0.351 mmol) in 2 mL of anhydrous CH_2Cl_2 dropwise. After 10 min, **13** (46 mg, 0.117 mmol) in 6 mL of anhydrous CH_2Cl_2 was added dropwise. After the addition, the reaction mixture was allowed to warm slowly to -10 °C over 2 h. The reaction mixture was quenched with the addition of saturated aqueous NH_4Cl and extracted with EtOAc. The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by PTLC (SiO_2 , 50% EtOAc/hexanes elution) yielding **15** (9 mg, 27%) as a tan solid. ^1H NMR (acetone- d_6 , 600 MHz) δ 8.04 (d, $J = 9$ Hz, 1H), 7.80 (d, $J = 7.2$ Hz, 1H), 7.79 (br, 1H), 7.66 (t, $J = 7.2$ Hz, 1H), 4.22–4.16 (m, 3H), 4.01 (dd, $J = 11.1$, 3.6 Hz, 1H), 3.79 (dd, $J = 8.1$, 11.4 Hz, 1H), 1.57 (s, 9H). ^{13}C NMR (THF- d_8 , 150 MHz) δ 161.9, 153.5, 152.8, 144.2, 130.5, 129.0, 126.5, 122.4, 118.9, 118.3, 116.1, 98.0, 81.6, 53.9, 47.3, 42.3, 28.6. IR (film) ν_{max} 2920, 1660, 1409, 1139, 758 cm^{-1} . ESI-TOF HRMS m/z 375.1120 ($\text{M}+\text{H}^+$, $\text{C}_{19}\text{H}_{19}\text{ClN}_2\text{O}_4$ requires 375.1106). The enantiomers were resolved on a semi-preparative Diacel ChiralCel OD column (0.46 cm $\times$ 25 cm) with 15% *i*-PrOH/hexanes elution. $\alpha = 1.19$.

(1*S*)-**15**: $[\alpha]^{23}_{\text{D}} -17$ (c 0.39, THF), natural enantiomer.

(1*R*)-**15**: $[\alpha]^{23}_{\text{D}} +19$ (c 0.30, THF), unnatural enantiomer.

Perfluorophenyl 5-(1*H*-Indole-2-carboxamido)-1*H*-indole-2-carboxylate (17)

Compound **16** (20 mg, 0.062 mmol), $\text{C}_6\text{F}_5\text{OH}$ (17.2 mg, 0.093 mmol), and EDCI (35.5 mg, 0.186 mmol) were combined, suspended in DMF (0.62 mL), and the mixture was stirred at 25 °C for 3 h. The reaction mixture was diluted with EtOAc and washed with H_2O . The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. Compound **17** (25 mg, 83%) was isolated as a tan solid. ^1H NMR ($\text{DMSO}-d_6$, 600 MHz) δ 12.44 (s, 1H), 11.72 (s, 1H), 10.24 (s, 1H), 8.29 (s, 1H), 7.74 (dd, $J = 10.8$, 1.8 Hz, 1H), 7.68 (d, $J = 8.4$ Hz, 1H), 7.62 (d, $J = 1.8$ Hz, 1H), 7.53 (d, $J = 9.0$ Hz, 1H), 7.48 (d, $J = 8.4$ Hz, 1H), 7.43 (d, $J = 1.2$ Hz, 1H), 7.22 (t, $J = 7.9$ Hz, 1H), 7.08 (t, $J = 7.8$ Hz, 1H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz) δ 159.5, 156.9, 141.6, 139.9, 138.3, 136.6, 135.6, 132.5, 131.6, 127.0, 126.4, 124.2, 123.5, 123.2, 121.6, 121.5, 119.7, 112.89, 112.88, 112.2, 111.9, 103.4. IR (film) ν_{max} 3360, 3256, 1721, 1646, 1515, 994 cm^{-1} . ESI-TOF HRMS m/z 486.0890 ($\text{M}+\text{H}^+$, $\text{C}_{24}\text{H}_{12}\text{F}_5\text{N}_3\text{O}_3$ requires 486.0872).

***N*-(2-(10-(Chloromethyl)-4-oxo-5,8,9,10-tetrahydro-4*H*-pyrrolo[3',2':5,6]naphtho[1,8-*de*][1,2]oxazine-8-carbonyl)-1*H*-indol-5-yl)-1*H*-indole-2-carboxamide (**7**)**

Compound **15** (3.2 mg, 0.008 mmol) was dissolved in 4 N HCl in EtOAc (0.3 mL) and the mixture was allowed to stir at room temperature for 10 min. The solvent was removed under a stream of nitrogen and the residue was redissolved in anhydrous DMF (0.2 mL). Et₃N (5.9 μ L, 0.042 mmol) and **17** (4.9 mg, 0.010 mmol) were added and the reaction mixture was allowed to stir at 25 °C for 20 h. The reaction mixture was quenched with the addition of H₂O and diluted with EtOAc. The organic phase was washed with saturated aqueous NaCl, dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by PTLC (SiO₂, 40% THF/toluene) providing **7** (1.07 mg, 22%) as a yellow solid. ¹H NMR (DMSO-*d*₆, 600 MHz) δ 11.83 (s, 1H), 11.73 (s, 1H), 10.18 (s, 1H), 8.24 (s, 1H), 8.16 (br, 1H), 8.02 (br, 1H), 7.71 (br, 1H), 7.68 (d, *J* = 18 Hz, 1H), 7.59 (dd, *J* = 9.3, 2.4 Hz, 1H), 7.49–7.46 (m, 2H), 7.43 (s, 1H), 7.28 (s, 1H), 7.21 (t, *J* = 6.6 Hz, 1H), 7.07 (t, *J* = 7.8 Hz, 1H), 4.89 (t, *J* = 9 Hz, 1H), 4.64 (d, *J* = 9.6 Hz, 1H), 4.35 (br, 1H), 4.08–3.99 (m, 2H). ¹³C NMR (DMSO-*d*₆, 150 MHz) δ 161.9, 160.3, 159.4, 143.2, 136.6, 133.4, 131.8, 131.7, 130.8, 128.6, 128.4, 127.9, 127.0, 124.8, 123.5, 121.6, 119.8, 119.5, 118.3, 112.8, 112.3, 112.2, 106.9, 106.1, 103.3, 98.7, 66.9, 66.5, 54.9, 47.3, 40.7 δ . ESI-TOF HRMS *m/z* 576.1423 (M+H⁺, C₃₂H₂₂ClN₅O₄ requires 576.1433).

(1*S*)-**7**: [α]_D²³ +18 (*c* 0.06, acetone), natural enantiomer.

(1*R*)-**7**: [α]_D²³ –16 (*c* 0.07, acetone), unnatural enantiomer.

***tert*-Butyl 1,2-Dihydro-1-(chloromethyl)-5-hydroxy-6-(methylcarbamoyl)benzo[*e*]indole-3-carboxylate (**18**)**

¹H NMR (acetone-*d*₆, 600 MHz) δ 10.66 (s, 1H), 8.26 (s, 1H), 7.96 (d, *J* = 7.8 Hz, 1H), 7.74 (br, 1H), 7.56 (d, *J* = 7.2 Hz, 1H), 7.46 (t, *J* = 7.8 Hz, 1H), 4.21 (d, *J* = 11.4 Hz, 1H), 4.14–4.09 (m, 2H), 3.95 (d, *J* = 10.8 Hz, 1H), 3.68 (t, *J* = 9.6 Hz, 1H), 3.01 (s, 3H), 1.58 (s, 9H). ¹³C NMR (DMSO-*d*₆, 150 MHz) δ 171.5, 154.7, 151.5, 141.7, 135.5, 130.6, 126.1, 123.1, 122.2, 117.0, 113.3, 99.6, 80.2, 52.3, 47.6, 40.2, 28.0, 26.1. IR (film) ν_{max} 2924, 1697, 1615, 1539, 1403, 1327, 1137, 760 cm^{–1}. ESI-TOF HRMS *m/z* 391.1422 (M+H⁺, C₂₀H₂₃ClN₂O₄ requires 391.1419).

3-(5-(1*H*-Indole-2-carboxamido)-1*H*-indole-2-carbonyl)-1-(chloromethyl)-5-hydroxy-*N*-methyl-1,2-dihydrobenzo[*e*]indole-6-carboxamide (19**)**

Compound **18** (15 mg, 0.038 mmol) was dissolved in 4 N HCl in EtOAc (1 mL) and the mixture was allowed to stir at room temperature for 15 min. The solvent was removed under a stream of nitrogen and the residue was redissolved in anhydrous DMF (0.76 mL). EDCI (21.9 mg, 0.115 mmol) and **16** (14.6 mg, 0.045 mmol) were added and the reaction mixture was allowed to stir at 25 °C for 22 h. The reaction mixture was quenched with the addition of H₂O and diluted with EtOAc. The organic phase was washed with 2 N aqueous HCl (3 $\times$), saturated aqueous NaHCO₃ (5 $\times$) and saturated aqueous NaCl. The organic extract was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by PTLC (SiO₂, 40% THF/toluene) providing **19** (7.1 mg, 31%) as a dark yellow solid. ¹H NMR (DMSO-*d*₆, 600 MHz) δ 11.74 (d, *J* = 16.2 Hz, 2H), 10.48 (s, 1H), 10.18 (s, 1H), 8.24 (s, 1H), 8.07–8.06 (m, 1H), 7.95 (br, 1H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.59 (d, *J* = 9 Hz, 1H), 7.50–7.47 (m, 2H), 7.43 (s, 1H), 7.24–7.18 (m, 3H), 7.07 (t, *J* = 7.8 Hz, 1H), 4.84 (t, *J* = 10.2 Hz, 1H), 4.59 (d, *J* = 10.8 Hz, 1H), 4.28 (m, 1H), 4.01 (d, *J* = 9 Hz, 1H), 3.88 (dd, *J* = 7.2, 10.8 Hz, 1H), 2.77 (s, 3H). ¹³C NMR (DMSO-*d*₆, 150 MHz) δ 171.2, 159.7, 159.1, 154.0, 142.2, 136.3, 135.3, 132.9, 131.5, 131.2, 130.8, 130.0, 126.74, 126.72, 125.9, 123.3, 123.1, 122.7, 121.2, 119.4, 118.9, 117.7, 114.9, 112.5, 112.0, 111.8, 105.4,

103.0, 101.4, 54.5, 47.3, 40.8, 25.9. IR (film) $\nu_{\max}$ 3293, 2953, 1613, 1516, 1402, 1241, 1138 cm^{-1} . ESI-TOF HRMS m/z 592.1744 ($\text{M}+\text{H}^+$, $\text{C}_{33}\text{H}_{26}\text{ClN}_5\text{O}_4$ requires 592.1746).

DNA Alkylation Studies

See reference 20a for full details of the DNA alkylation assay. The DNA alkylation reactions were conducted at 25 °C with 5'- ^{32}P -end-labeled w794 DNA, which contains a single major alkylation site for the natural products. The glutathione reductive activation of compound (+)-**7** and (+)-**3c** was conducted for the specified time with 1 M L-glutathione at 37 °C.

Cell Growth Inhibition Assay

Compounds were tested for their cell growth inhibition of L1210 (ATCC CCL-219) mouse lymphocytic leukemia cells. A population of cells ($>1 \times 10^6$ cells/mL as determined with a hemocytometer) was diluted with an appropriate amount of Dulbecco-modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) to a final concentration of 30,000 cells/mL. To each well of a 96-well plate (Corning Costar), 100 μL of the cell-media solution was added with a multichannel pipette. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air for 24 h. The test compounds were added to the plate as follows: test substances were diluted in DMSO to a concentration of 1 mM and 10-fold serial dilutions were performed on a separate 96-well plate. Fresh culture medium (100 μL) was added to each well of cells to constitute 200 μL of medium per well followed by 2 μL of each test agent. Compounds were tested in duplicate (2 times) at six concentrations between 0–100 nM or 0–1000 nM. Following addition, cultures were incubated for an additional 72 h.

A phosphatase assay was used to establish the IC_{50} values as follows: the media in each cell was removed and 100 μL of phosphatase solution (100 mg phosphatase substrate in 30 mL 0.1 M NaOAc, pH 5.5, 0.1% Triton X-100 buffer) was added to each well. The plates were incubated at 37 °C for 5 min. After the given incubation time, 50 μL of 0.1 N NaOH was added to each well and the absorption at 405 nm was determined using a 96 well plate reader. As the absorption is directly proportional to the number of living cells, the IC_{50} values were calculated and the reported values represent of the average of 4 determinations ($\text{SD} \pm 10\%$).

The Sulforhodamine B (SRB) assay was used to determine the activity of the cyclic carbamate prodrug **20**^{12h} of (+)-**4** and the prodrug (+)-**6** under aerobic (37°C, 5% CO_2 and 95% humidified air) and hypoxic (37°C, 5% CO_2 , and 0.5% O_2) conditions. The SRB assay was conducted as described by the Developmental Therapeutics Program at NCI (dtp.cancer.gov/branches/btb/ivclsp.html). Prior to conducting these studies, A549 and PC3/MM2 cells were cultured under hypoxia (New Brunswick Galaxy 170 R) for one week, sub-culturing twice to replenish media and nutrients to ensure the cells were viable and healthy. Cell viability of A549 and PC3/MM2 was 94% and 96%, respectively as determined by trypan blue (In Vitrogen Countess automated cell counter) under these conditions. Subsequently, cells were plated, returned to the aerobic or hypoxic incubator and 24 h later the cyclic carbamate prodrug **20**^{12h} of (+)-**4** and the prodrug (+)-**6** were run in ten concentration dose-response experiments against lung A549 and prostate PC3/MM2 cancer cell lines under aerobic and hypoxic conditions for 72 h. Percentage growth was calculated for each drug concentration using the absorbance raw data, and percentage of growth inhibition was calculated using the formula $[(T_1 - T_2)/(C - T_2)] \times 100\%$. Data from $n = 3$ independent experiments were plotted by sigmoidal dose-response using non-linear regression (GraphPad Prism) and IC_{50} values were determined for each drug under aerobic and hypoxic conditions. The hypoxia cytotoxicity ratio (HCR) reported was determined by

dividing the IC₅₀ value under aerobic conditions by the IC₅₀ value under hypoxic conditions.

In Vivo Antitumor Activity

B6D2F1 mice were injected intraperitoneal (i.p.) with syngeneic L1210 cells (1×10^6) on day 0. Ten mice were randomly assigned to control vehicle or treatment groups for compounds (+)-**4** and (+)-**6** at doses of 60, 100, 250, and 500 $\mu\text{g/kg/inj}$ for (+)-**4** or 60, 200, 500, 1000, and 2500 $\mu\text{g/kg/inj}$ for (+)-**6**. Compounds (+)-**4** and (+)-**6** were formulated in 100% DMSO. Both compounds were injected i.p. on days 1, 5, and 9. Following injection of tumor cells, animals were monitored daily and weighed two times per week. Percent survival (T/C) for treated and control groups were determined by dividing the total survival days for each treatment group by the total survival days for the control group and multiplying $\times 100$. All animal studies were carried out in the animal facilities of The University of Kansas Medical Center with strict adherence to the guidelines of the IACUC Animal Welfare Committee of KUMC (IACUC approval # 2009-1837).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

CBI	1,2,9,9a-tetrahydrocyclopropa[<i>c</i>]benz[<i>e</i>]indol-4-one
DPPA	diphenylphosphoryl azide
LiHMDS	lithium bis(trimethylsilyl)amide
THF	tetrahydrofuran
BnSH	benzyl thiol
BnOH	benzyl alcohol
BnNH₂	benzylamine
EtOAc	ethyl acetate
DMF	dimethylformamide
HCR	hypoxia cytotoxicity ratio
MeOH	methanol

References

1. Ichimura M, Ogawa T, Takahashi K, Kobayashi E, Kawamoto I, Yasuzawa T, Takahashi I, Nakano H, Duocarmycin SA. A New Antitumor Antibiotic From Streptomyces sp. *J. Antibiot.* 1990; 43:1037–1038. [PubMed: 2211354]
2. Martin DG, Biles C, Gerpheide SA, Hanka LJ, Krueger WC, McGovren JP, Mizsak SA, Neil GL, Stewart JC, Visser J. CC-1065 (NSC 298223), A Potent New Antitumor Agent. Improved Production and Isolation, Characterization and Antitumor Activity. *J. Antibiot.* 1981; 34:1119–1125. [PubMed: 7328053]

3. Takahashi I, Takahashi K, Ichimura M, Morimoto M, Asano K, Kawamoto I, Tomita F, Nakano H. Duocarmycin, A New Antitumor Antibiotic From Streptomyces. *J. Antibiot.* 1988; 41:1915–1917. [PubMed: 3209484]
4. Igarashi Y, Futamata K, Fujita T, Sekine A, Senda H, Naoki H, Furumai T. Yatakemycin, A Novel Antifungal Antibiotic Produced by Streptomyces sp. TP-A0356. *J. Antibiot.* 2003; 56:107–113. [PubMed: 12715869]
5. For duocarmycin SA, see: Boger DL, Johnson DS, Yun W. (+)- and *ent*-(–)-Duocarmycin SA and (+)- and *ent*-(–)-N-BOC-DSA DNA Alkylation Properties. Alkylation Site Models That Accommodate the Offset AT-Rich Adenine N3 Alkylation Selectivity of the Enantiomeric Agents. *J. Am. Chem. Soc.* 1994; 116:1635–1656. For yatakemycin, see: Parrish JP, Kastrinsky DB, Wolkenberg SE, Igarashi Y, Boger DL. DNA Alkylation Properties of Yatakemycin. *J. Am. Chem. Soc.* 2003; 125:10971–10976. [PubMed: 12952479] Trzuppek JD, Gottesfeld JM, Boger DL. Alkylation of Duplex DNA in Nucleosome Core Particles by Duocarmycin SA and Yatakemycin. *Nat. Chem. Biol.* 2006; 2:79–82. [PubMed: 16415862] Tichenor MS, MacMillan KS, Trzuppek JD, Rayl TJ, Hwang I, Boger DL. Systematic Exploration of the Structural Features of Yatakemycin Impacting DNA Alkylation and Biological Activity. *J. Am. Chem. Soc.* 2007; 129:10858–10869. [PubMed: 17691783] For CC-1065, see: Hurley LH, Lee C-S, McGovren JP, Warpehoski MA, Mitchell MA, Kelly RC, Aristoff PA. Molecular Basis for Sequence-Specific DNA Alkylation by CC-1065. *Biochemistry.* 1988; 27:3886–3892. [PubMed: 3408734] Boger DL, Johnson DS, Yun W, Tarby CM. Molecular Basis for Sequence Selective DNA Alkylation by (+)- and *ent*-(–)-CC-1065 and Related Agents: Alkylation Site Models that Accommodate the Offset AT-Rich Adenine N3 Alkylation Selectivity. *Bioorg. Med. Chem.* 1994; 2:115–135. [PubMed: 7922122] Boger DL, Munk SA, Zarrinmayeh H. (+)-CC-1065-DNA Alkylation: Key Studies Demonstrating a Noncovalent Binding Selectivity Contribution to the Alkylation Selectivity. *J. Am. Chem. Soc.* 1991; 113:3980–3983. Boger DL, Zarrinmayeh H, Munk SA, Kitos PA, Suntornwat O. Demonstration of a Pronounced Effect of Noncovalent Binding Selectivity on the (+)-CC-1065 DNA Alkylation and Identification of the Pharmacophore of the Alkylation Subunit. *Proc. Nat. Acad. Sci. U.S.A.* 1991; 88:1431–1435. Boger DL, Coleman RS, Invergo BJ, Sakya SM, Ishizaki T, Munk SA, Zarrinmayeh H, Kitos PA, Thompson SC. Synthesis and Evaluation of Aborted and Extended CC-1065 Functional Analogs: (+)- and (–)-CPI-PDE-I₁, (+)- and (–)-CPI-CDPI₁, and (+)- and (–)-CPI-CDPI₃. Preparation of Key Partial Structures and Definition of an Additional Functional Role of the CC-1065 Central and Right-Hand Subunits. *J. Am. Chem. Soc.* 1990; 112:4623–4632. Boger DL, Munk SA, Zarrinmayeh H. (+)-CC-1065 DNA Alkylation: Key Studies Demonstrating a Noncovalent Binding Selectivity Contribution to the Alkylation Selectivity. *J. Am. Chem. Soc.* 1991; 113:3980–3983. For duocarmycin A, see: Boger DL, Ishizaki T, Zarrinmayeh H, Munk SA, Kitos PA, Suntornwat O. Duocarmycin-Pyrindamycin DNA Alkylation Properties and Identification, Synthesis, and Evaluation of Agents Incorporating the Pharmacophore of the Duocarmycin-Pyrindamycin Alkylation Subunit. Identification of the CC-1065 Duocarmycin Common Pharmacophore. *J. Am. Chem. Soc.* 1990; 112:8961–8971. Boger DL, Ishizaki T, Zarrinmayeh H. Isolation and Characterization of the Duocarmycin-Adenine DNA Adduct. *J. Am. Chem. Soc.* 1991; 113:6645–6649. Boger DL, Yun W, Terashima S, Fukuda Y, Nakatani K, Kitos PA, Jin Q. DNA Alkylation Properties of the Duocarmycins: (+)-Duocarmycin A, *epi*-(+)-Duocarmycin A, *ent*-(–)-Duocarmycin A and *epi*, *ent*-(–)-Duocarmycin A. *Bioorg. Med. Chem. Lett.* 1992; 2:759–765. Boger DL, Yun W. Reversibility of the Duocarmycin A and SA DNA Alkylation Reaction. *J. Am. Chem. Soc.* 1993; 115:9872–9873. Boger DL, Invergo BJ, Coleman RS, Zarrinmayeh H, Kitos PA, Thompson SC, Leong T, McLaughlin LW. A Demonstration of the Intrinsic Importance of Stabilizing Hydrophobic Binding and Noncovalent van der Waals Contacts Dominant in the Noncovalent CC-1065/B-DNA Binding. *Chem.-Biol. Interactions.* 1990; 73:29–52.
6. Reviews: Boger DL, Johnson DS. CC-1065 and the Duocarmycins: Understanding Their Biological Function Through Mechanistic Studies. *Angew. Chem. Int. Ed. Engl.* 1996; 35:1438–1474. Boger DL. The Duocarmycins: Synthetic and Mechanistic Studies. *Acc. Chem. Res.* 1995; 28:20–29. Boger DL, Johnson DS. CC-1065 and the Duocarmycins: Unraveling the Keys to a New Class of Naturally Derived DNA Alkylating Agents. *Proc. Natl. Acad. Sci. U.S.A.* 1995; 92:3642–3649. [PubMed: 7731958] Boger DL, Garbaccio RM. Shape-Dependent Catalysis: Insights into the Source of Catalysis for the CC-1065 and Duocarmycin DNA Alkylation Reaction. *Acc. Chem. Res.* 1999; 32:1043–1052. Tichenor MS, Boger DL. Yatakemycin: Total Synthesis, DNA Alkylation, and Biological Properties. *Natural Prod. Rep.* 2008; 25:220–226. MacMillan KS, Boger DL.

Fundamental Relationships Between Structure, Reactivity, and Biological Activity for the Duocarmycins and CC-1065. *J. Med. Chem.* 2009; 52:5771–5780. [PubMed: 19639994] Searcey M. Duocarmycins: Nature's Prodrugs? *Curr. Pharm. Des.* 2002; 8:1375–1389. [PubMed: 12052214] Tse WC, Boger DL. Sequence-Selective DNA Recognition: Natural Products and Nature's Lessons. *Chem. Biol.* 2004; 11:1607–1617. [PubMed: 15610844]

7. (a) Boger DL, Coleman RS. Total Synthesis of (+)-CC-1065 and *ent*-(–)-CC-1065. *J. Am. Chem. Soc.* 1988; 110:1321–1323. (b) Boger DL, Coleman RS. Total Synthesis of CC-1065, and the Precise, Functional Agent CPI-CDPI₂. *J. Am. Chem. Soc.* 1988; 110:4796–4807. (c) Boger DL, Machiya K. Total Synthesis of (+)-Duocarmycin SA. *J. Am. Chem. Soc.* 1992; 114:10056–10058. (d) Boger DL, Machiya K, Hertzog DL, Kitos PA, Holmes D. Total Synthesis and Preliminary Evaluation of (+)- and *ent*-(–)-Duocarmycin SA. *J. Am. Chem. Soc.* 1993; 115:9025–9036. (e) Boger DL, McKie JA, Nishi T, Ogiku T. Enantioselective Total Synthesis of (+)-Duocarmycin A, *epi*-(+)-Duocarmycin A, and Their Unnatural Enantiomers. *J. Am. Chem. Soc.* 1996; 118:2301–2302. (f) Boger DL, McKie JA, Nishi T, Ogiku T. Total Synthesis of (+)-Duocarmycin A and *epi*-(+)-Duocarmycin A and Their Unnatural Enantiomers: Assessment of Chemical and Biological Properties. *J. Am. Chem. Soc.* 1997; 119:311–325. (g) Boger DL, Boyce CW, Garbaccio RM, Goldberg JA. CC-1065 and the Duocarmycins: Synthetic Studies. *Chem. Rev.* 1997; 97:787–828. [PubMed: 11848889] (h) Tichenor MS, Kastrinsky DB, Boger DL. Total Synthesis, Structure Revision, and Absolute Configuration of (+)-Yatakemycin. *J. Am. Chem. Soc.* 2004; 126:8396–8398. [PubMed: 15237994] (i) Tichenor MS, Trzupsek JD, Kastrinsky DB, Shiga F, Hwang I, Boger DL. Asymmetric Total Synthesis of (+)- and *ent*-(–)-Yatakemycin and Duocarmycin SA. Evaluation of Yatakemycin Key Partial Structures and Its Unnatural Enantiomer. *J. Am. Chem. Soc.* 2006; 128:15683–15696. [PubMed: 17147378] (j) MacMillan KS, Nguyen T, Hwang I, Boger DL. Total Synthesis and Evaluation of *iso*-Duocarmycin SA and *iso*-Yatakemycin. *J. Am. Chem. Soc.* 2009; 131:1187–1194. [PubMed: 19154178]
8. (a) Boger DL, Hertzog DL, Bollinger B, Johnson DS, Cai H, Goldberg J, Turnbull P. Duocarmycin SA Shortened, Simplified, and Extended Agents: A Systematic Examination of the Role of the DNA Binding Subunit. *J. Am. Chem. Soc.* 1997; 119:4977–4986. (b) Boger DL, Bollinger B, Hertzog DL, Johnson DS, Cai H, Mesini P, Garbaccio RM, Jin Q, Kitos PA. Reversed and Sandwiched Analogs of Duocarmycin SA: Establishment of the Origin of the Sequence-Selective Alkylation of DNA and New Insights into the Source of Catalysis. *J. Am. Chem. Soc.* 1997; 119:4987–4998. (c) Boger DL, Garbaccio RM. Catalysis of the CC-1065 and Duocarmycin DNA Alkylation Reaction: DNA Binding Induced Conformational Change in the Agent Results in Activation. *Bioorg. Med. Chem.* 1997; 5:263–276. [PubMed: 9061191]
9. (a) Boger DL, Ishizaki T, Wysocki RJ Jr, Munk SA, Kitos PA, Suntornwat O. Total Synthesis and Evaluation of (±)-N-(*tert*-Butoxycarbonyl)-CBI, (±)-CBI-CDPI₁, and (±)-CBI-CDPI₂: CC-1065 Functional Agents Incorporating the Equivalent 1,2,9,9a-Tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) Left-Hand Subunit. *J. Am. Chem. Soc.* 1989; 111:6461–6463. (b) Boger DL, Ishizaki T, Kitos PA, Suntornwat O. Synthesis of N-(*tert*-Butyloxycarbonyl)-CBI, CBI, CBI-CDPI₁, and CBI-CDPI₂: Enhanced Functional Analogs of CC-1065 Incorporating the 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI) Left-Hand Subunit. *J. Org. Chem.* 1990; 55:5823–5832. (c) Boger DL, Ishizaki T. Resolution of a CBI Precursor and Incorporation into the Synthesis of (+)-CBI, (+)-CBI-CDPI₁, (+)-CBI-CDPI₂: Enhanced Functional Analogs of (+)-CC-1065. A Critical Appraisal of a Proposed Relationship Between Electrophile Reactivity, DNA Binding Properties, and Cytotoxic Potency. *Tetrahedron Lett.* 1990; 31:793–796. (d) Boger DL, Ishizaki T, Zarrinmayeh H, Kitos PA, Suntornwat O. A Potent, Simple Derivative of an Analog of the CC-1065 Alkylation Subunit. *Bioorg. Med. Chem. Lett.* 1991; 1:55–58. (e) Boger DL, Munk SA. DNA Alkylation Properties of Enhanced Functional Analogs of CC-1065 Incorporating the 1,2,9,9a-Tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) Alkylation Subunit. *J. Am. Chem. Soc.* 1992; 114:5487–5496. (f) Boger DL, Yun W. Role of the CC-1065 and Duocarmycin N² Substituent: Validation of a Direct Relationship Between Solvolysis Chemical Stability and in vitro Biological Potency. *J. Am. Chem. Soc.* 1994; 116:5523–5524. (g) Boger DL, Yun W, Cai H, Han N. CBI-CDPBO₁ and CBI-CDPBI₁: CC-1065 Analogs Containing Deep-Seated Modifications in the DNA Binding Subunit. *Bioorg. Med. Chem.* 1995; 3:761–775. [PubMed: 7582954] (h) Boger DL, Yun W. CBI-TMI: Synthesis and Evaluation of a Key Analog of the Duocarmycins. Validation of a Direct Relationship between Chemical Solvolytic Stability and Cytotoxic Potency and Confirmation of the Structural Features Responsible for the Distinguishing Behavior of

Enantiomeric Pairs of Agents. *J. Am. Chem. Soc.* 1994; 116:7996–8006.(i) Parrish JP, Katrinsky DB, Stauffer F, Hedrick MP, Hwang I, Boger DL. Establishment of Substituent Effects in the DNA Binding Subunit of CBI Analogues of the Duocarmycins and CC-1065. *Bioorg. Med. Chem.* 2003; 11:3815–3838. [PubMed: 12901927] (j) Boger DL, Wysocki RJ, Ishizaki T. Synthesis of N-(Phenylsulfonyl)-CI, N-(tert-Butyloxy carbonyl)-CI, CI-CDPI1, and CI-CDPI2: CC-1065 Functional Analogs Incorporating the Parent 1,2,7,7a-Tetrahydrocycloprop[1,2-*c*]indol-4-one (CI) Left-Hand Subunit. *J. Am. Chem. Soc.* 1990; 112:5230–5240.

10. (a) Boger DL, Yun W, Teegarden BR. An Improved Synthesis of 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI): A Simplified Analog of the CC-1065 Alkylation Subunit. *J. Org. Chem.* 1992; 57:2873–2876.(b) Boger DL, McKie JA. An Efficient Synthesis of 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI): An Enhanced and Simplified Analog of the CC-1065 and Duocarmycin Alkylation Subunits. *J. Org. Chem.* 1995; 60:1271–1275.(c) Boger DL, McKie JA, Boyce CW. Asymmetric Synthesis of the 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI) Alkylation Subunit of CC-1065 and Duocarmycin Analogues. *Synlett.* 1997:515–517.(d) Kastrinsky DB, Boger DL. Effective Asymmetric Synthesis of 1,2,9,9a-Tetrahydrocyclopropa[*e*]benz[*e*]indol-4-one (CBI). *J. Org. Chem.* 2004; 69:2284–2289. [PubMed: 15049620] (e) Lajiness JP, Boger DL. Asymmetric Synthesis of 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI). *J. Org. Chem.* 2010; 76:583–587. [PubMed: 21192653] (f) Drost KJ, Cava MP. A Photochemically Based Synthesis of the Benzannelated Analog of the CC-1065 A Unit. *J. Org. Chem.* 1991; 56:2240–2244.(g) Aristoff PA, Johnson PD. Synthesis of CBI-PDE-I-Dimer, the Benzannelated Analog of CC-1065. *J. Org. Chem.* 1992; 57:6234–6239.(h) Mohamadi F, Speez MM, Staten GS, Marder P, Kipka JK, Johnson DA, Boger DL, Zarrinmayeh H. Total Synthesis and Biological Properties of Novel Antineoplastic (Chloromethyl)furanoidindolines. An Asymmetric Hydroboration Mediated Synthesis of the Alkylation Subunits. *J. Med. Chem.* 1994; 37:232–239. [PubMed: 8295210] (i) Ling L, Xie Y, Lown JW. A Practical Route to Optically Active CBI, A Potent Analog of the CC-1065 Alkylation Subunit. *Heterocycl. Commun.* 1997; 3:405–408.
11. (a) Boger DL, Yun W, Han N. 1,2,9,9a-Tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI) Analogs of CC-1065 and the Duocarmycins: Synthesis and Evaluation. *Bioorg. Med. Chem.* 1995; 3:1429–1453. [PubMed: 8634824] (b) Boger DL, Ishizaki T, Sakya S, Munk SA, Kitos PA, Jin Q, Besterman JM. Synthesis and Preliminary Evaluation of (+)-CBI-indole₂: An Enhanced Functional Analog of CC-1065. *Bioorg. Med. Chem. Lett.* 1991; 1:115–120.
12. For Carzelesin, see: Li L, DeKoning TF, Kelly RC, Krueger WC, McGovren JP, Padbury GE, Petzold GL, Wallace TL, Ouding RJ, Prairie MD, Gebhard I. Cytotoxicity and Antitumor Activity of Carzelesin, a Prodrug Cyclopropylpyrroloindole Analogue. *Cancer Res.* 1992; 52:4904–4913. [PubMed: 1516047] van Tellingen O, Nooijen WJ, Schaaf LJ, van der Valk M, van Asperen J, Henrar REC, Beijnen JH. Comparative Pharmacology of the Novel Cyclopropylpyrroloindole-Prodrug Carzelesin in Mice, Rats, and Humans. *Cancer Res.* 1998; 58:2410–2416. [PubMed: 9622082] For KW-2189, see: Kobayashi E, Okamoto A, Asada M, Okabe M, Nagamura S, Asai A, Saito H, Gomi K, Hirata T. Characteristics of Antitumor Activity of KW-2189, A Novel Water-Soluble Derivative of Duocarmycin, Against Murine and Human Tumors. *Cancer Res.* 1994; 54:2404–2410. [PubMed: 8162588] Nagamura S, Kanda Y, Kobayashi E, Gomi K, Saito H. Synthesis and Antitumor Activity of Duocarmycin Derivatives. *Chem. Pharm. Bull.* 1995; 43:1530–1535. [PubMed: 7586077] For other CBI carbamate prodrugs: Boger DL, Boyce CW, Garbaccio RM, Searcey M, Jin Q. CBI Prodrug Analogs of CC-1065 and the Duocarmycins. *Synthesis.* 1999:1505–1509. Wang Y, Li L, Tian Z, Jiang W, Larrick J. Synthesis and Antitumor Activity of CBI-Bearing Ester and Carbamate Prodrugs of CC-1065. *Bioorg. Med. Chem.* 2006; 14:7854–7861. [PubMed: 16908174] Li LS, Sinha SC. Studies Toward the Duocarmycin Prodrugs for the Antibody Prodrug Therapy Approach. *Tetrahedron Lett.* 2009; 50:2932–2935. Wolfe AL, Duncan KK, Parelkar NK, Weir SJ, Vielhauer GA, Boger DL. A Novel, Unusually Efficacious Duocarmycin Carbamate Prodrug that Releases No Residual Byproduct. *J. Med. Chem.* 2012; 55:5878–5886. [PubMed: 22650244]
13. For glycosidic prodrugs: Tietze LF, Lieb M, Herzig T, Haunert F, Schuberth I. A Strategy for Tumor-Selective Chemotherapy by Enzymatic Liberation of seco-duocarmycin SA-derivatives from Nontoxic Prodrugs. *Bioorg. Med. Chem.* 2001; 9:1929–1939. [PubMed: 11425596] Tietze LF, Major F, Schuberth I. Antitumor Agents: Development of Highly Potent Glycosidic Duocarmycin Analogues for Selective Cancer Therapy. *Angew. Chem. Int. Ed.* 2006; 45:6574–

6577. Tietze LF, Schuster HJ, Schmuck K, Schuberth I, Alves F. Duocarmycin-based Prodrugs for Cancer Prodrug Monotherapy. *Bioorg. Med. Chem.* 2008; 16:6312–6318. [PubMed: 18524605]
14. For reductively activated prodrugs: Hay MP, Anderson RF, Ferry DM, Wilson WR, Denny WA. Synthesis and Evaluation of Nitroheterocyclic Carbamate Prodrugs for Use with Nitroreductase-Mediated Gene-Directed Enzyme Prodrug Therapy. *J. Med. Chem.* 2003; 46:5533–5545. [PubMed: 14640560] Hay MP, Sykes BM, Denny WA, Wilson WR. A 2-Nitroimidazole Carbamate Prodrug of 5-Amino-1-(chloromethyl)-3-[(5,6,7-trimethoxyindol-2-yl)carbonyl]-1,2-dihydro-3*H*-benz[e]indole (Amino-seco-CBI-TMI) for Use With ADEPT and GDEPT. *Bioorg. Med. Chem. Lett.* 1999; 9:2237–2242. [PubMed: 10465553] Tercel M, Atwell GJ, Yang S, Ashoorzadeh A, Stevenson RJ, Botting KJ, Gu Y, Mehta SY, Denny WA, Wilson WR, Pruijn FB. Selective Treatment of Hypoxic Tumor Cells In Vivo: Phosphate Pre-Prodrugs of Nitro Analogues of the Duocarmycins. *Angew. Chem. Int. Ed.* 2011; 50:2606–2609. Townes H, Summerville K, Purnell B, Hooker M, Madsen E, Hudson S, Lee M. Investigation of a Novel Reductively-Activatable Anticancer Prodrug of *seco*-CBI-TMI, an Analog of Duocarmycin SA. *Med. Chem. Res.* 2002; 11:248–253. Boger DL, Garbaccio RM. A Novel Class of CC-1065 and Duocarmycin Analogues Subject to Mitomycin-Related Reductive Activation. *J. Org. Chem.* 1999; 64:8350–8362. [PubMed: 11674758]
15. For additional prodrugs: Zhao RH, Erickson HK, Leece BA, Reid EE, Goldmacher VS, Lambert JM, Chari RVJ. Synthesis and Biological Evaluation of Antibody Conjugates of Phosphate Prodrugs of Cytotoxic DNA Alkylators for the Targeted Treatment of Cancer. *J. Med. Chem.* 2012; 55:766–782. [PubMed: 22148292] Pors K, Loadman PM, Shnyder SD, Sutherland M, Sheldrake HM, Guino M, Kiakos K, Hartley JA, Searcey M, Patterson LH. Modification of the Duocarmycin Pharmacophore Enables CYP1A1 Targeting for Biological Activity. *Chem. Commun.* 2011; 47:12062–12064.
16. Wolkenberg SE, Boger DL. Mechanisms of in situ Activation for DNA Targeting Antitumor Agents. *Chem. Rev.* 2002; 102:2477–2495. [PubMed: 12105933]
17. (a) Jin W, Trzupek JD, Rayl TJ, Broward MA, Vielhauer GA, Weir SJ, Hwang I, Boger DL. A Unique Class of Duocarmycin and CC-1065 Analogues Subject to Reductive Activation. *J. Am. Chem. Soc.* 2007; 129:15391–15397. [PubMed: 18020335] (b) Lajiness JP, Robertson WM, Dunwiddie I, Broward MA, Vielhauer GA, Weir SJ, Boger DL. Design, Synthesis, and Evaluation of Duocarmycin *O*-Amino Phenol Prodrugs Subject to Tunable Reductive Activation. *J. Med. Chem.* 2010; 53:7731–7738. [PubMed: 20942408]
18. (a) Ahmad S, Okine L, Le B, Najarian P, Vistica DT. Elevation of Glutathione in Phenylalanine Mustard-resistant Murine L1210 Leukemia Cells. *J. Biol. Chem.* 1987; 262:15048–15053. [PubMed: 3667623] (b) Balendiran GK, Dabur R, Fraser D. The Role of Glutathione in Cancer. *Cell Biochem. Func.* 2004; 22:343–352.
19. (a) Paz MM, Hopkins PB. DNA-DNA Interstrand Crosslinking by FR66979: Intermediates in the Activation Cascade. *J. Am. Chem. Soc.* 1997; 119:5999–6005. (b) Williams RM, Rajski SR, Rollins SB. FR 900482, A Close Cousin of Mitomycin C that Exploits Mitosene-based DNA Crosslinking. *Chem. Biol.* 1997; 4:127–137. [PubMed: 9190287] (c) Tepe JJ, Kosogof C, Williams RM. DNA Interstrand Cross-link Formation by Reductive Activation of Dehydropyrrolizidine Progenitors. *Tetrahedron.* 2002; 58:3553–3559.
20. (a) Boger DL, Munk SA, Zarrinmayeh H, Ishizaki T, Haught J, Bina M. An Alternative and Convenient Strategy for Generation of Substantial Quantities of Singly 5'-P32-End-Labeled Double-Stranded DNA for Binding Studies. Development of a Protocol for Examination of Functional Features of (+)-CC-1065 and the Duocarmycins That Contribute to Their Sequence-Selective DNA Alkylation Properties. *Tetrahedron.* 1991; 47:2661–2682. (b) Boger DL, Fink BE, Brunette SR, Tse WC, Hedrick MP. A Simple High-Resolution Method for Establishing DNA Binding Affinity and Sequence Selectivity. *J. Am. Chem. Soc.* 2001; 123:5878–5891. [PubMed: 11414820]

Figure 1.
Natural products and structure of CBI.

Figure 2.
Initial N-O prodrugs.

Figure 3.
Cyclic N-O prodrugs.

Entry	Solvent	Nucleophile ^a	Base ^a	2 h ^b	24 h ^b	48 h ^b	72 h ^b
1	THF ^c	BnSH	NaHCO ₃	28%	33%	39%	46%
2	MeOH	BnSH	NaHCO ₃	33%	58%	75%	83%
3	THF ^c	BnNH ₂	NaHCO ₃	2%	3%	4%	3%
4	MeOH	BnNH ₂	NaHCO ₃	stable	9%	15%	16%

^aexcess reagents used; ^bpercent of starting material consumed as determined by LCMS absorption at 254 nM; ^cstable under non basic conditions

Figure 4. Nucleophilic cleavage of **15** and plausible mechanisms for the thiol-promoted reductive cleavage of weak N-O bond and subsequent spirocyclization.

Figure 5.

DNA alkylation by (+)-7 in w794 DNA. No DNA alkylation by (+)-7 is observed in the absence of activation (lane 7), whereas thiol addition (L-glutathione) releases the free drug and results in DNA alkylation (lanes 8 and 9) at sites observed with (+)-CBI-indole₂ (lane 6). DNA-agent incubation at 23 °C for 2 h, removal of unbound agent by EtOH precipitation, and 30 min thermolysis (100 °C) followed by 8% denaturing PAGE and autoradiography. Lane 1, control DNA; lanes 2–5, Sanger G, C, A, and T sequencing reactions; lane 6, (+)-4 (1×10^{-5} M); lane 7, (+)-7 (1×10^{-5} M); lanes 8–9, (+)-7 (1×10^{-5} M) incubated at 37 °C with 1 M L-glutathione for 12 h and 24 h.

Figure 6.

DNA alkylation by **3c** in w794 DNA. No DNA alkylation by (+)-**3c** is observed in the absence of activation (lane 7) even at concentrations 100-fold higher than (+)-**4**, whereas thiol addition (L-glutathione) releases the free drug and results in DNA alkylation (lanes 8 and 9) at sites observed with (+)-CBI-indole₂ (lane 6). DNA-agent incubation at 23 °C for 2 h, removal of unbound agent by EtOH precipitation, and 30 min thermolysis (100 °C) followed by 8% denaturing PAGE and autoradiography. Lane 1, control DNA; lanes 2–5, Sanger G, C, A, and T sequencing reactions; lane 6, (+)-**4** (1×10^{-5} M); lane 7, (+)-**3c** ($1 \times$

10^{-3} M); lanes 8–9, (+)-**3c** (1×10^{-5} M) incubated at 37 °C with 1 M L-glutathione for 12 h and 48 h.

Compd	IC ₅₀ L1210	
	natural (nM)	unnatural (nM)
1 , duocarmycin SA	0.010	0.100
2 , CC-1065	0.020	0.020
4 , seco-CBI-indole ₂	0.030	0.900
6	0.99	740
7	0.30	65
(±)- 19		0.073
(+)-N-Boc-CBI	80	900
12	850	>1000
15	650	>1000

Figure 7.
In vitro cytotoxic activity.

Compd	Dose $\mu\text{g/kg}^{\text{a}}$	MSP days ^b	T/C ^c	Surviving Mice ^d
none	0	17.6	100	0/10
4	60	>50	>284	1/10
4	100	6.4	36	0/10
4	250	3.7	21	0/10
4	500	3.0	17	0/10
6	60	28	159	0/10
6	200	>68	>386	1/10
6	500	>201	>1142	4/10
6	1000	>199	>1130	4/10
6	2500	>264	>1500	6/10

^aDose ($\mu\text{g/kg}$ wt. of animal) administered i.p. on days 1, 5, and 9.

^bMSP = Mean Survival Period (days). ^cT/C = Treated/Control (MSP) x 100. ^dNo. of live animals after 366 days (terminated).

Figure 8.
In vivo antitumor activity (L1210, ip).

Compd	A549, IC ₅₀ (nM)			PC3/MM2, IC ₅₀ (nM)		
	aerobic	hypoxic	HCR ^a	aerobic	hypoxic	HCR ^a
(+)-20	90	90	1	320	170	1.9
(+)-6	25	5	5	350	35	10

^aHCR = hypoxic cytotoxicity ratio, aerobic/hypoxic IC₅₀.

Figure 9.
Hypoxia-selective cytotoxic activity.

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.