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Novel Furano Analogues of Duocarmycin C1 and C2: Design, Synthesis, and Biological Evaluation of *seco*-iso-Cyclopropylfurano[2,3-*e*]indoline (*seco*-iso-CFI) and *seco*-Cyclopropyltetrahydrofurano[2,3-*f*]quinoline (*seco*-CFQ) Analogues

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This paper is dedicated to Professor Gordon L. Lange on the occasion of his retirement.

Abstract—The design, synthesis and biological evaluation of novel *seco*-iso-cyclopropylfurano[2,3-*e*]indoline (*seco*-iso-CFI) and the *seco*-cyclopropyltetrahydrofurano[2,3-*f*]quinoline (*seco*-CFQ) analogues of the duocarmycins are described. These novel analogues (**4**–**7**) were designed on the premise that the lone pair of electrons on the furano-oxygen atom could enter into conjugation with the isocyclopropylfurano[*e*]indolone (iso-CFI) alkylating moiety, formed from the loss of HCl in compounds **4**–**7**. The *seco*-iso-CFI DNA alkylating pharmacophore was synthesized through a well precedented approach of *5-exo-trig* aryl radical cyclization with a vinyl chloride. In our studies, in addition to the formation of the *seco*-iso-CFI product, an equal amount of an unexpected *seco*-CFQ product was also generated during the radical cyclization reaction. Like CC-1065 and adozelesin, using *Taq* DNA polymerase stop and thermal cleavage assays, the *seco*-iso-CFI compounds (**4** and **6**) and the *seco*-CFQ compounds (**5** and **7**) were shown to preferentially alkylate the adenine-N3 position within the minor groove of long stretches of A residues. A MM2 energy optimized molecular model of a 1:1 complex of compound **6** with DNA reveals that the *iso*-CFI compound fits snugly within the minor groove. Using a MTT based experiment, the cytotoxicity of compounds **4**–**7** were determined against the growth of murine leukemia (L1210), mastocytoma (P815) and melanoma (B16) cell lines. The concentrations of compounds required to inhibit the growth of these tumor cells by 50% is in the range of 10^{-8} M. These compounds were also tested against a panel of human cancer cells by the National Cancer Institute, demonstrating that the compounds exhibited a high level of activity against selected solid tumors. At a concentration of 0.0084 μM (based on the IC_{50} of compound **17** (*seco*-CBI-TMI) against the growth L1210 cells), while compounds **4** and **17** were toxic against murine bone marrow cells as judged by a colony forming study of freshly isolated murine progenitor hematopoeitic cells, compound **5**, a *seco*-CFQ compound, was significantly less toxic. Flow cytometric analysis of P815 cells that had been incubated for 24 h with compounds **4** and **5** at their cytotoxic IC_{50} concentrations indicated the induction of apoptosis in a large percentage of cells, thereby suggesting that this might be the mechanism by which the iso-CFI compounds kill cells.

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Introduction

The duocarmycins, such as duocarmycin A (**1**), C1 (**2**) and C2 (**3**) as depicted in Figure 1, and CC-1065 are highly potent anticancer agents, and the development of analogues of these compounds into potential drugs for cancer treatment has received much attention.[1] Several analogues of these natural products have entered clinical trials: adozelesin,[2] carzelesin,[3] KW2189,[4] and bizelesin.[5] While these compounds demonstrated significant anticancer activity, their clinical efficacy was significantly limited by their bone marrow toxicity, and currently only bizelesin is still under clinical evaluation (phase II).[5c] Analogues of the duocarmycins and CC-1065 derive their cytotoxic potency by covalently reacting with the adenine-N3 position within the minor groove of AT-rich DNA sequences, including a preferred site 5′-AGTTA-3′ for CC-1065,[1] and their mechanism of cytotoxic action has been suggested to be a result of drug induced apoptosis of cancer cells.[6]

In attempts to design novel analogues of the duocarmycins and CC-1065 with reduced toxicity to bone marrow cells, a wide range of analogues of the cyclopropylpyrrolo[*e*]indolone (CPI) subunit were synthesized and tested.[1] Examples of such analogues, cyclopropylbenzo[*e*]indolone (CBI),[1,7] cyclopropylpyrazolo[*e*]indolone (CPzI),[8] cyclopropylfurano[*e*]indolone (CFI),[9] and cyclopropylindolone (CI),[10] are given in Figure 2. From studies of these analogues several trends of structure–activity relationships became apparent.[1] First, the cytotoxicity of the analogues is directly related to the chemical stability of the molecules in aqueous acidic solutions.[1d–h] The stability is believed to arise from vinylogous resonance conjugation of the lone pair of electrons on the nitrogen atom of the carboxamido moiety and the cyclopropylindolone unit.[1d,11] It has also been demonstrated that the electron withdrawing capability of the carboxamido unit will further stabilize the cyclopropylindolone unit against solvolysis.[12] Second, inclusion of an extended aromatic unit to the CI unit, as in the case of CBI, significantly enhances chemical stability and cytotoxicity.[1,7,11] Third, attachment of an electron withdrawing group to the extended aromatic moiety also improves the chemical stability of the pharmacophore.[1d,13] The extensive SAR studies have further revealed that the *seco*-prodrugs are equally reactive with DNA and as cytotoxic as the cyclopropane containing drugs.[1,10] The *seco*-prodrugs readily lose HCl in cells or in the cell culture media to generate the active cyclopropane containing drugs to elicit the biological activity. It has also been shown that both the six- and five-membered ring containing *seco*-prodrugs, duocarmcyin C1 and C2, respectively, undergo a loss of HCl to generate Duocarmycin A.[14]

In the present study, novel *seco*-prodrugs of iso-CFI analogues of duocarmycins were synthesized and investigated. The ultimate alkylating agent iso-CFI, as depicted in the iso-CFI-TMI molecule given in Figure 1, was designed on the basis that the lone pair of electrons of the furan-oxygen atom would enter into conjugation (+R effect) with the cyclopropylindolone moiety. In addition to the carboxamido moiety, the furano-oxygen should provide a second set of vinylogous conjugation to the pharmacophore and stabilize the cyclopropylindolone funtionality toward nucleophiles. Another attractive feature of the iso-CFI pharmacophore is that furans are known to be stable towards biological degradation.[9a] In this paper, the synthesis, DNA binding properties, cytotoxicity, and bone marrow toxicity of two classes of *seco*-prodrugs of iso-CFI compounds, **4**–**7** as depicted in Figure 3, are described. The *seco*-iso-CFI compounds **4** and **6** are analogous to duocarmycin C2, and the *seco*-CFQ agents **5** and **7** are congeners of duocarmycin C1. While extensive studies have been done on *seco*-chloromethylindoline precursors of the cyclopropane-containing drugs,[1] only limited work has been done on *seco*-chloroquinoline analogues that are related to duocarmycin C1.[15] This paucity of work might be partly due to the lack of available synthetic methodologies. In this paper, formation of the *seco*-CFQ products represents a direct and efficient synthesis of chloroquinoline precursors of the cyclopropaneindolone compounds.

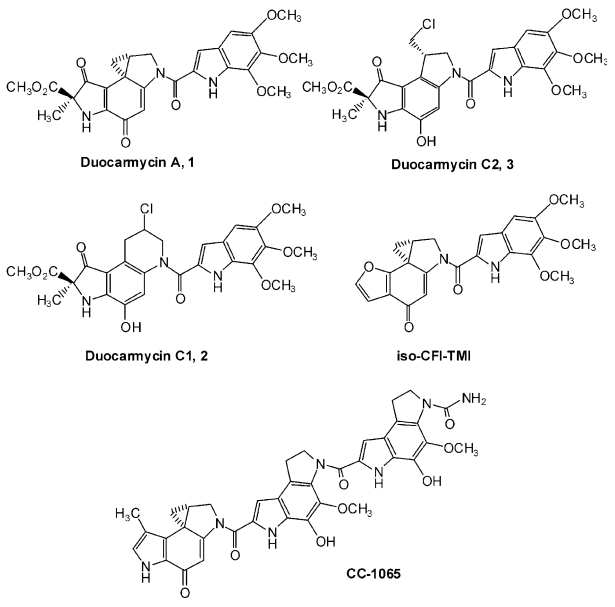


Figure 1. Structures of duocarmycin A, C1, C2, CC-1065 and iso-CFI-TMI.

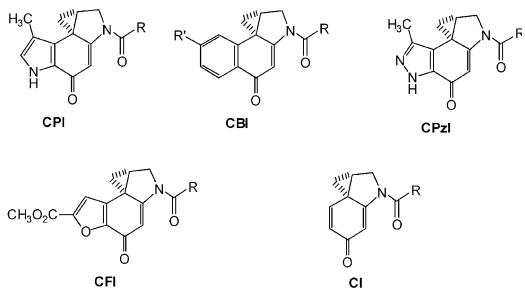


Figure 2. Examples of known DNA alkylating subunits of the duocarmycins and CC-1065.

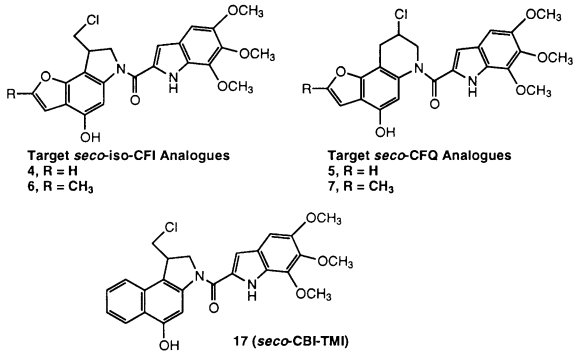
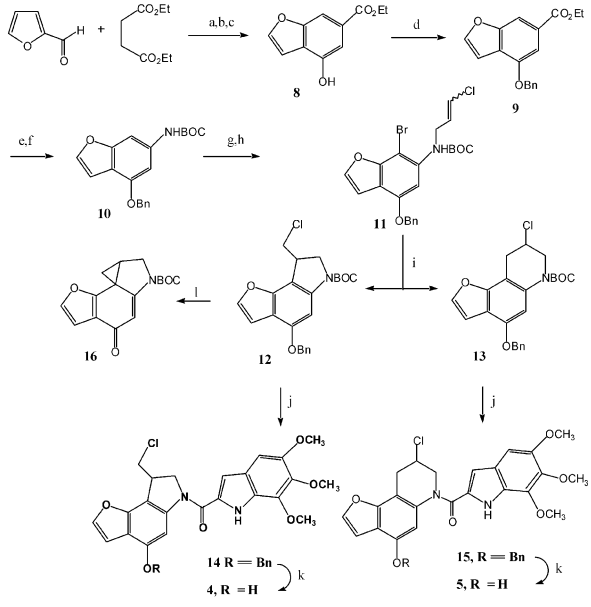


Figure 3. Target molecules of this study: *seco*-iso-CFI analogues (**4** and **6**) and *seco*-CFQ analogues (**5** and **7**). Structure of the reference compound *seco*-CBI-TMI (**17**).[20]



Scheme 1. (a) Potassium *t*-butoxide, reflux; (b) acetic anhydride, sodium acetate, reflux; (c) K_2CO_3, ethanol, reflux; (d) benzyl bromide, NBu_4I, DMF, K_2CO_3, rt; (e) (i) NaOH; (ii) HCl; (f) DPPA; *t*-butanol, Et_3N, heat; (g) NBS, *p*TSOH, methanol/THF, −78 °C; (h) NaH, DMF, 1,3-dichloro-2-propene; (i) AIBN, Bu_3SnH, toluene, heat; (j) (i) HCl, THF; (ii) TMI–CO_2H, EDCI, DMF, rt; (k) ammonium formate, 10% Pd/C, aq THF, H_2; (l) (i) H_2, 10% Pd/C, THF; (ii) NaH, THF.

Results and Discussion

Synthesis

The synthetic approach utilized for the preparation of the *seco*-CFI compounds is indicated in Scheme 1, and it follows the general methodology reported by Boger and coworkers.[1d–h,7,13] A Stobbe condensation of 2-furaldehyde with diethyl succinate in the presence of potassium *t*-butoxide afforded an isomeric mixture of half-esters in 56% yield. The half-esters were converted into the substituted benzofuran by Friedel–Crafts acylation (AcONa, Ac_2O, reflux), followed by removal of the acetate group with ethanolic sodium carbonate to provide phenol **8** in 54% yield. Protection of the hydroxy group with benzyl bromide gave product **9** in 95% yield. Hydrolysis of the ester group in compound **9** afforded a carboxylic acid intermediate in 93% yield, which upon treatment with the Shioiri–Yamada reagent (DPPA)[16] in *t*-butanol and triethylamine produced the BOC protected amine **10** in quantitative yield. Treatment of compound **10** with NBS and *p*-toluenesulfonic acid at −78 °C generated a bromide (55%), which was alkylated with (*E*/*Z*)-1,3-dichloropropene in the presence of sodium hydride to give compound **11** in 90% yield. A degassed solution of compound **11** in toluene was heated at reflux in the presence of 2.7 equivalents of tri-*n*-butlytin hydride and 0.6 equivalents of AIBN to produce the 5-*exo*-*trig* aryl radical/vinyl chloride cyclization reaction[9b,17] and to give the desired *seco*-iso-CFI alkylating subunit **12** in 40% yield after silica gel column purification. In addition to compound **12**, a new and slightly less polar product was also obtained in 48% yield. The structure of compound **13** was assigned as the protected *seco*-CFQ (*seco*-cyclopropanefuranoquinoline) on the basis of its 500 MHz ^{1}H NMR and mass spectral data. The proton signals of the chloroquinoline subunit, 4.17 (t, 9.5, 2H), 3.5–3.65 (m, 3H), are consistent with the pattern observed for duocarmycin C1.[14] It is worthy to note that variation of the reaction temperature (80–110 °C) as well as the concentration (1–2 dilutions) did not significantly alter the chemical yields and proportions of the isomers produced.

The formation of equal amounts of the *seco*-CFQ product **13** in addition to the 5-*exo*-*trig* (*seco*-iso-CFI) product **12** is note worthy because this type of radical cyclization reactions on a diverse range of structures have been widely reported, including the formation of *seco*-CFI.[9b] However, in all cases, the five-membered ring or 5-*exo*-*trig* product was obtained exclusively.[9b,17] Under a similar reaction condition for the radical promoted formation of compounds **12** and **13**, and consistent with literature precedence,[17] the reaction of a corresponding substituted naphthalene reactant gave the *seco*-CBI structure exclusively. As indicated in Figure 4, we propose that the formation of the *seco*-CFQ is most likely influenced by the furano-oxygen atom. The lone pair of electrons provides stabilization on the 5-membered ring radical intermediate, formed via a 5-*exo*-*trig* ring closure reaction. Instead of immediately reacting with tributyltin hydride, the radical intermediate undergoes a ring expansion reaction to produce a more stable intermediate, which has a six-membered ring (*seco*-CFQ). The *seco*-CFQ radical is further stabilized by conjugation of the radical at the benzylic position with the benzofuran moiety. Subsequent reaction of the *seco*-CFQ radical with tributyltin hydride affords product **13**.

To complete the synthesis of the target molecules **4** and **5**, the BOC protecting group on compounds **12** and **13** were removed by treatment with HCl. The resulting amines were directly coupled with 5,6,7-trimethoxyindole-2-carboxylic acid in the presence of EDCI to produce amides **14** and **15** in 64 and 80% yields, respectively. Removal of the benzyl moieties of compounds **14** and **15** by catalytic hydrogenolysis provided racemic mixtures of compounds **4** and **5** in almost quantitative yields. The *seco*-iso-methyl-CFI (**6**) and

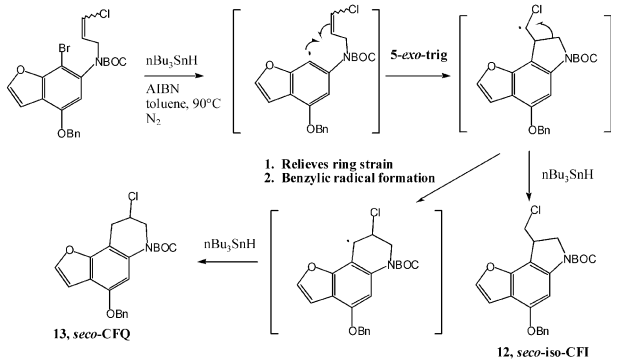


Figure 4. Formation of the *seco*-CFQ and *seco*-iso-CFI structures via a 5-*exo*-*trig* radical/vinyl chloride cyclization mechanism.

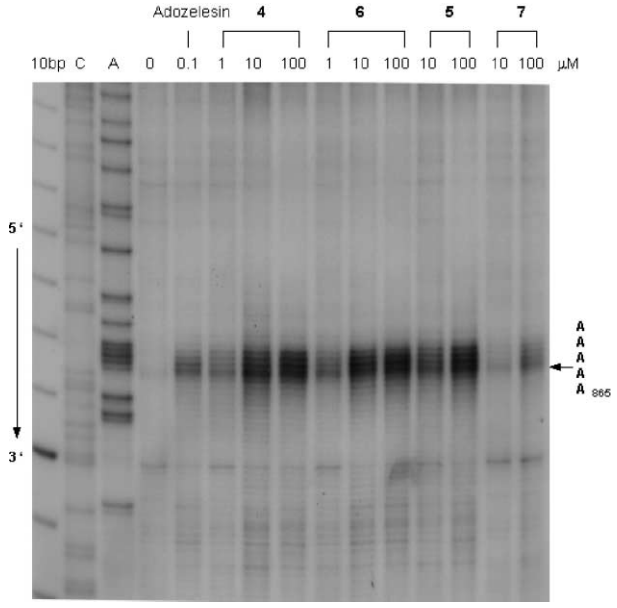


Figure 5. *Taq* polymerase gel examining damage on the bottom strand of a mixed AT/GC sequence (defined in the Experimental). 10 bp: molecular weight marker; C and A: sequencing lanes specific for cytosines and adenines; 0: control; adozelesin: 0.1 μM; compound **4**: 1, 10, and 100 μM; compound **6**: 1, 10, and 100 μM; compound **5**: 10 and 100 μM; compound **7**: 10 and 100 μM (arrows indicate bases alkylated and their position on the plasmid sequence.

seco-methyl-CFQ (**7**) compounds were prepared in a similar fashion as compounds **4** and **5**, except the synthesis began with 5-methylfurfural.

In order to test the relative solvolytic stability of the iso-CFI pharmacophore, the cyclopropane-containing product **16** was generated. Removal of the benzyl protecting group in compound **12** was achieved by catalytically hydrogenation in quantitative yield. Reaction of the resulting phenol with sodium hydride in THF gave compound **16** in 23% yield. A room temperature 0.1 mM solution of *N*-BOC protected **16** in a 1:1 methanol/water mixture at pH 3 was monitored by UV–vis spectrophotometry. Following the disappearance of the absorbance for the indolone moiety at 320 nm with time, a half-life for compound **16** was found to be 410 h. A similar study was undertaken on *N*-BOC-CBI, and its half-life was found to be 235 h, indicating that the iso-CFI molecule was somewhat more stable under the conditions studied. The reported half-life for *N*-BOC-CBI is 133 h.[12a]

DNA binding studies

The *Taq* DNA Polymerase stop assay developed by Hartley and co-workers is very sensitive in determining the sequence selective alkylation of DNA.[18] This PCR based assay works on the premise that *Taq* DNA polymerase activity will stop DNA synthesis at the site of alkylation. Using a singly radiolabeled primer 5′-^{32}P-CTCACTCAAAGGCGGTAATAC-3′ and a *Hind III* linearized pUC18 plasmid DNA, the region of the plasmid starting from position 749 was linearly amplified. The autoradiograph shown in Figure 5 demonstrates that, like adozelesin,[2] compounds **4**–**7** could stop the activity of *Taq* DNA polymerase at sites with contiguous adenine residues, and all four compounds showed similar covalent sequence selectivity at the A(865) cluster. These results are not surprising since both the *seco*-iso-CFI and *seco*-CFQ prodrugs would undergo a loss of HCl to generate an identical iso-CFI-TMI compound (see Fig. 1). It is worthy to note that CC-1065 was more reactive with the A(865) cluster than compounds **4**–**7**. This observation could be a result of the enhanced binding of CC-1065 to the minor groove due to the larger PDE units, when compared to the trimethoxyindole group in compounds **4**–**7**. Even though compounds **4**–**6** produced similar alkylation efficiencies, compound **7** was somewhat less reactive for reasons that are unclear at this stage.

The thermally induced DNA sequence selective strand break assay is a commonly used method for determining the covalent sequence selectivity of compounds provided that the alkylation occurs with purine N3 nucleophiles in the minor groove.[1,19] For this study, the probe sequence was obtained from PCR amplification of base pairs 749–956 of plasmid pUC18 that was linearized with *Hind III*. In addition to the above primer 1, a second primer 2 (CTGTCGGGTTT) fragment was used as a forward primer so that the final DNA fragment was singly end labeled. Results from the thermal cleavage assay for compounds **4**, **5** and **17** (*seco*-CBI-TMI),[20] shown in Figure 6, revealed that these agents could covalently interact with the N3 position of adenine residues in the minor groove. Consistent with results from the *Taq* stop assay, all three of these agents recognized and alkylated the sequence 3′-AAAAA-5′ at position 865, with approximately the same efficiency. All agents also recognized secondary sites, for example, at A764 and A772 within AT-rich sequences.

Molecular modeling studies

The MM2 energy minimized complex of iso-CFI-TMI with duplex dGGCGGAGTTAGG in the B-form, and alkylating at the adenine N3 position of the underlined A residue, is shown in Figure 7. This oligonucleotide was used by Hurley and coworkers in their NMR and molecular modeling studies of a DNA complex with

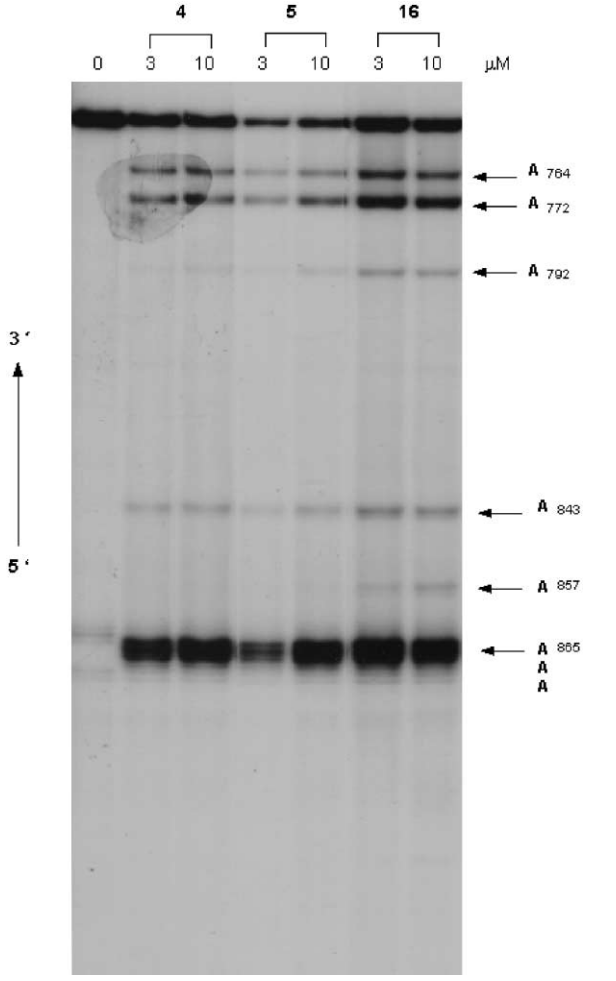


Figure 6. Thermal cleavage gel showing purine–N3 lesions on the upper strand of the mixed AT/GC region. 0: control; Compound **4**: 3 and 10 μM; Compound **5**: 3 and 10 μM; Compound **17** (*seco*-CBI-TMI): 3 and 10 μM.

CC.[1c] As depicted in Figure 7, the iso-CFI-TMI molecule fits snugly and isohelically in the minor groove. The angular plane between the iso-CFI and TMI units exhibits a twist of 6° in order to accommodate the natural curvature of the DNA.

Cytotoxicity studies

Although it is important to characterize the interaction of potential anticancer agents with their DNA target, the goal is to design compounds which are capable of killing tumor cells. Using a MTT based growth inhibition assay, the IC_{50} values for compounds **4**–**7** against murine L1210 leukemia, murine P815 mastocytoma, and murine B16 melanoma cells were determined.[21] Compound **17**, which has a reported IC_{50} value of 30 pM against L1210 cells (3 days' exposure),[20] was used as a reference compound for the cytotoxicity studies. The results given in Table 1 demonstrate that the novel *seco*-iso-CFI (**4** and **6**) and *seco*-CFQ (**5** and **7**) agents, which contain an additional electron donating furan-oxygen compared to a benzo moiety in CBI units, have IC_{50} values in the 10^{-8} M range. This level of cytotoxicity is almost comparable to the average value for

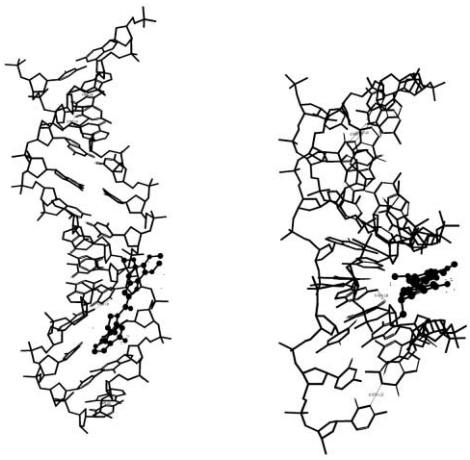


Figure 7. MM2 energy optimized (steepest descend) structure of a complex of a covalent adduct between the chloromethyl moiety of compound **4** and adenine–N3 of the underlined residue of this oligonucleotide dGGCGGAGTTAAGG.

Table 1. In vitro cytotoxicity (IC_{50} in μM) of compounds **4**–**7** and **17** against murine cancer cells (the cells were incubated with the compounds continuously for 3 days)

Compd/cells	L1210	P815	B16
4	0.053	0.051	0.040
5	0.064	0.061	0.062
6	0.030	0.064	0.092
7	0.032	0.018	0.064
17	0.0084	0.0086	0.076

compound **17**, and for B16 melanoma cells compounds **4**, **5**, and **7** are slightly more cytotoxic. Furthermore, compounds **6** and **7**, the methylated analogues of the *seco*-iso-CFI and *seco*-CFQ compound, have nearly indistinguishable cytotoxic properties with their parent compounds **4** and **5**, respectively.

The cytotoxic properties of compounds **4**–**7** were also examined at the National Cancer Institute, against a panel of 60 different human cancer cell lines.[22] The viability of the cells after 48 h of continuous exposure of the compounds was determined using a sulforhodamine B assay. The LC_{50} values (concentration for killing 50% of the cells) for compounds **4** and **5** against the 60 different tumor cells are given in Figure 8. Bars extending to the right indicate cells more sensitive than the average to the particular compound, whereas bars to the left indicate less sensitive cells. One of the interesting features that are apparent from these studies is that agents **4**–**7** are not indiscriminately toxic to cells, and they show unique patterns of cytotoxicity primarily against cells derived from solid tumors such as breast, prostate, renal, melanoma, CNS and colon cancers. Even though these compounds demonstrated subtly different patterns of cytotoxicity, they tend to be more active against melanoma and renal cancer cells. Specific reasons for the unique patterns of cytotoxicity for compounds **4**–**7** remain unknown, and experiments are underway to address this issue.

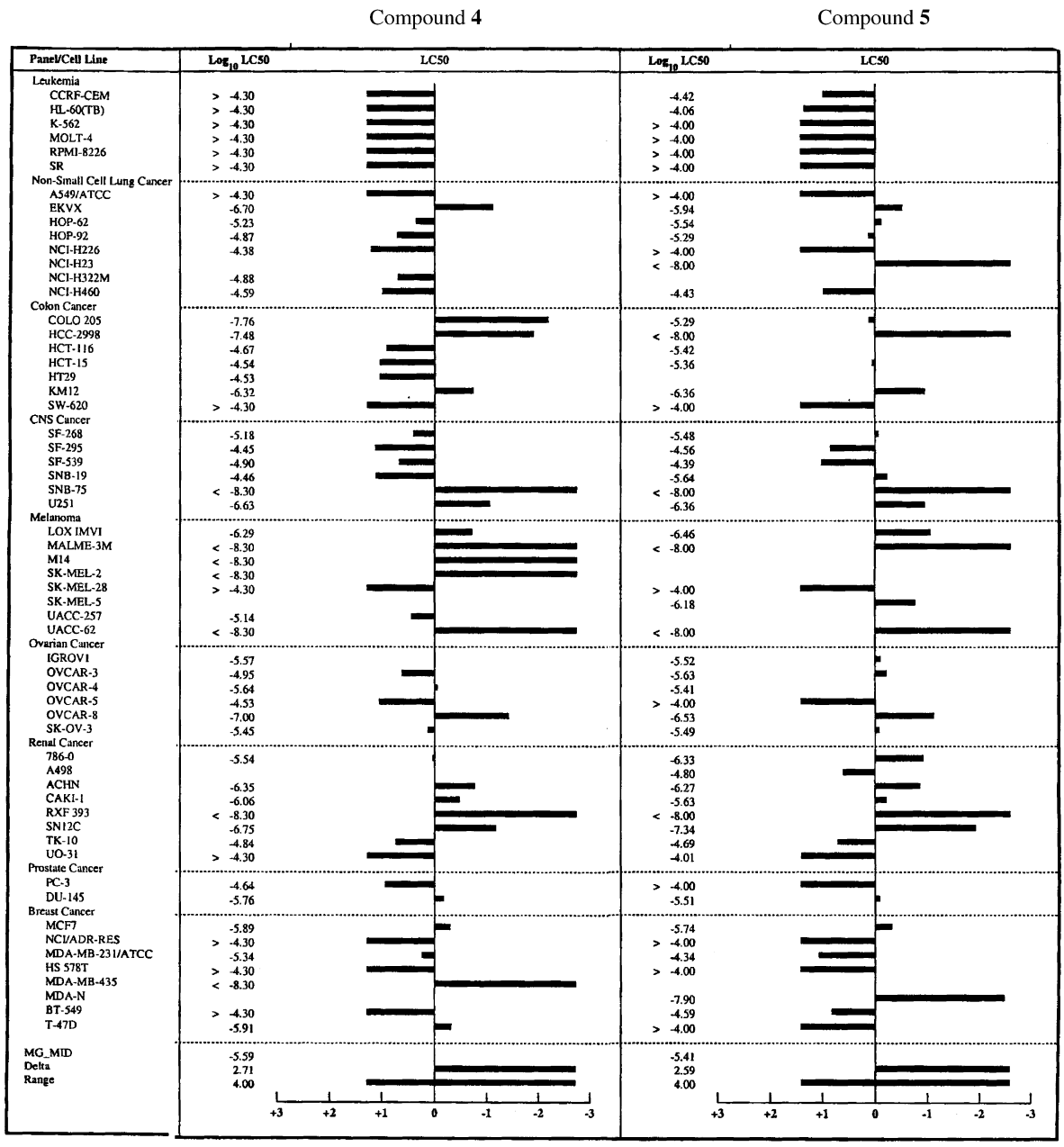


Figure 8. NCI mean graph (log LC_{50} values) for a 48-h in vitro assay on compounds **4** and **5**.

Cell-cycle studies

Evidence that duocarmycin analogues cause cellular death through apoptosis was obtained through microscopic examination of Molt-4 leukemia cells that had been incubated with duocarmycin SA. Those cells demonstrated morphological abnormalities and genomic DNA degradation associated with apoptosis.[6] Flow cytometry is another way in which apoptotic cells can be detected. It has been suggested that the sub-G_0 peak is indicative of the presence of apoptotic cells,[23] that can be determined by a propidium iodide staining method. Following a 24-h incubation of P815 cells at the cytotoxic IC_{50} concentration of compound **5**, the results showed a substantial increase in the percentage of sub-G_0 stage (22% compared to $<1\%$ for untreated control cells), indicating that compound **5** was capable of damaging DNA and inducing the cells to undergo apoptosis. For comparison, cisplatin at 100 μM, produced 17% of sub-G_0 population of cells, which is consistent with a literature precedent.[6] Results from our studies suggest that iso-CFI compounds are likely to exert their cytotoxic activity through the induction of apoptosis.

Bone marrow toxicity studies

One of the most severe limitations in the development of analogues of CC-1065 and the duocarmycins into useful therapeutic agents is their severe non-selective toxicity toward the bone marrow.[2–5] For this reason, the toxicity of compounds **4** and **5** against hematopoietic progenitor cells were assessed using a commercially available colony forming assay (StemCell Technologies,

Inc., Vancouver, Canada). In our studies, bone marrow cells were taken from the femurs of healthy mice that had not been treated with drug. The cells were then incubated in the Methocult™ media (StemCell, Inc.), with continuous exposure to the drugs, where equal concentrations (0.0084 μM, IC_{50} for compound **17** for L1210 cells) were used for each of the compounds. On day 12, the colonies were counted. The formation of colonies indicates that the stem cells retain their activity and thus stimulate the formation of progenitor cells. The absence of colonies either indicates that exposure to the drug solutions completely kills the bone marrow cells or makes these cells non-viable. The results from this study indicate that while compound **17** and **4** are toxic (no colonies were observed in duplicated experiments), the *seco*-CFQ analogue **5** displayed significantly less toxicity (45 ± 10 colonies compared to 60 ± 3 colonies for untreated control). Overall, the favorable activity against tumor cell lines and low toxicity to murine bone marrow cells of the *seco*-CFQ pharmacophore to murine bone marrow cells makes it a worthy candidate for further drug development studies.

Experimental

Ethyl 4-hydroxybenzofuran-6-carboxylate, (8). Potassium *t*-butoxide (1 M in *t*-butanol) (18.0 mL, 0.018 mol) was added to a solution of 2-furaldehyde (11.0 mL, 0.13 mol) and diethyl succinate (32.0 mL, 0.19 mol). The resulting dark brown mixture was kept under a nitrogen atmosphere and heated to reflux in an oil bath (150 °C). After 45 min, another aliquot of diethyl succinate (32.0 mL) and potassium *t*-butoxide (18.0 mL) were added. The solution was allowed to reflux overnight. After cooling, the dark solution was chilled on an ice bath and carefully acidified (6 M HCl) to pH 1. The mixture was then immediately extracted with ether (3 × 150 mL). The combined ether layers were extracted with saturated Na_2CO_3 (5 × 75 mL). The Na_2CO_3 layers were combined and then slowly acidified to a pH of 1 (6 M HCl) and extracted with ether (4 × 100 mL). The ether layers were combined, washed with brine (100 mL), and then dried over sodium sulfate. The solvent was removed in vacuo to yield a dark brown oil (15.19 g, 73 mmol, 56%), which was used directly in the next step.

The viscous brown oil was dissolved in acetic anhydride (100 mL) and anhydrous sodium acetate (5.3 g, 64.6 mmol) was added. The solution was kept under a positive pressure of nitrogen and heated in an oil bath at 150 °C overnight. The solvent was removed by vacuum flash distillation at about 80–100 °C. The resulting residue was partitioned with Na_2CO_3 (200 mL) and ethyl acetate (200 mL), and the aqueous layer was further extracted with ethyl acetate (2 × 150 mL). The combined organic layers were dried with anhydrous sodium sulfate and concentrated to yield a dark brown oil (8.0 g) which was immediately dissolved in ethanol (200 mL). To the solution was added K_2CO_3 (12 g, 86.8 mmol). The resulting reaction mixture was heated to reflux overnight, then the solvent was removed in vacuo. The brown residue was then treated with water (200 mL) and acidified to pH 6 with 6 M HCl. The solution was then extracted with ethyl acetate (4 × 100 mL), and the combined organic layers were dried and concentrated under reduced pressure to produce a brown oil. This crude material was purified on a silica gel column. A gradient eluent system was used beginning with 10% EtOAc/hexane and ending with 30% EtOAc/hexane to produce phenol **8** as a yellow solid (1.7 g, 8.23 mmol, 54%). Mp 100–104 °C. TLC (2.5% MeOH/$CHCl_3$) R_f 0.44. IR (neat) 3323, 3139, 1652, 1600, 1430, 1380, 1323, 1241. ^{1}H NMR ($CDCl_3$) 7.78 (t, 1.0, 1H), 7.61 (d, 2.5, 1H), 7.33 (d, 1.0, 1H), 6.83 (dd, 1.0, 2.5, 1H), 5.44 (s br, 1H), 4.33 (q, 7.0, 2H), 1.35 (t, 7.0, 3H). EI–MS (*m*/*z*, rel. intensity) 206 (M^+, 72), 191 (10), 178 (30), 161 (100). Accurate mass for $C_{11}H_{10}O_4$: calcd 206.0579, obsd 206.0574.

Ethyl 4-benzyloxybenzofuran-6-carboxylate (9). Compound **8** (3.38 g, 16.4 mmol) was combined with K_2CO_3 (2.9 g, 23.3 mmol) and Bu_4NI (20 mg), and to the mixture was added dry DMF (35 mL). Benzyl bromide (2.3 mL, 20.8 mmol) was added to this suspension. The reaction mixture was stirred overnight under positive N_2 pressure. The DMF was removed under vacuum (0.1 mmHg, 60 °C) using a kugelrohr apparatus and the resulting residue was dissolved in $CHCl_3$. The crude product was purified using a silica gel column and $CHCl_3$ as the eluent to yield the product **9** as an orange solid (5.04 g, 17.0 mmol, 95%). Mp 52–58 °C. TLC ($CHCl_3$) R_f 0.59. IR (Neat) 3035, 1708, 1600, 1445, 1328, 1308, 1236. ^{1}H NMR ($CDCl_3$) 7.82 (t, 1.0, 1H), 7.59 (d, 2.5, 1H), 7.43 (d, 7.5, 2H), 7.40 (d, 1.0, 1H), 7.34 (t, 7.5, 2H), 7.28 (t, 7.5, 1H), 6.87 (dd, 1.0, 2.5, 1H), 5.18 (s, 2H), 4.33 (q, 7.0, 2H), 1.35 (t, 7.0, 3H). EI–MS (*m*/*z* rel. intensity) 296 (M^+, 15), 91 (100). Accurate mass for $C_{18}H_{16}O_4$: calcd 296.1049, obsd 296.1042

4-Benzyloxy-6-(*N*-(*tert*-butyloxycarbonyl)amino)benzofuran (10). Compound **9** (5.04 g, 17.0 mmol) was dissolved in EtOH (200 mL) and 10% NaOH (200 mL) was added. The yellow solution was heated to reflux for 3 h. The ethanol was removed in vacuo and the remaining aqueous suspension was stirred in an ice bath and acidified to pH 1 using 3 M HCl. The resulting yellow precipitate was filtered and washed with water. The 5-benzyloxybenzofuran-7-carboxylic acid was then dried in a vacuum oven (70 °C at 0.1 mmHg). The filtrate was extracted with $CHCl_3$ (3 × 75 mL), and the organic layers were combined and dried with sodium sulfate. The solvent was removed in vacuo to produce more of the carboxylic acid as a yellow solid. The total yield was 4.2 g, 15.7 mmol, 93%. Mp 192–196 °C. TLC ($CHCl_3$) R_f 0.05. IR (neat) 2985, 1677, 1595, 1426, 1405, 1374, 1313, 1256. ^{1}H NMR ($CDCl_3$) 7.90 (s, 1H), 7.64 (d, 2.5, 1H), 7.45 (s, 1H), 7.44 (d, 7.0, 2H), 7.35 (t, 7.0, 2H), 7.30 (t, 7.5, 1H), 6.90 (dd, 0.5, 2.0, 1H), 5.20 (s, 1H). EI–MS (*m*/*z* rel. intensity) 268 (M^+, 30), 91 (100).

The above carboxylic acid (2.0 g, 7.46 mmol) was suspended in dry *t*-butanol (over molecular sieves 3A), and diphenylphosphorylazide (DPPA) (2.3 mL, 10.6 mmol)

and dry triethylamine (1.5 mL,10.8 mmol) were added. The resulting suspension was heated to reflux overnight under a N_2 atmosphere, at which time a clear light-brown solution formed. The solution was concentrated in vacuo. The resulting dark brown oil was purified using a silica gel column with $CHCl_3$ as the eluent to provide product **10** as a yellow solid (2.52 g, 7.46 mmol, 100%). Mp 30–35 °C. TLC ($CHCl_3$) R_f 0.73. IR (neat) 3317, 3050, 1728, 1710, 1608, 1550, 1497, 1453, 1413, 1368, 1311, 1239. ^{1}H NMR ($CDCl_3$) 7.40 (d, 8.0, 2H), 7.32 (t, 8.0, 2H), 7.27 (t, 8.0, 1H), 7.20 (s, 1H), 7.17 (d, 1.0, 1H), 6.76 (s, 1H), 6.74 (dd, 1.0, 2.5, 1H), 6.44 (s br, 1H), 5.11 (s, 2H), 1.46 (s, 9H). EI–MS (*m*/*z* rel. intensity) 339 (M^+, 20), 283 (42), 91 (100). Accurate mass for $C_{20}H_{21}NO_4$: calcd 339.1471, obsd 339.1464.

4-Benzyloxy-7-bromo-6-[*N*-(3-chloro-2-propenyl)-*N*-(*tert*-butyloxycarbonyl)amino]benzofuran (11). Compound **10** (500 mg, 1.47 mmol) was dissolved in a 1:1 solution of dry THF/MeOH (30 mL) and stirred under N_2 at −78 °C. A solution of *p*-toluenesulfonic acid (20 mg, 0.105 mmol, dissolved in 1 mL of dry THF) and *N*-bromosuccinimide (287 mg, 1.61 mmol, dissolved in 5 mL of dry THF) were slowly added to the solution. The solution was allowed to stir at −78 °C for about 2 h, then 5% $NaHCO_3$ (8 mL) was added at −78 °C. The reaction mixture was partitioned with EtOAc (50 mL) and water (50 mL). The aqueous layer was further extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with brine, dried with sodium sulfate, and concentrated in vacuo on a rotary evaporator. The resulting viscous yellow oil was purified on a silica gel column that was pretreated with 0.5% triethylamine/hexane and followed by a hexane wash. The product was eluted with a gradient EtOAc/hexane solvent, beginning with 1.0% EtOAc and gradual increase of 0.5% EtOAc every 100 mL. The bromide intermediate was isolated as a viscous clear oil which solidified upon refrigeration (340 mg, 813 mmol, 55%). TLC (10% EtOAc/hexane) R_f 0.47. Mp 86–90 °C. IR (neat) 3415, 3362, 3069, 3033, 1736, 1652, 1599, 1581, 1534, 1481, 1454, 1410, 1374, 1330, 1268, 1237, 802. ^{1}H NMR ($CDCl_3$) 7.77 (s, 1H), 7.45 (d, 2.0, 1H), 7.42 (d, 7.5, 1H), 7.33 (t, 7.5, 2H), 7.28 (t, 7.5, 1H), 6.96 (s br, 1H), 6.82 (d, 1.5, 1H), 5.13 (s, 2H), 1.97 (s, 9H). FAB-MS (*m*/*z* rel. intensity) 419 ($M^+ + 2$, 5), 417 (M^+, 5). Accurate mass for $C_{20}H_{20}NO_4^{79}Br$: calcd 417.0576, obsd 417.0574.

To a chilled (ice bath) and freshly washed sample (with hexanes) of NaH (134 mg of 60% suspension in mineral oil; 80.4 mg, 3.35 mmol) was added a solution of the above bromo compound (350 mg, 0.84 mmol) in DMF (9 mL). After 45 min, the flask was removed from the ice bath and 1,3-dichloropropene (0.23 mL, 2.51 mmol) was added to the solution, which was allowed to stir overnight under N_2. The DMF was removed under vacuum (0.1 mmHg, 60 °C) using a kugelrohr apparatus to produce a brown oil which was dissolved in CH_2Cl_2 and purified on an silica gel column. A gradient solvent system was used to elute the product, beginning with 1% EtOAc/hexane and ending with 5% EtOAc/hexane. The fractions containing either the *cis* or *trans* isomer were combined and concentrated in vacuo to produce clear viscous oils. *cis* isomer: (200 mg, 0.28 mmol, 45%). TLC (10% EtOAc/hexane) R_f 0.38. IR (neat) 3033, 1701, 1630, 1594, 1528, 1488, 1457, 1404, 1368, 1328, 1302, 1253, 770. ^{1}H NMR ($CDCl_3$) 7.56 (s, 1H), 7.37 (d, 8.0, 1H), 7.32 (t, 7.5, 2H), 7.27 (t, 7.5, 1H), 6.89 (s, 1H), 6.51 (s, 1H), 5.93 (d, 7.0, 1H), 5.87 (q, 7.0, 1H), 5.13 (d, 11.0, 1H), 5.08 (d, 11.0, 1H), 4.41 (dd, 5.5, 15.5, 1H), 4.26 (dd, 6.0, 15.5, 1H), 1.26 (s, 9H). EI–MS (*m*/*z* rel. intensity) 491 (M^+, 15), 356 (100). Accurate mass for $C_{23}H_{23}NO_4^{79}Br^{35}Cl$: calcd 491.0499, obsd 491.0491. *trans* isomer: (200 mg, 0.28 mmol, 35%). TLC (10% EtOAc/hexane) R_f 0.35. IR (Neat) 3122, 3069, 3025, 1701, 1590, 1484, 1453, 1404, 1364, 1320, 1258, 770. ^{1}H NMR ($CDCl_3$) 7.57 (s, 1H), 7.38 (d, 6.5, 2H), 7.33 (t, 7.5, 2H), 7.27 (t, 7.0, 1H), 6.91 (s, 1H), 6.45 (s, 1H), 5.94 (m, 1H), 5.87 (d, 13.5), 5.13 (d, 11.0, 1H), 5.08 (d, 11.0, 1H), 4.30 (d, 6.5, 15, 1H), 4.27 (dd, 7.0, 15, 1H), 1.25 (s, 9H). EI–MS (*m*/*z* rel. intensity) 491 (M^+, 12), 356 (100).

5-Benzyloxy-3-(*tert*-butoxycarbonyl)-1-chloromethyl-1,2-dihydro-3*H*-furano[2,3-*e*]indole (12) and 6-benzyloxy-4-(*tert*-butoxycarbonyl)-2-chloro-1,2,3,4-tetrahydro-4*H*-furano[2,3-*f*]quinoline (13). To a mixture of *cis* and *trans* **11** (1.37 g, 2.59 mmol) was added 2,2′-azobisisobutyronitrile (AIBN) (250 mg, 1.52 mmol). The reagents were then dissolved in freshly distilled toluene (140 mL), and nBu$_3$SnH (1.87 mL, 6.95 mmol) was added. The solution was degassed with nitrogen and heated in an oil bath (90 °C) for 2 h. The toluene was removed in vacuo to produce a light-yellow oil which was dissolved in CH_2Cl_2 and loaded on a hexane/silica gel column. The products were eluted with 1% EtOAc/hexane with the composition of EtOAc gradually increased to 5%. Fractions containing the products were concentrated in vacuo. The expected five-membered ring product **12** (*seco*-iso-CFI) was obtained as a colorless clear oil (430 mg, 1.03 mmol, 40%). TLC (10% EtOAc/hexane) R_f 0.52. IR (neat) 3070, 3024, 1701, 1652, 1634, 1617, 1559, 1541, 1493, 1368, 1342, 1257, 788. ^{1}H NMR ($CDCl_3$) 7.57 (s br, 1H), 7.41 (d br, 8.0, 2H), 7.35 (d, 2.5, 1H), 7.33 (t, 8.0, 2H), 7.28 (t, 8.0, 1H), 6.77 (d, 2.5, 1H), 5.13 (s, 2H), 4.15 (dd, 9.5, 10.0, 1H), 4.02 (dd, 4.0, 11.0, 1H), 3.98 (m, 1H), 3.92 (dt, 4.0, 10.0, 1H), 3.54 (t, 10.5, 1H), 1.51 (s, 9H). EI–MS (*m*/*z* rel. intensity) 413 (M^+, 14). Accurate mass for $C_{23}H_{24}NO_4^{35}Cl$: calcd 413.1394, obsd 413.1381.

In addition to the *seco*-iso-CFI product **12**, an unexpected 6-membered ring substance **13** (*seco*-CFQ) was also isolated as a colorless clear oil (513 mg, 1.24 mmol, 48%). TLC (10% EtOAc/hexane) R_f 0.51. IR (neat) 3122, 3069, 1701, 1639, 1612, 1493, 1457, 1417, 1368, 1342, 1258, 1222, 756. ^{1}H NMR ($CDCl_3$) 7.57 (s br, 1H), 7.41 (d br, 7.5, 2H), 7.36 (d, 2.0, 1H), 7.33 (t, 7.5, 2H), 7.27 (t, 7.5, 1H), 6.76 (d, 2.0, 1H), 5.13 (s, 2H), 4.17 (t, 9.5, 2H), 3.5.65 (m, 3H), 1.49 (s, 9H). EI–MS (*m*/*z* rel. intensity) 413 (M^+, 4), 379 ($M^+ +$ H-Cl, 20). Accurate mass for $C_{23}H_{24}NO_4^{35}Cl$: calcd 413.1394, obsd 413.1384.

Note: The radical cyclization of pure *cis* or *trans* isomer of compound **11** gave similar mixtures of products **12** and **13**.

5-Benzyloxy-1-(chloromethyl)- 1,2-dihydro-3-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-3*H*-furano[2,3-*e*]indoline (14). Compound **12** (178 mg, 0.430 mmol) was dissolved in dry THF (3.2 mL) and chilled in an ice bath. Concentrated HCl (1.3 mL) was slowly added to the solution. After 15 min the reaction flask was removed from the ice bath and allowed to stir at room temperature under N_2 for 4 h. The reaction was quenched with 2.5 M Na_2CO_3 (25 mL) and saturated $NaHCO_3$ (10 mL), followed by an extraction with EtOAc (2 × 75 mL). The organic layer was dried with sodium sulfate and concentrated in vacuo to produce the amine as a brown oil. To the amine was added EDCI (247 mg, 1.29 mmol) and 5,6,7-trimethoxyindole-2-carboxylix acid (130 mg, 0.51 mmol). The reaction mixture was suspended in DMF (8.5 mL) and stirred under N_2 for 3 days. The DMF was removed in vacuo and the resulting residue was dissolved in CH_2Cl_2 and loaded on a hexane/silica gel column. The product was eluted with 15% EtOAc/hexane with the percentage of EtOAc slowly increased to 35%. Fractions containing product were concentrated in vacuo to produce compound **14** as an off white solid (150 mg, 64%). Mp 170–174 °C. TLC (30% EtOAc/hexane) R_f 0.45. IR (neat) 3433, 3050, 1639, 1621, 1590, 1528, 1460, 1413, 1382, 1226, 694. ^{1}H NMR ($CDCl_3$) 9.31 (s br, 1H), 8.01 (s, 1H), 7.443 (d, 7.0, 2H), 7.445 (d, 2.0, 1H), 7.35 (t, 7.0, 2H), 7.28 (t, 7.0, 1H), 6.92 (d, 2.0, 1H), 6.85 (d, 2.0, 1H), 6.82 (s, 1H), 5.19 (s, 2H), 4.71 (t, 10.0, 1H), 4.53 (dd, 4.5, 10.0, 1H), 4.10 (m, 1H), 4.02 (s, 3H), 4.01 (d, 4.0, 1H), 3.88 (s, 3H), 3.85 (s, 3H), 3.61 (t, 9.8, 1H). Uv (ethanol) 222 (ε 7.8 × 10^7). EI–MS (*m*/*z* rel. intensity) 546 (M^+, 44), 512 (M^+ + H–Cl, 58). Accurate mass for $C_{30}H_{27}N_2O_6^{35}Cl$: calcd 546.1558, obsd 546.1560.

6-Benzyloxy-2-chloro-4-[(5,6,7-trimethoxy-1*H*-indol-2-yl)-carbonyl]-1,2,3,4-tetrahydro-4*H*-furano[2,3-*f*]quinoline (15). The synthesis of compound **15** followed a procedure similar to that used in the synthesis of compound **14**, except compound **13** (168 mg, 0.41 mmol) was used. The desired product **15** was isolated as an off white solid (178 mg, 80%). Mp 186–190 °C. TLC (30% EtOAc/hexane) R_f 0.53. IR (neat) 3459, 3080, 1621, 1586, 1524, 1413, 1377, 1311, 1222, 739. ^{1}H NMR ($CDCl_3$) 9.32 (s br, 1H), 7.99 (s, 1H), 7.449 (d, 6.5, 2 h), 7.448 (d, 2.0, 1H), 7.35 (t, 6.5, 2H), 7.28 (t, 6.5, 1H), 6.86 (d, 2.0, 1H), 6.84 (d, 2.5, 1H), 6.79 (s, 1H), 5.19 (s, 2H), 4.72 (t, 10.0, 1H), 4.10 (dd, 6.0, 9.5, 1H), 4.01 (s, 3H), 4.00 (m, 1H), 3.88 (s, 3H), 3.92 (m, 1H), 3.85 (m, 1H), 3.84 (s, 3H). UV (ethanol) 222 (ε 6.3 × 10^7). EI–MS (*m*/*z* rel. intensity) 546 (M^+, 12) 512 (M^+ + H–Cl, 60). Accurate mass for $C_{30}H_{27}N_2O_6^{35}Cl$: calcd 546.1558, obsd 546.1562.

1-(Chloromethyl)-5-hydroxy-1,2-dihydro-3-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-3*H*-furano[2,3-*e*]indoline (4). To a mixture of 10% Pd/C (52 mg) and compound **14** (152 mg, 0.278 mmol) was added in 25% aqueous NH_4HCO_2 (1.27 mL) and ice-cold THF (7.5 mL). The suspension was sealed with a rubber septum and flushed with H_2 then stirred for 5 h at ambient temperature. The reaction mixture was filtered over Celite. The resulting clear solution was concentrated in vacuo to produce compound **4** as a white powder (0.135 g, 0.296 mmol, 100%). Mp 250 °C (dec). TLC (2.5% MeOH/$CHCl_3$) R_f 0.27; (30% EtOAc/hexanes) R_f 0.13. IR (neat) 3433, 3090, 1630, 1595, 1523, 1426, 1377, 1386, 1220, 739. ^{1}H NMR ($CDCl_3$) 9.42 (s br, 1H), 7.99 (s, 1H), 7.92 (s br, 1H), 7.52 (d, 2.0, 1 h), 7.08 (d, 2.0, 1H), 6.88 (s, 1H), 6.80 (d, 2.0, 1H), 4.76 (t, 10.0, 1H), 4.59 (dd, 4.5, 10.0, 1H), 4.18 (dd, 3.5, 11.0, 1H), 4.13 (s, 3H), 4.11 (d, 5.0, 1H), 3.96 (s, 3H), 3.92 (s, 3H), 3.67 (t, 10.0, 1H). Uv (ethanol) 226 (ε 8.0 × 10^7). EI–MS (*m*/*z*, rel. intensity) 456 (M^+, 20), 422 (M^+ + H–Cl, 40). Accurate mass for $C_{23}H_{21}N_2O_6^{35}Cl$: calcd 456.1088, obsd 456.1091.

2-Chloro-6-hydroxy-4-[(5,6,7-trimethoxy-1*H*-indol-2-yl)-carbonyl]-1,2,3,4-tetrahydro-4*H*-furano[2,3-*f*]quinoline (5). The synthesis of compound **5** followed a procedure similar to that used in the synthesis of compound **4**, except compound **15** (169 mg, 0.309 mmol) was used as a starting material. Compound **5** was isolated as a creamy white powder (0.131 g, 0.287 mmol, 93%). Mp 260–264 °C. TLC (30% EtOAc/hexanes) R_f 0.15. (Neat) 3433, 3123, 3008, 1621, 1586, 1523, 1431, 1382, 1311, 1258, 1222, 797. ^{1}H NMR ($CDCl_3$) 9.38 (s, 1H), 7.95 (s, 1H), 7.46 (d, 2.0, 1H), 6.91 (s, 1H), 6.89 (d, 2.5, 1H), 6.80 (d, 2.0, 1H), 6.79 (s, 1H), 4.72 (t, 9.5, 1H), 4.11 (dd, 6.0, 9.5, 1H), 4.07 (s, 3H), 4.05 (m, 1H), 3.93 (m, 1H), 3.89 (s, 3H), 3.85 (s, 3H), 3.82 (m, 1H). Uv (ethanol) 222 (ε 7.4 × 10^7). EI–MS (*m*/*z* rel. intensity) 456 (M^+, 4), 422 (M^+ + H–Cl, 64). Accurate mass for $C_{23}H_{21}N_2O_6^{35}Cl$: calcd 456.1088, obsd 456.1088.

1-(Chloromethyl)-5-hydroxy-1,2-dihydro-3-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-3*H*-5-methylfurano[2,3-*e*]indoline (6). Compound **6** was prepared following a similar scheme used in the synthesis of compound **4**, except 5-methylfurfural was used as a starting material. Compound **6** was isolated as a white powder (0.136 g, 0.289 mmol, 99% in the last hydrogenation step). TLC (5% MeOH/$CHCl_3$) R_f 0.50. Mp 263–267 °C. IR (neat) 3433, 3100, 1963, 1927, 1634, 1586, 1524, 1457, 1391, 1311, 1262, 1220, 800. ^{1}H NMR ($CDCl_3$) 9.45 (s, 1H), 9.42 (s, 1H), 7.82 (s, 1H), 7.64 (s, 1H), 6.98 (d, 2.0, 1H), 6.89 (s, 1H), 6.46 (d, 2.0, 1H), 4.75 (t, 10.0, 1H), 4.59 (dd, 4.5, 10.0, 1H), 4.19 (dd, 3.5, 11.0, 1H), 4.11 (s, 3H), 3.96 (s, 3H), 3.93 (s, 3H), 3.64 (t, 10.0, 1H), 2.46 (s, 3H). FAB-MS (NBA) (*m*/*z* rel. intensity) 471 (M + H, 4). Accurate mass (FAB-NBA) for $C_{24}H_{24}N_2O_6^{35}Cl$: calcd 471.1323, obsd 471.1307.

2-Chloro-6-hydroxy-4-[(5,6,7-trimethoxy-1*H*-indol-2-yl)-carbonyl]-1,2,3,4-tetrahydro-4*H*-5-methylfurano[2,3-*f*]-quinoline (7). Compound **7** was prepared following a similar scheme used in the synthesis of compound **5**, except 5-methylfurfural was used as a starting material. Compound **7** was isolated as a light-yellow powder (167 mg, 0.356 mmol, 72% in the final hydrogenation step). TLC (25% EtOAc/hexane) R_f 0.49. Mp 258–260 °C. IR (neat) 3424, 1683, 1621, 1586, 1523, 1493, 1382, 1306, 1217. ^{1}H NMR ($CDCl_3$) 9.41 (s, 1H), 7.97 (s, 1H), 6.99 (s br, 1H), 6.94 (s, 1H), 6.86 (s, 1H), 6.45 (s, 1H), 4.76 (t, 10.0, 1H), 4.14 (dd, 6.0, 9.5, 1H), 4.13 (s, 3H), 3.96 (s, 3H), 3.92 (s, 3H), 3.87 (m, 3H), 2.47 (s, 3H). EI–MS *m*/*z* (rel. intensity) 470 (M^+,5), 436 (55),

203 (100). Accurate mass (EI–MS) for $C_{24}H_{23}N_2O_6^{35}Cl$: calcd 470.1245, obsd 470.1250.

Note: Complete detailed procedures and characterization for compounds **6** and **7** can be obtained from the authors.

***N*-BOC-iso-CFI (16).** To a flame dried 25 mL round bottom flask was added NaH (11.5 mg, 0.477 mmol), which was washed with dry hexane (2 mL). A solution of the phenol generate from catalytic hydrogenation of compound **12** (51 mg, 0.16 mmol) was dissolved in dry THF (2 mL) and added to the reaction flask, which was kept in an ice bath. The reaction mixture was stirred for 3 h, at which time the THF was removed in vacuo resulting in a brown solid which was dissolved in water (20 mL) and EtOAc (20 mL). The water layer was extracted with EtOAc (3 × 20 mL) and the organic layers were collected and concentrated to produce a brown oil, which was purified by TLC (1% EtOAc/hexane). The product was isolated as a clear oily residue (14 mg, 0.037 mmol, 23%). IR (neat) 3020, 1662, 1645, 1483, 1458, 1437, 1391, 1366, 1262. UV–vis (MeOH/water, pH 3.0) 325 (ε 222), 272 (ε 833), 242 (ε 1477). ^{1}H NMR ($CDCl_3$) 6.98 (d, 1.5, 1H), 6.55 (d, 1.5, H), 6.54 (s, 1H), 3.75 (m, 1H), 3.71 (t, 7.5, 1H), 3.47 (m, 1H), 1.54 (s, 2H), 1.42 (s, 9H). FABMS (NBA) *m*/*z* (rel. intensity) 288 (M + H, 10).

Cytotoxicity studies

Compounds **4**–**7** and **17** were dissolved in DMSO to obtain 1.75×10^{-2} M stock solutions, which were diluted with DMSO to prepare standard solutions from concentrations of 1.75×10^{-3} to 1.75×10^{-10} M. These solutions were then further diluted with DMEM (24 μL standard solution in 176 μL media). Addition of these new drug solutions (5 μL) to wells containing 100 μL media/cell suspension resulted in final drug concentrations ranging from 1.0×10^{-12} to 1.0×10^{-4} M.

The murine mastocytoma P815 and murine leukemia L1210 cell lines were obtained from American Type Tissue Culture Collection (ATCC). The murine melanoma B16-F_0 cell line was obtained from the Cancer Center of the Greenville Hospital System. The cell lines were grown in Delbecco's Modified Eagle Medium (DMEM, Atlanta Biochemicals) supplemented with 10% fetal bovine serum, Hepes Buffer (2 mM, Mediatech Cellgro, 25–060-Cl), L-glutamine (2 mM, Mediatech Cellgro), and penicillin/streptomycin (50,000 units penicillin, 50,000 μg streptomycin, Atlanta Biologicals). Cells were maintained at 37 °C in a 5% humidified CO_2 atmosphere.

Cultured cells were counted using a hemocytometer and were resuspended in fresh DMEM at a concentration of 8×10^4 cells/mL. The cell suspension (100 μL) was added to 96-well flat-bottom cell culture plates. At this concentration, 8000 cells were seeded in each well. The drug solutions diluted in DMEM (detailed above) were added to each well (5 μL/well). Quadruplicate wells were prepared for each drug concentration. The plates were incubated for 72 h at 37 °C in a 5% CO_2 atmosphere. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS (5 mg/mL). After the indicated cell incubation period, 10 μL stock MTT solution was added to each well, and the plates were further incubated for 4 h at 37 °C in a 5% CO_2 atmosphere. After this final incubation, 100 μL acidic isopropanol solution (16 μL 12.1 N HCl in 5 mL isopropanol) was added to each well. The contents of the wells were mixed thoroughly by pipetting the cell suspension up and down and the plates were allowed to sit at room temperature for 15 min in order to allow for full development of the purple color. The plates were then read on a Dynatech Plate Reader, utilizing Dynex Revelation 3.2 software, with a test wavelenth of 570 nm and a reference wavelength of 630 nm. The dose inhibiting the growth by 50% (IC_{50}) was extrapolated from curves generated based on the averages of the absorbance data (4 points/concentration).

Hematopoietic progenitor assay

For direct incubation of the drug solutions with the bone marrow, the concentration of drug solution was chosen to be 0.0084 μM (the IC_{50} value for the *seco*-CBI-TMI compound **17** against L1210 cells). Bone marrow cells were harvested from the femurs of healthy DBA mice by flushing the femur with IMEM media (~1 mL). These cells were counted and diluted to a concentration of 2.0×10^5 cells/mL in additional IMEM media. The drug solutions were prepared by making a 2.52×10^{-2} M stock solution of the drug in DMSO. In order to make duplicate cultures, 0.3 mL of cells in IMEM were added to 3.0 mL of the Methocult™ M3434 media (StemCell Technologies) in a 15 mL conical tube. The tube was then vortexed to ensure complete mixing of the cells and media. Then, 11 μL of the drug stock solution was added to the tube and thoroughly mixed via vortexing. This cell suspension (1 mL) was then transferred (via 16G needle) into the wells of a six-well plate. The plates were incubated for 12 days at 37 °C and 5% CO_2. The colonies were counted using a dissecting microscope with a blue filter film attached to add contrast to the cells.

Flow cytometry studies

The P815 cell line was used for this experimental procedure. The cells were incubated with drugs in a final concentration equaling their IC_{50} value. The IC_{50} concentrations for cisplatin and compound **4** were 100 and 0.061 μM, respectively. Cisplatin was used as a positive control. The drug stock solutions were made in DMSO so that the addition of 5 μL of the solution into 5 mL of media would result in the desired concentrations listed above. Cells were counted and re-suspended at a concentration of 1.5×10^5 cells/mL, and 5 mL of this cell suspension were pipetted into a small culture flask. The drug solutions were added (5 μL), and the cells were incubated at 37 °C and 5% CO_2 for 24 h. The cells were then harvested via centrifugation (1000 rpm, 10 min, 4 °C) and washed twice with sample buffer (0.5 g glucose in 500 mL Ca^{2+}/Mg^{2+} free PBS; Electron Microscopy

Sciences, Fort Washington, PA, USA). To fix the cells, the cells were vortexed vigorously for ~10 s, after which the vortexing was continued at a slower rate during the dropwise addition of 1 mL of ice-cold 70% ethanol. The tubes were capped and stored at 4 °C until the day of the flow cytometry analysis.

Approximately 1 h before the desired time of analysis, the cells were stained with propidium iodide (PI). The PI staining solution was prepared by the addition of 0.6 mL of the PI stock solution (made from 0.8 mg of PI with 0.8 mL of water) to 12 mL of sample buffer. RNase A (EC 3.2.27.5; Sigma) was added (1200 Kunitz units, ~17 mg). This solution was then thoroughly mixed before it (1 mL) was added to the cells, which were pelleted by centrifugation at 3000 rpm for 5 min and at 4 °C. The cells were kept on ice until they were analyzed by flow cytometry. The analysis was completed on a Becton Dickinson FACScan.

Drug–DNA reactions

All drug–DNA reactions were performed in 25 mM triethanolamine, 1 mM EDTA, pH 7.2, at 37 °C for 5 h. Following incubation, DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate and 3 volumes of 95% ethanol and washed with 70% ethanol. The resulting pellet was dried by lyophilization.

Taq polymerase stop assay

The procedure employed was previously described by Ponti et al.[18] Prior to drug/DNA incubation, plasmid pUC18 was linearised with the *Hind* III enzyme, which cuts at only one site in the plasmid (position 399), providing a stop for the *Taq* polymerase downstream from the primer. The synthetic primer 5′-CTCACT-CAAAGGCGGTAATAC-3′ binds to the complementary (bottom) strand at positions 749–769 and was used to examine the alkylation patterns on the bottom strand. The oligodeoxynucleotide primer was 5′ end labeled prior to amplification using T4 polynucleotide kinase and [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham, UK). The labeled primer was purified by elution through Bio-Rad spin columns. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 5 pmol of labeled primer, 250 μM of each dNTP, 10 U *Taq* polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl and 0.1% Triton® X-100, 2.5 mM $MgCl_2$ and 0.01% Gelatin.

After an initial denaturation at 94 °C for 4 min, the cycling conditions were 94 °C for 1 min, 58 °C for 1 min, 74 °C for 1 min + 1 s per cycle, for a total of 30 cycles. After amplification the samples were ethanol precipitated and washed with 70% ethanol.

Sequence selectivity of purine–N3 alkylation

The sites of covalent binding for the compounds tested, as determined by the polymerase stop assay, were confirmed as minor groove purine N3 lesions using the thermal cleavage assay. The region of the plasmid containing the prominent sites of damage on the bottom strand was PCR amplified, using the pUC1 primer and the synthetic primer 5′-TGGTATCTTTATAGTCCTGTCG-3′, 5′-end labeled and binding on the complementary (upper) strand at positions 956–935. The 166 base pairs singly end-labeled fragment generated, was purified by agarose gel electrophoresis and isolated using a Bio101 kit according to the manufacturer's instruction.

The dry DNA pellets from the drug–DNA incubations were re-suspended in sodium citrate buffer (pH 7.2) and heated to 90 °C for 30 min to thermally cleave at sites of adenine– or guanine–N3 lesions as described by Reynolds.[24] Samples were chilled, precipitated and dried.

Acrylamide gel electrophoresis

Samples were dissolved in formamide loading dye, heat-denatured for 3 min at 95 °C, cooled on ice and electrophoresed at 2000 V for ≈2 h on a 6% acrylamide denaturing gel (Sequagel, National Diagnostics). The gels were dried under vacuum at 80 °C and exposed to film (X-OMAT, Kodak). Densitometry was performed on a Bio-Rad GS-670 imaging densitometer.

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