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A novel class of achiral *seco*-analogs of CC-1065 and the duocarmycins: design, synthesis, DNA binding, and anticancer properties

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Abstract—The synthesis, DNA binding properties, and in vitro and in vivo anticancer activity of fifteen achiral *seco*-cyclopropylindoline (or achiral *seco*-CI) analogs (**5a**–**o**) of CC-1065 and the duocarmycins are described. The achiral *seco*-CI analogs contain a 4-hydroxyphenethyl halide moiety that is attached to a wide range of indole, benzimidazole, pyrrole, and pyridyl-containing noncovalent binding components. The 4-hydroxyphenethyl halide moiety represents the simplest mimic of the *seco*-cyclopropylpyrroloindoline (*seco*-CPI) pharmacophore found in the natural products, and it lacks a chiral center. The sequence and minor groove specificity of the achiral compounds was ascertained using a *Taq* DNA polymerase stop assay and a thermal induced DNA cleavage experiment using either a fragment of pBR322 or pUC18 plasmid DNA. For example, *seco*-CI-InBf (**5a**) and *seco*-CI-TMI (**5c**) demonstrated specificity for AT-rich sequences, particularly by reacting with the underlined adenine-N3 position of 5′-AAAA<u>A</u>(865)-3′. This is also the sequence that CC-1065 and adozelesin prefer to alkylate. The achiral *seco*-CI compounds were subjected to cytotoxicity studies against several human (K562, LS174T, PC3, and MCF-7) and murine cancer cell lines (L1210 and P815). Following continuous drug exposure, the achiral compounds were found to be cytotoxic, with IC_{50} values in the μM range. Interestingly, the carbamate protected compound **5p** was significantly less cytotoxic than agent **5c**, supporting the hypothesis that loss of HCl and formation of a spiro[2,5]cyclopropylcyclohexadienone intermediate is necessary for biological activity. The achiral *seco*-CI compounds **5a** and **5c** were submitted to the National Cancer Institute for further cytotoxicity screening against a panel of 60 different human cancer cell lines. Both compounds showed significant activity, particularly against several solid tumor cell lines. Flow cytometry studies of P815 cells that were incubated with compound **5c** at its IC_{50} concentration for 24 h showed induction of apoptosis in a large percentage of cells. Compounds **5a** and **5c** were selected by the NCI for an in vivo anticancer hollow-fiber test, and received composite scores of 18 and 22, respectively. These two compounds were subsequently evaluated for in vivo anticancer activity against the growth of a human advanced stage SC UACC-257 melanoma in skid mice. At a dose of 134 mg/kg administered IP, compound **5c** gave a T/C value of 40% (for day 51), and the median number of days of doubling tumor growth was 27.7, versus 15.8 for untreated animals. For compound **5a**, at 200 mg/kg, the T/C was 58% and the median number of days of doubling tumor growth was 20.0 versus 8.7 for untreated animals. At these doses no toxicity or weight loss was observed for either compound. Furthermore, compound **5c** was not toxic to murine bone marrow cell growth in culture, at a dose that was toxic for the previously reported *seco*-CBI (cyclopropylbenzoindoline)-TMI (**4**).

1. Introduction

Minor groove and AT sequence selective alkylating agents,[1a,b] such as (+)-CC-1065 (**1**)[1c–e,2] and the duocarmycins,[2,3] exemplified by (+)-duocarmycin SA (or DUMSA, **2**) and depicted in Figure 1, belong to a group

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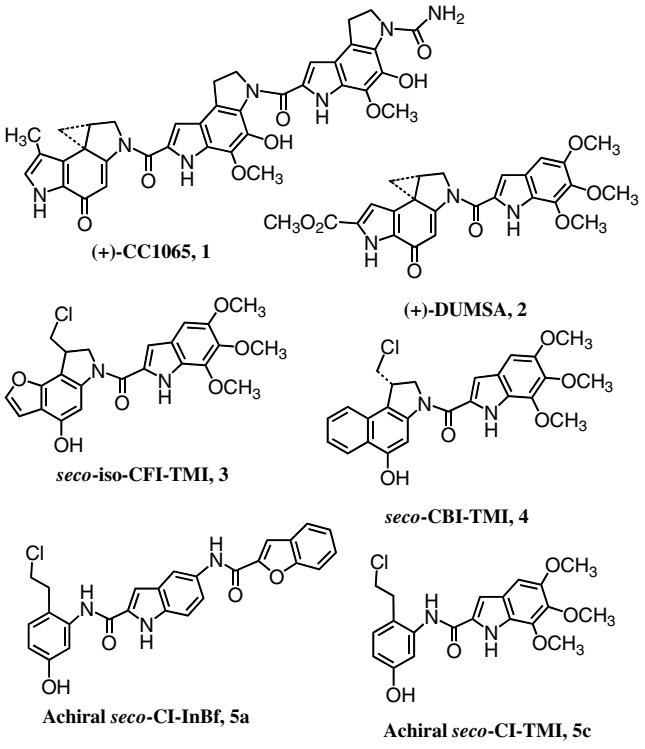


Figure 1. Structures of (+)-CC-1065 (**1**), (+)-duocarmycin SA (DUMSA, or **2**), *seco*-iso-CFI-TMI (**3**), and *seco*-CBI-TMI (**4**).

of natural products that have potent cytotoxic properties. These compounds, which were isolated from the fermentation broth of *Streptomyces zelensis*[4] and *Streptomyces* sp.,[5] respectively, derive their anticancer activity by reaction of the cyclopropylpyrroloindole or CPI moiety with adenine-N3 groups in the minor groove of specific sequences of DNA. This mechanism of action was confirmed by the isolation of adenine-CC-1065[6] and adenine–duocarmycin SA products[7] from thermally cleaved DNA-drug adducts. (+)-CC-1065 preferentially binds to 5′-PuNTTA-3′ and 5′-AAAAA-3′ sequences and reacts with the adenine-N3 position of the underlined residue.[6,8] (+)-DUMSA shows similar sequence selectivity, but, some subtle differences were observed, such as the lack of alkylation at 5′-GCAAA by CC-1065.[2,3,7] The cytotoxic potency of the CPI pharmacophore is directly related to its solvolytic stability. DUMSA, one of the most cytotoxic analogs, is the most solvolytically stable member of this class of compounds.[2,3,9]

Even though (+)-CC1065 has potent cytotoxic properties, its usefulness as an anticancer drug is hampered by delayed lethal toxicity to the animals at therapeutic doses.[4b] DUMSA is devoid of this toxicity,[2a] but it is toxic to the bone marrow.[5] As a result, several analogs of CC-1065 and the duocarmycins have been developed for clinical evaluation, including adozelesin,[1b,4c,10] carzelesin,[11] bizelesin,[12] and KW2189.[13] Presently, only bizelesin remains under clinical evaluation.[12] Clinical studies on the other three compounds were discontinued due to adverse toxicity to the bone marrow.[10–12] Consequently there is a strong interest in the design and development of novel analogs of CC-1065 and the duocarmycins that effectively kill cancer cells and have reduced toxicity to the host. In attempts to design novel analogs of the duocarmycins and CC-1065 with reduced toxicity to bone marrow cells, a wide range of analogs of the cyclopropylpyrrolo[*e*]indolone (CPI) subunit were synthesized and tested.[1] Examples of such analogs are, cyclopropylbenzo[*e*]indolone (CBI),[2,14] cyclopropylpyrazolo[*e*]indolone (CPzI),[15] cyclopropylfurano[*e*]indolone (CFI),[16] and cyclopropylindolone (CI).[17] Our laboratories have recently reported that *seco*-iso-CFI-TMI (isocyclopropylfurano[*e*]indoline) analog **3** is endowed with potent anticancer activity and is relatively nontoxic to murine bone marrow cells, when compared to *seco*-CBI-TMI **4**.[18]

As part of our efforts to design novel analogs of CC-1065 and the duocarmycins, we are undertaking a program to investigate whether the chiral center present in the natural products is needed for DNA sequence recognition and biological activity. To date, there has been little activity on the development of achiral analogs of CC-1065 and the duocarmycins. One report describes the synthesis and DNA binding studies of a bischloromethyl *seco*-CBI-TMI analog.[19] The bulky prochiral molecule was found to alkylate DNA poorly and demonstrated weak cytotoxicity due to the inefficient production of interstrand crosslinks. Another report described the synthesis of a spiro[2,5]cyclopropanecyclohexadienone derivative, but no biochemical studies were conducted.[20]

As illustrated in Figure 2, we have designed an achiral analog **5** (*seco*-CI-TMI, **5c**) that contains a 4-hydroxyphenylethyl halide functionality. Similar to the loss of HCl in *seco*-CC-1065 and *seco*-duocarmycin compounds in biological media,[2,3] compound **5c** should lose HCl to produce the putative spiro[2,5]cyclopropanecyclohexadienone DNA alkylating agent **6**, which should react with an adenine-N3 group in AT-rich sequences. Hydroxyphenethyl halides are known to readily lose a hydrogen halide to generate the corresponding spiro[2,5]cyclopropanecyclohexadienones, which react with DNA and possess cytotoxic properties.[21] In this

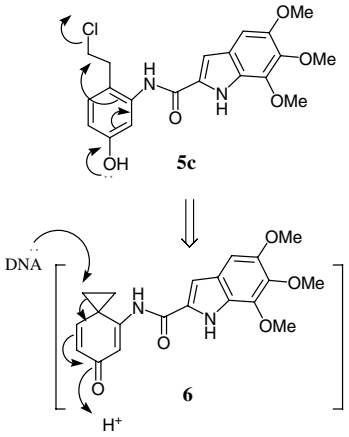


Figure 2. Proposed mechanism of activation and DNA alkylation of the achiral *seco*-CI moiety in compound **5**.

report, we will describe the synthesis of fifteen *seco*-CI compounds (**5a–o)** along with their DNA sequence specific alkylation and anticancer properties.

2. Results and discussion

2.1. Synthesis

Fifteen novel achiral *seco*-CI analogs of CC-1065 and the duocarmycins were designed and synthesized in this study, and the structures are given in Figure 3. Compounds **5a** and **5b** are reminiscent of adozelesin, which contain an indole-benzofuran noncovalent binding moiety. Compounds **5c–f**,**l** are mimics of the duocarmycins, which contain a 5,6,7-trimethoxyindole noncovalent binding moiety. Analogs **5g–i** contain a cinnamoyl group;[22] **5j**,**k** contain a substituted pyridyl group. Agent **5m** was designed to test the influence of interstrand crosslink formation on cytotoxicity. Compounds **5n** and **5o** are hybrids of achiral *seco*-CI and bisbenzimide[23] and netropsin,[24] respectively.

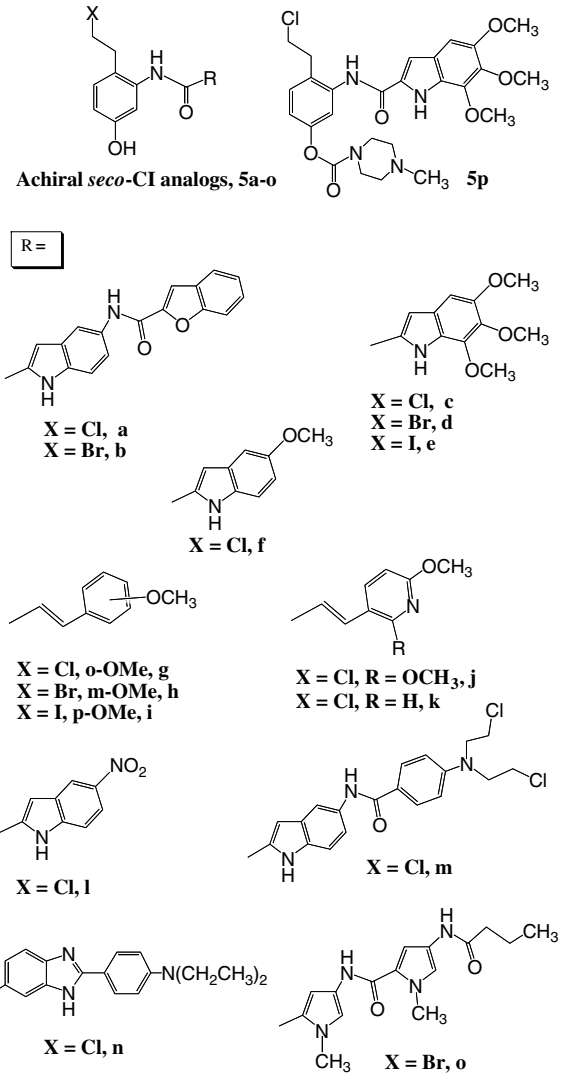


Figure 3. Fifteen analogs of achiral *seco*-CI prepared in this study, **5a–o**, and a carbamate derivative **5p**.

Synthesis of the achiral *seco*-CI analogs started from malonate diester **7**, which was synthesized according to a literature procedure.[4c] Hydrolysis of the esters, followed by acid-promoted decarboxylation afforded a substituted acetic acid intermediate in 95% yield. The carboxylic acid functional group was reduced to the primary alcohol (**8**) with borane in THF in 95% yield. Treatment of compound **8** with carbon tetrachloride or carbon tetrabromide and triphenylphosphine afforded the corresponding chloride **9** or bromide **10** in excellent yields. Catalytic hydrogenation of compounds **9** and **10** with 10% Pd–C in THF gave the aminophenols **11** and **12**, which were unstable and used immediately in the subsequent step. Coupling of the aminophenols with the corresponding carboxylic acids in presence of 3 mol equivalents EDCI in DMF gave the target compounds **5a–l**,**n**,**o** in reasonable yields. For synthesis of a potential interstrand crosslinking agent **5m**, aminophenol **11** was condensed with 5-nitrobenzofuran-2-carboxylic acid and EDCI. The amide intermediate **5l** was collected in 14% yield, which was converted to the desired compound **5m** in 24% yield by reduction of the nitro group with 5% Pd–C, followed by coupling with *N*,*N*-bis-(2-chloroethyl)aminobenzoic acid in EDCI.

Due to the interesting biological activity of achiral *seco*-CI-TMI **5c**, a different strategy was needed to improve its synthetic efficiency from 11% yield using the EDCI coupling method. Selective reduction of the nitro group of compound **9** was achieved by catalytic hydrogenation over Adams catalyst (PtO_2) and 55 PSI of hydrogen. The resulting amine was coupled to 5,6,7-trimehtoxyindole-2-carboxylic acid in presence of PyBOP and Hunig's base. From this reaction an amide intermediate was obtained in 66% yield. Catalytic hydrogenation of this intermediate over 10% Pd–C afforded the desired compound **5c** in 50% yield, after silica gel column chromatography. The overall yield for the synthesis of **5c**, from compound **9**, using the PyBOP method was 33%, which is 3 times more efficient than the EDCI method. All intermediates and products were characterized by FT-IR, 500 MHz ^{1}H NMR, MS, and accurate mass measurements. Elemental analysis was done for **5c**.

2.2. Taq polymerase stop assay

This PCR-based assay determines the sequence selectivity of alkylation in DNA.[25] This assay works on the premise that Taq DNA polymerase activity is blocked at the site of covalent binding. Using a singly radiolabeled primer 5′-^{32}P-5′-GCATTGGTAACTGTCAGACC-3′ and a *Pvu*II linearized pBR322 plasmid DNA, the region starting from 3303 was linearly amplified. Taq DNA polymerase stop studies were performed on compounds **5a**, **5b**, **5l**, **5m**, and **5o** along with CC-1065 and adozelesin, and the results are given in Figure 4. Concentrations of 10 μM of the achiral *seco*-CI compounds were used for the studies, but a concentration of 0.005 μM was used for CC-1065 and adozelesin to give similar levels of alkylation. The results clearly show that all compounds interact strongly at a site containing a run of seven adenine residues [5′-AAAAAAA(3114)-3′]. This finding is interesting because major changes

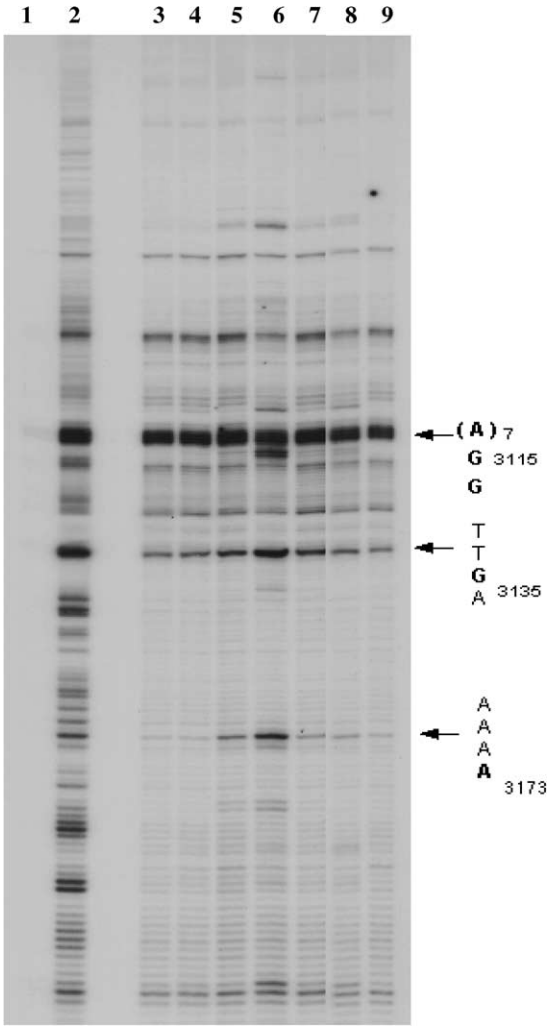


Figure 4. *Taq* DNA Polymerase Stop Assay using the *Pvu*II primer; lane 1, control DNA; lane 2, chlorambucil; lane 3, 0.005 μM CC-1065; lane 4, 0.005 μM adozelesin; lane 5, compound **5l** 10 μM; lane 6, compound **5a** 10 μM; lane 7, compound **5b** 10 μM; lane 8, compound **5m** 10 μM; lane 9, compound **5o** 10 μM.

to the noncovalent binding portion of the molecules had little effect on sequence specificity. A significantly weaker alkylation site was seen at 5′-TTG(3135)A-3′, with alkylation at the underlined G residue. It is worthy to note that CC-1065 produced the highest level of alkylation at the weaker site. For compound **5a**, an additional weak alkylation band was observed at 5′-AAAA(3173)-3′ and 5′-A_7G(3115)-3′.

The covalent sequence specificity of compound **5c** was ascertained using a Taq DNA polymerase stop assay using a singly radiolabeled primer 5′-^{32}P-CTCACT-CAAAGGCGGTAATAC-3′ and a *Hin*dIII linearized pUC18 plasmid DNA. The region of the plasmid starting from position 749 was linearly amplified. The autoradiograph shown in Figure 5B demonstrates that, like adozelesin,[2] compound **5c** could stop the activity of *Taq* DNA polymerase at sites with contiguous adenine residues. The sequence specific covalent reaction of compound **5c** with the adenine-N3 position was confirmed using a thermal cleavage experiment, as depicted in Figure 5A, thereby providing evidence of binding of **5c** to the minor groove. This gel demonstrated an additional alkylation site for adozelesin, A(843), which was absent for compound **5c**, suggesting an improved sequence preference for the latter compound for the DNA sequence studied.

2.3. Molecular modeling studies

The MM2 energy minimized complex of achiral CI-InBf (**5a**) with duplex 5′-dGGCGG*AGTTA*GG-3′ in the B-form and alkylating at the adenine N3 position of the underlined A residue, is shown in Figure 6. This oligonucleotide was used by Hurley and co-workers in their NMR and molecular modeling studies of a DNA complex with CC-1065.[26] As depicted, the molecule **5a** fits snugly and isohelically in the minor groove. The angular plane between the achiral CI and InBf units exhibits a twist of 5° in order to accommodate the natural curvature of the DNA.

2.4. 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 **5a**–**p** against a range of human K562 (chronic myeloid leukemia cells), and murine L1210 leukemia and P815 mastocytoma cells were determined.[27] Cytotoxicity studies on LS174T (human colorectal adenocarcinoma), PC3 (human prostate adenocarcinoma), MCF7 (human breast adenocarcinoma) were probed using a sulforhodamine B assay.[28] The cells were treated continuously for three days, except for the K562 cells, which were treated for four days. The cytotoxicity of compounds **5a**–**p**, expressed as IC_{50} values are given in Table 1. For comparison, the reported IC_{50} value of CC-1065 against L1210 cells (three days exposure) was 20 pM[29] The results reveal several interesting trends. First, compounds **5a**–**o** inhibited the growth of the tumor cells at μM concentrations. For comparison, continuous exposure of the racemic *seco*-CI-TMI (**13**) and *seco*-CI-InBf (**14**) [the structures of these compounds are given in Scheme 1] gave IC_{50} values of 0.10 and 0.14 μM, respectively, against the growth of K562 cells.[30] Since their achiral counterparts **5c** (1.43 μM) and **5a** (1.47 μM) retain activity, the results suggest that the chiral center in compounds **13** and **14**, as well as the natural products, is not essential for cytotoxicity.

Second, protection of the hydroxyl group in compound **5c** led to a dramatic decrease in cytotoxicity. Carbamate **5p** gave IC_{50} values of 75 μM and >100 μM against L1210 and P815 cells. In contrast, the achiral *seco*-CI-TMI agent **5c** produced IC_{50} values for these cell lines of 1.5 and 5.6 μM, respectively. These results suggest that the achiral hydroxyphenethyl halide compounds eliminate HCl to form spiro[2,5]cyclopropanecyclohexadienone intermediates that react with DNA and elicit the cytotoxic activity. This observation is consistent

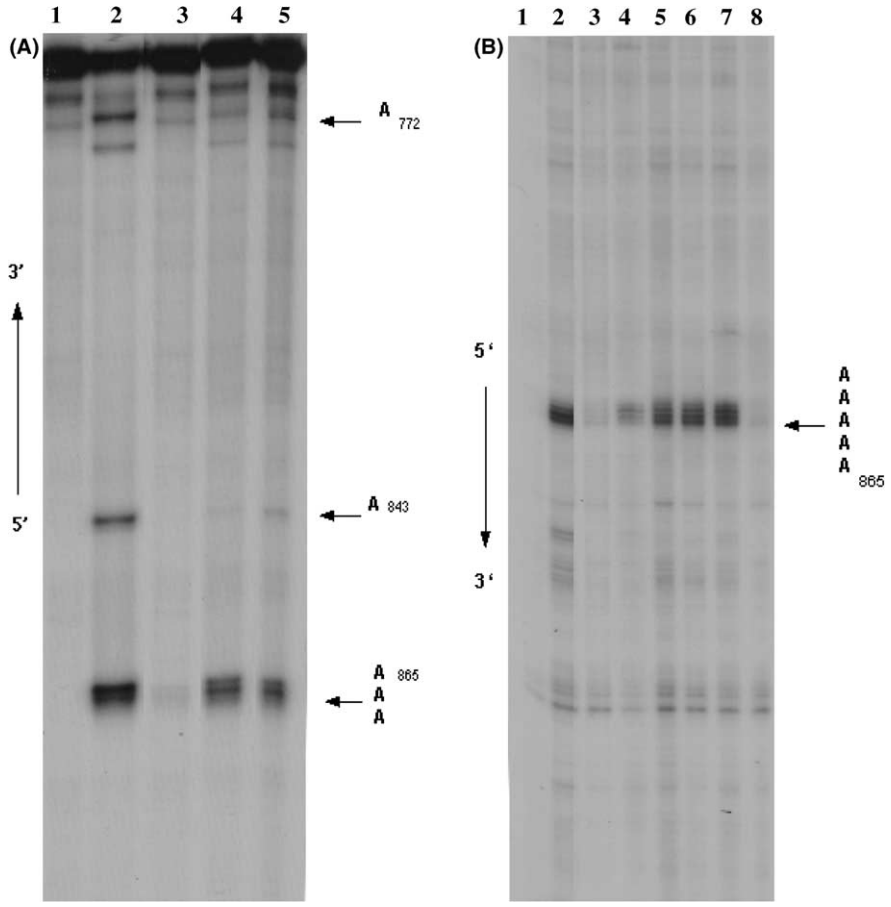


Figure 5. (A) Thermal cleavage gel showing purine-N3 lesions on the upper strand of a region from the *Hin*dIII linearized fragment of plasmid pUC18. Lane 1, control; lane 2, 0.1 μM adozelesin; lanes 3–5 correspond to 1, 10, 100 μM of compound **5c**. (B) Taq DNA polymerase stop assay on the same fragment of DNA. Lane 1, control; lane 2, 0.1 μM adozelesin; lanes 3-8 correspond to 1, 3, 10, 30, 100, and 300 μM of compound **5c**.

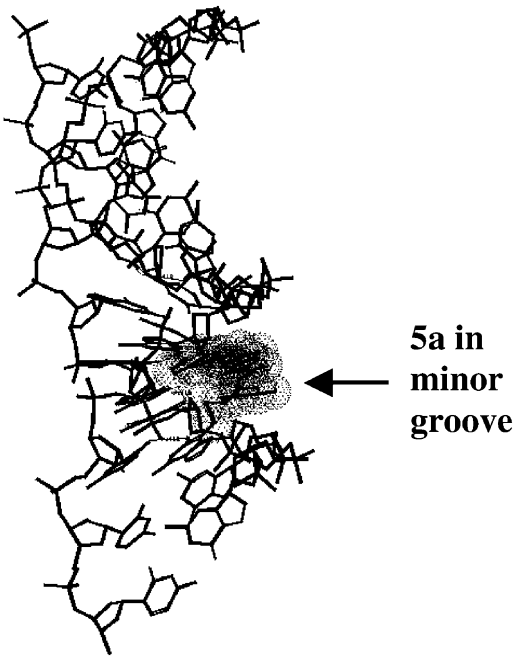


Figure 6. MM2 energy optimized (steepest descend) structure of a complex of a covalent adduct between the chloromethyl moiety of compound **5a** and adenine-N3 of the underlined residue of duplex dGGCGGAGTTAGG in the B-form.

with the biological activity of *seco*-analogs of CC-1065 and duocarmycins.[2,3] Third, even though the noncovalent binding portions of compounds **5a**–**o** are very different, their IC_{50} values are quite similar. This discovery, while not surprising because compounds **5a**–**o** have very similar DNA covalent sequence specificity, suggests that the DNA alkylation event is responsible for the cytotoxic activity. Lastly, comparing the cytotoxicity of bromo (**5d**, **5h**) and iodo (**5e**) compounds to the chloro compound, the results indicate that the nature of the leaving group has minimal effects on biological activity.

Compounds **5a** and **5c** were subjected to further cytotoxicity testing at the National Cancer Institute, against a panel of 60 human cancer cell lines.[31] The viability of the cells after 48 h of continuous exposure of the compounds was determined using a sulforhodamine B assay. Both compounds **5a** and **5c** gave comparable cytotoxicity results, and Figure 7 shows the LC_{50} (concentration for killing 50% of the cells) values for compound **5c** against the 60 different tumor cell lines. 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. Several observations were made on the results. First, they exhibit activity in the micromolar range. Second, agents **5a** and **5c** are not

Table 1. Cytotoxicity of compounds **5a**–**p** against the growth of cancer cells grown in culture

Compound	K562	LS174T	PC3	MCF7	L1210	P815
5a	1.47	2.27	3.0	3.43	23	43
5b	1.61					
5c	1.43	0.39	0.46	0.94	1.5	5.6
5d	—	1.25				
5e	—	0.29				
5f	1.11					
5g	2.56	4.04				
5h	1.41	1.14				
5i	1.52	1.62				
5j	2.22					
5k	2.86					
5l	2.37					
5m	2.70					
5n	1.81					
5o	3.64					
5p	—				75	>100

The IC_{50} values are given in μM, and the cells were continuously exposed to the compounds for 3 days continuously, except 4 days for K562 cells.

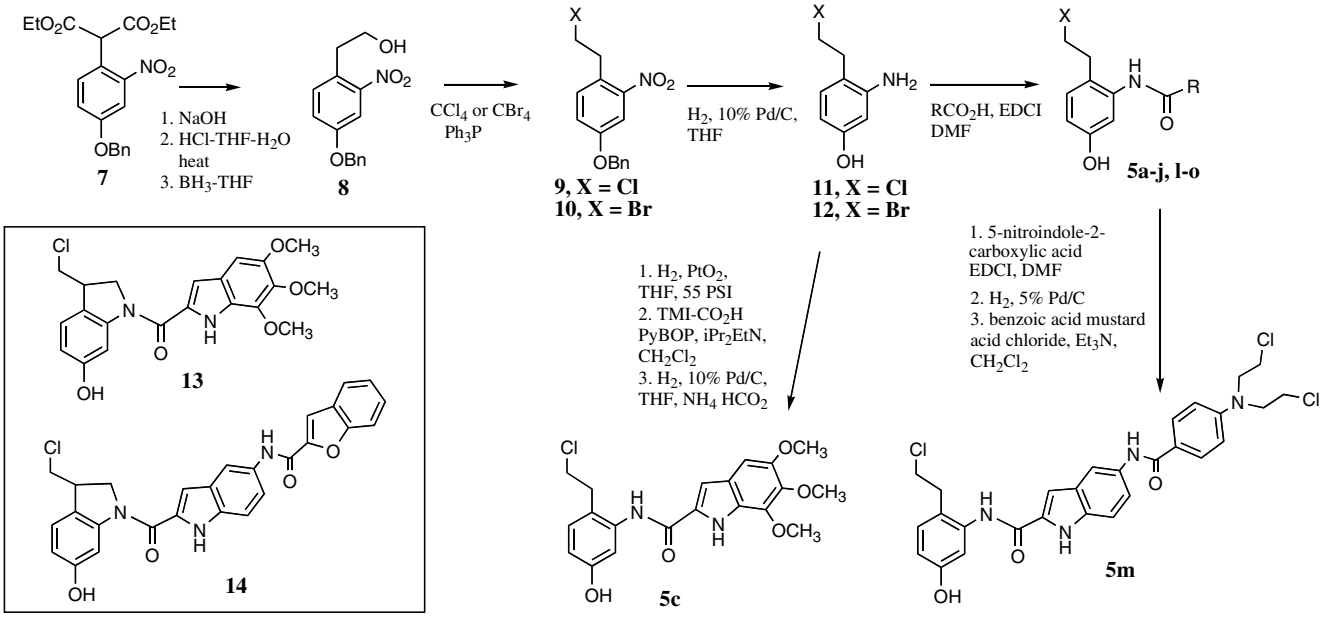
indiscriminately toxic to cells, for example, they were not active against leukemic cells. Third, these compounds show activity against cells from many solid tumors, and in particular **5c** appears to be very active against melanoma cells. Specific reasons for the unique patterns of cytotoxicity for these compounds are unknown, and experiments are underway to address this issue.

2.5. Cell cycle studies

Duocarmycin analogs have been demonstrated to induce leukemic cells to undergo apoptosis. The cells demonstrated morphological abnormalities and genomic DNA degradation associated with apoptosis.[32] Flow cytometry was used to detect apoptotic cells as a sub-G_0 peak[33] using propidium iodide staining. Following a 24 h incubation of P815 cells at 10-times the IC_{50} concentration of compound **5c** (56 μM), the results given in Figure 8 showed a substantial increase in the percentage of sub-G_0 stage (67%), compared to 0.7% for untreated control cells, indicating that compound **5c** was capable of damaging DNA and inducing the cells to undergo apoptosis. For comparison, at a concentration of 1000 μM, cisplatin produced 59% of sub-G_0 population of cells, which is consistent with a literature report.[33] Results from our studies suggest that like their *seco*-CC-1065 and *seco*-duocarmycin counterparts, achiral *seco*-CI compounds are likely to exert their cytotoxic activity through the induction of apoptosis.

2.6. Hollow fiber assay for preliminary in vivo testing[34]

In this study conducted at the NCI, human tumor cells were cultivated in polyvinylidene fluoride (PVDF) hollow fibers, and a sample of each cell line was implanted into each of two physiologic compartments (intraperitoneal and subcutaneous) in mice. Each test mouse received a total of six fibers (three intraperitoneally and three subcutaneously) representing three distinct cancer cell lines. A total of 12 different tumor cell lines were used for these studies. Three mice were treated with potential antitumor compounds (dissolved in DMSO) at each of two test doses by the intraperitoneal route using a QD × 4 treatment schedule. Vehicle controls consisted of 6 mice receiving the compound diluent only. The fiber cultures were collected on the day following the last day of treatment. To assess anticancer effects, viable cell mass was determined for each of the cell lines using a formazan dye (MTT) conversion assay. From this, the %T/C values were calculated using the average optical density of the compound treated samples divided by the average optical density of the vehicle controls. The results were converted to scores of IP and SC, representing activity against cells implanted by the two



Scheme 1. Synthesis of the achiral *seco*-CI analogs of CC-1065 and the duocarmycins.

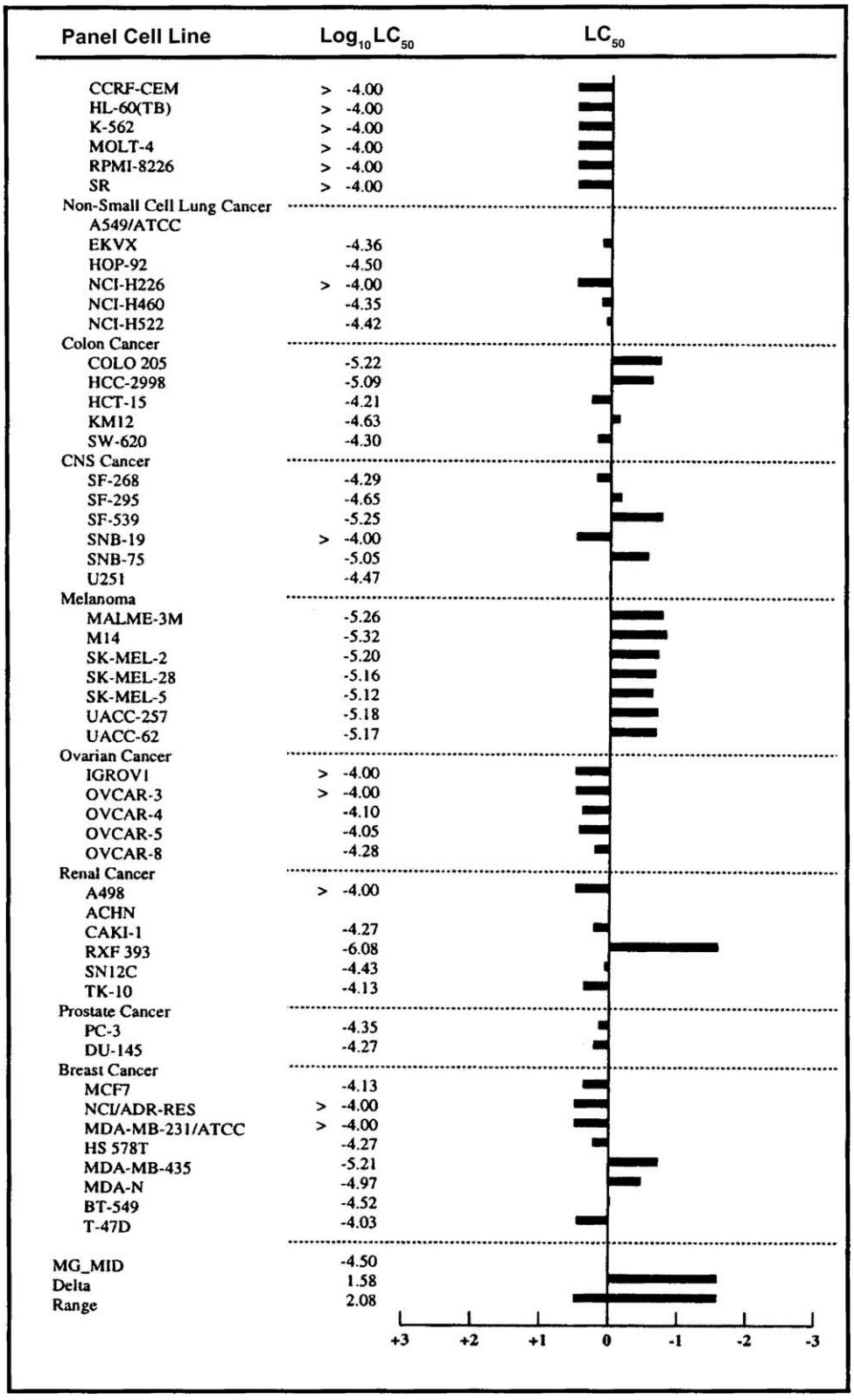


Figure 7. LC_{50} values of compound **5c** against the NCI's panel of 60 human cancer cell lines.

routes. Compounds with a combined IP + SC score $\geqslant 20$, a SC score $\geqslant 8$, or a net cell kill of one or more cell lines were referred for xenograft testing. The composite IP+SC score for compound **5a** was 18, and cell kill was observed. For compound **5c**, the IP+SC score was 22, and no cell kill was observed. Based on these composite scores as well as the unique structure of the achiral *seco*-CI compounds, both compounds were selected by the NCI for further in vivo testing using human tumor xenografts grown in nude mice.

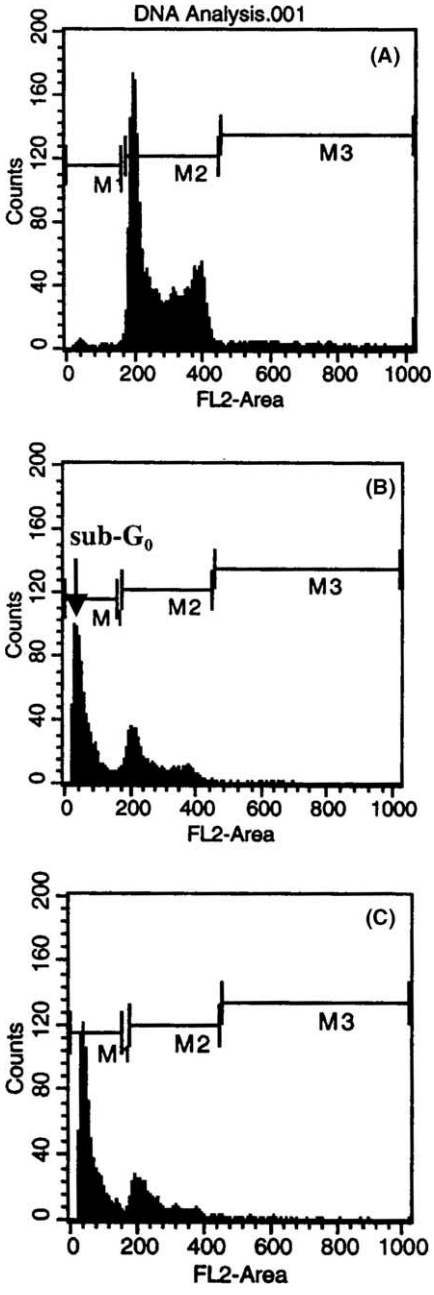


Figure 8. Propidium iodide stained flow cytometry analysis of P815 mastocytoma cells that were incubated for 24 h with compounds **5c** (B) and cisplatin (C) at 10 times their IC_{50} concentrations. Part A represents untreated cells.

2.7. In vivo tumor xenograft studies[35]

This in vivo tumor model assay involved the subcutaneous implantation of the human UACC-257 melanoma fragments (30 mg) into the axillary region of pathogen-free immunodeficient mice on day 0 of the experiment. Drug treatments were initiated on day 10 for compound **5a** and day 16 for compound **5c**. In both cases the compounds were dissolved in DMSO and were administered via an IP route on a QD × 3 schedule. The volume of injected was adjusted according to a ratio of 0.02 mL for every 10 g of body weight. Compounds **5a** and **5c** were administered at their maximum tolerated doses of 200 and 134 mg/kg/dose, respectively. Tumor size and body weights are obtained approximately two times per week.

The results for compounds **5a** and **5c** obtained from the xenograft studies are given in Table 2. It is apparent that both compounds were able to inhibit the growth of the melanoma. For the achiral *seco*-CI-InBf compound **5a**, a T/C value was obtained at 58% at day 51. The mean time for doubling of the tumor weight was 20.0 days, compared to 8.7 days for the control untreated animals. For compound **5c**, the T/C value was 40% at day 51, which according the NCIs evaluation criteria is deemed an 'active' compound. The mean time for the tumor to double in weight was 27.7 days, compared to 15.8 days for the untreated control. The results provide evidence that compound **5c** possesses anticancer activity, and more importantly, at the dose administered to the animals, no toxicity was observed. Compound **5c** was also found to be nontoxic to murine bone marrow cells in culture at a dose that produced no colonies with the previously reported compound **4** (0.0084 μM, the IC_{50} value for **4** against L1210 cells).[14,18]

3. Conclusion

A novel class of achiral *seco*-CI analogs of CC-1065 and the duocarmycins has been designed and synthesized. Overall, the favorable cytotoxicity of the achiral *seco*-CI compounds against tumor cell lines, their low toxicity, and their activity against a human melanoma xenograft strongly indicated that the chiral center present in the CC-1065 and the duocarmycins is not needed for antitumor activity. Furthermore, elimination of the chiral center makes the achiral analogs a simpler

Table 2. In vivo anticancer properties of compounds **5a** and **5c** against the growth of human advanced stage SC UACC-257 melanoma grown in skid mice

Compound	Dose	T/C (%)	Mean doubling time	Net log cell kill
5a[a]	200 mg/kg/dose Q4D × 3, day 10	58 (at day 51)	20.0 days versus 8.7 days for control	−0.60
5c[a]	134 mg/kg/dose Q4D × 3, day 16	40 (at day 51)	27.7 days versus 15.8 days for control	0.10

The compounds were administered via an intraperitoneal (IP) route.

[a] Both compounds did not cause significant weight loss in the animals indicating that they were relatively not toxic. Twenty skid mice were used in the control group, and six mice were used in the test experiments. The compounds were dissolved in DMSO, and the injections were made using volumes that corresponded to 0.02 mL/10 g of body weight.

platform from which more active and potentially useful medicinal compounds could be developed in the future.[36] Such studies are in progress, and the results will be reported when they become available.

4. Experimental

4.1. 2-(4-Benzyloxy-2-nitrophenyl)ethanol (8)

To a round-bottom flask containing diethyl 2-(4-benzyloxy-2-nitrophenyl)malonate (**7**)[4c] (2.00 g, 5.17 mmol) was added EtOH (45 mL) and 10% NaOH (60 mL) producing a dark brown solution, which was refluxed for 3 h. The solvent was removed under reduced pressure to form a yellow suspension and THF (30 mL) was added to produce a clear yellow solution, which was placed in an ice bath and stirred. 6 M HCl (30 mL) was slowly added to the solution to reach pH 1. The light orange solution was refluxed for another hour, at which time two layers formed. The top THF layer was collected and the aqueous was extracted twice with EtOAc (50 mL each). The combined organic extracts were dried with anhydrous sodium sulfate, and concentrated to produce 4-benzyloxy-2-nitrophenylacetic acid as an orange solid (1.41 g, 4.91 mmol, 95%). Mp 145–152 °C; TLC ($CHCl_3$) $R_f = 0.15$; ^1H NMR ($CDCl_3$, 300 MHz) 7.76 (d, 2.7, 1H), 7.41 (m, 5H), 7.26 (d, 8.7, 1H), 7.21 (dd, 2.7, 8.7, 1H), 5.13 (s, 2H), 4.00 (s, 2H); IR ($CHCl_3$ cast) 3400–2600 (br), 3100, 1698, 1601, 1526, 1523, 1453, 1376, 1349; EI-MS *m/z* (rel. intensity) 287 (M^+, 10). Accurate mass (EI-MS) for $C_{15}H_{13}NO_5$: calcd. 287.0794, obsd. 287.0799.

4-Benzyloxy-2-nitrophenylacetic acid (6.38 g, 22.2 mmol) was dissolved in freshly distilled and dry THF (60 mL) was kept under an atmosphere of N_2 and stirred in an ice bath. Borane (55.6 mL, 55.5 mmol, 1 M solution in THF) was slowly added to the solution, producing much effervescence. The red solution was left stirring in the ice bath for 10 min and then allowed to stir at room temperature for 4 h. Water (150 mL) was slowly added to the flask. The THF layer was collected, and the aqueous layer was extracted twice with EtOAc (75 mL each). The combined organic extracts were dried with anhydrous sodium sulfate and concentrated to give a brown oil. Column chromatography (silica gel, 10–60% EtOAc/5% $CHCl_3$/hexane gradient elution) afforded 2-(4-benzyloxy-2-nitrophenyl)ethanol (**8**) (5.72 g, 21.0 mmol, 95%) as a brownish solid. Mp 48–52 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.61$; ^1H NMR ($CDCl_3$, 500 MHz) 7.54 (d, 2.5, 1H), 7.42 (m, 5H), 7.31 (d, 8.5, 1H), 7.17 (dd, 2.5, 8.5, 1H), 5.10 (s, 2H), 3.91 (br q, 6.5, 2H), 3.65 (br t, 6.5, OH), 3.10 (t, 6.5, 2H); IR (neat) 3400, 3050, 2965, 2921, 2858, 1622, 1523, 1453, 1344. EI-MS *m/z* (rel. intensity) 273 (M^+, 14). Accurate mass (EI-MS) for $C_{15}H_{15}NO_4$: calcd. 273.1001, obsd. 273.0992.

4.2. 2-(4-Benzyloxy-2-nitrophenyl)ethyl chloride (9)

Triphenylphosphine (1.98 g, 7.55 mmol), CCl_4 (2.18 mL, 22.62 mmol), and dry CH_2Cl_2 (13.7 mL) were added to 2-(4-benzyloxy-2-nitrophenyl)ethanol (**8**) (1.03 g, 3.77 mmol). The solution was allowed to stir overnight under a positive pressure of nitrogen and then concentrated under reduced pressure. The brown oily residue was purified using silica gel column chromatography and a 10% ethyl acetate, 5% $CHCl_3$, and 85% petroleum ether solvent system. 2-(4-Benzyloxy-2-nitrophenyl)ethyl chloride (**9**) was isolated as a thick yellowish oil, which solidified upon standing in the refrigerator (1.06 g, 3.64 mmol, 97% yield). Mp 38–40 °C; TLC (20% ethyl acetate/hexane) $R_f = 0.53$; ^1H NMR (500 MHz, $CDCl_3$) 7.51 (d, 2.5, 1H), 7.33 (m, 5H), 7.23 (d, 8.5, 1H), 7.10 (dd, 2.5, 8.5, 1H), 5.02 (s, 2H), 3.70 (t, 7.0, 2H), 3.20 (t, 7.0, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 158.1, 149.5, 135.7, 143.0, 128.7, 128.4, 127.5, 125.1, 120.4, 110.7, 70.6, 44.0, 35.8; IR (neat) 3092, 3038, 2962, 2919, 2865, 1618, 1570, 1532, 1499, 1451, 1343, 696; EI-MS *m/z* (rel. intensity) 291 (M^+, 10); isotope pattern of M^+ and M^++2 agrees with the presence of one chlorine atom. Accurate mass (EI-MS) for $C_{15}H_{14}{}^{35}ClNO_3$: calcd. 291.0662, obsd. 291.0667.

4.3. 2-(4-Benzyloxy-2-nitrophenyl)ethyl bromide (10)

The procedure was similar to that used for the synthesis of compound **9**, except 2-(4-benzyloxy-2-nitrophenyl) ethanol (**8**) (1.00 g, 3.66 mmol), CBr_4 and dry CH_3CN were used. Column chromatography (SiO_2, 10% EtOAc/5% $CHCl_3$/85% petroleum ether gradient elution) afforded 2-(4-benzyloxy-2-nitrophenyl)ethyl bromide as a brownish solid (1.33 g, 3.56 mmol, 92%). Mp 30–34 °C; TLC (20% EtOAc/hexane) $R_f = 0.48$; ^1H NMR ($CDCl_3$, 500 MHz) 7.52 (d, 2.5, 1H), 7.34 (m, 5H), 7.24 (d, 8.5, 1H), 7.11 (dd, 2.5, 8.5, 1H), 5.04 (s, 2H), 3.57 (t, 7.0, 2H), 3.30 (t, 7.0, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) 158.0, 149.4, 135.6, 133.8, 128.7, 128.4, 127.5, 125.8, 120.5, 110.8, 70.6, 35.9, 32.0; IR (neat) 3070, 3027, 2919, 2855, 1618, 1570, 1526, 1499, 1451, 1408, 1381, 1343, 695; EI-MS *m/z* (rel. intensity) 335 (M^+, 9); isotope pattern of M^+ and M^++2 agrees with the presence of one bromine atom. Accurate mass (EI-MS) for $C_{15}H_{14}NO_3Br$: calcd. 335.0157, obsd. 335.0160.

4.4. 2-(2-Amino-4-hydroxyphenyl)ethyl chloride (11)

Pd/C 10% (45 mg) was added to 2-(4-benzyloxy-2-nitrophenyl)ethyl chloride(132 mg, 0.453 mmol). The mixture was suspended in chilled THF (30 mL), degassed and purged three times with hydrogen, and then reduced under atmospheric pressure at room temperature. After 3 h the solution the solution was filtered by suction over Celite. The filtrate was concentrated under reduced pressure to give 2-(2-amino-4-hydroxyphenyl)ethyl chloride as a white solid (60.7 mg, 0.354 mmol, 78%). Mp 96–100 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.34$; ^1H NMR ($CDCl_3$ in 1 drop DMSO-d_6, 500 MHz) 8.20 (br s, 1H), 6.85 (d, 8.0, 1H), 6.27 (dd, 2.5, 8.0, 1H), 6.26 (d, 2.5, 1H), 4.00 (br s, 2H), 3.66 (t, 8.5, 2H), 2.90 (t, 8.5, 2H); IR (neat) 3139, 3020, 2954, 1622, 1594, 1513, 1464, 1560, 1306, 722; EI-MS *m/z* (rel. intensity) 171 (M^+, 28); isotope pattern of M^+ and M^++2 agrees with the presence of one chlorine atom. Accurate mass (EI-MS) for $C_8H_{10}ClNO$: calcd. 171.0451, obsd. 171.0444.

4.5. 2-(2-Amino-4-hydroxyphenyl)ethyl bromide (12)

The procedure was similar to that used for the synthesis of compound **11**, except 2-(4-benzyloxy-2-nitrophenyl)ethyl bromide (540 mg, 1.6 mmol) was used. 2-(2-Amino-4-hydroxyphenyl)ethyl bromide was isolated as an off-white solid (540 mg, 2.50 mmol, 100%). Mp 160–166 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.27$; ^1H NMR ($CDCl_3$ in 1 drop DMSO, 500 MHz) 7.21 (br s, 1H), 7.14 (d, 8.0, 1H), 7.10 (d, 2.0, 1H), 6.87 (dd, 2.0, 8.0), 3.83 (t, 7.5, 2H), 3.17 (t, 7.5, 2H); IR (neat) 3335, 3008, 2921, 1622, 1561, 1502, 1464, 1442, 698; EI-MS *m/z* (rel. intensity) 135 (M^+−HBr, 85). Accurate mass (EI-MS) for C_8H_9NO: calcd. 135.0684, obsd. 135.0678.

4.6. 4-(2-Chloroethyl)-3-[5-(5-benzofuran-2-carboxamido)indole-2-carboxamido]phenol (5a)

2-(2-amino-4-hydroxyphenyl)ethyl chloride (**11**), produced from catalytic hydrogenation of compound **10** (0.4068 g, 1.40 mmol) with 10% Pd/C (0.200 g), was mixed with 5-(Benzofuran-2-carboxamido)indole-2-carboxylic acid (0.36 g, 1.12 mmol) and EDCI (0.81 g, 4.20 mmol). The mixture was dissolved in dry DMF (15 mL) and stirred under a N_2 atmosphere at room temperature for 4 days. The DMF was removed using a Kugelrohr apparatus (40 °C, 0.1 mmHg). The oily residue was dissolved in chloroform (200 mL) and washed with water (50 mL) and 5% sodium bicarbonate (50 mL). The organic layer was collected, dried with sodium sulfate, and concentrated under reduced pressure. The product was purified on a silica gel column. The column was eluted with ethyl acetate (0–50%)/chloroform to give the product as an off-white solid (299 mg, 0.632 mmol, 28%). Mp = 233–235 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.37$; ^1H NMR (DMSO-d_6, 500 MHz) 11.74 (d, 1.5, 1H, 1H), 10.44 (s, 1H), 9.87 (s, 1H), 9.48 (s, 1H), 8.17 (d, 1.0, 1H), 7.83 (d, 8.0, 1H), 7.76 (s, 1H), 7.73 (d, 8.5, 1H), 7.58 (dd, 2.0, 8.5, 1H), 7.51 (dt, 1.0, 8.0, 1H), 7.44 (d, 8.0, 1H), 7.38 (t, 8.0, 1H), 7.33 (d, 1.0, 1H), 7.17 (d, 9.0, 1H), 6.79 (d, 2.5, 1H), 5.67 (dd, 2.5, 9.0 1H), 3.75 (t, 7.5, 2H),2.98 (t, 7.5, 2H); IR (neat) 3401, 3259, 3041, 2965, 2921, 1643, 1594, 1534, 1446, 1301, 739; UV–vis (ethanol) 218 ($\varepsilon = 1.1 \times 10^6 M^{-1} cm^{-1}$), 290 ($\varepsilon = 1.7 \times 10^4 M^{-1} cm^{-1}$); FAB-MS (NBA) *m/z* (rel. intensity) 474 ($M+H^+$, 2). Accurate mass for $C_{26}H_{21}N_3O_4{}^{35}Cl$: calcd. 474.1221, obsd. 474.1219.

4.7. 4-(2-Bromoethyl)-3-[5-(5-benzofuran-2-carboxamido)indole-2-carboxamido]phenol (5b)

The procedure was similar to that used for the synthesis of compound **5a**, except 2-(4-benzyloxy-2-nitrophenyl)ethyl bromide (300 mg, 0.893 mmol) was used. Product **5b** was isolated as an off-white solid (8.1 mg, 0.016 mmol, 2%). Mp = 285–290 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.50$; ^1H NMR (DMSO-d_6, 500 MHz) 11.70 (s, 1H), 10.45 (s, 1H), 9.32 (s, 1H), 8.18 (d, 1.5, 1H), 7.83 (d, 8.0, 1H), 7.76 (s, 1H), 7.73 (dd, 1.0, 8.0, 1H), 7.60 (dd, 2.0, 8.5, 1H), 7.51 (dt, 1.0, 8.0, 1H), 7.47 (d, 8.5, 1H), 7.38 (dt, 1.0, 8.0, 1H), 7.15 (d, 2.0 1H), 7.07 (d, 8.0, 1H), 6.47 (dd, 2.0, 8.0, 1H), 4.52 (t, 7.5, 2H), 3.12 (t, 7.5, 2H); IR (neat) 3346, 3120, 2943, 1660, 1616, 1589, 1512, 1423, 1414, 1305, 739, 619; FAB-MS (NBA) *m/z* (rel. intensity) 438 ($M+H^+$−HBr, 3). Accurate mass for $C_{26}H_{20}N_3O_4$: calcd. 438.1454, obsd. 438.1440.

4.8. 4-(2-Chloroethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenol (5c)

A mixture of 2-(2-amino-4-hydroxyphenyl)ethyl chloride (**11**) (449 mg, 1.72 mmol), 5,6,7-trimethoxyindole-2-carboxylic acid (429.0 mg, 1.71 mmol), and EDCI (987.7 mg, 5.16 mmol) was dissolved in dry DMF (15 mL). The solution was stirred under a N_2 atmosphere at room temperature for 3 days. The DMF was removed using a Kugelrohr apparatus (60 °C, 1 mmHg), and the oily residue was partitioned between $CHCl_3$ (200 mL) and water (75 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated. Purification of the residue by column chromatography (1–5% MeOH/$CHCl_3$ gradient elution) afforded compound **5c** as an off-white solid (77.8 mg, 11% yield). Mp = 48–50 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.43$; ^1H NMR (500 MHz, $CDCl_3$) 9.71 (s br, 1H), 8.32 (s, 1H), 7.99 (d, 2.5, 1H), 7.18 (s br, 1H), 7.09 (d, 8.5, 1H), 6.95 (d, 2.0, 1H), 6.86 (s, 1H), 6.72 (dd, 2.5, 8.5, 1H), 4.11 (s, 3H), 3.96 (s, 3H), 3.91 (s, 3H), 3.83 (t, 6.0, 2H), 3.09 (t, 6.0, 2H); IR (KBr) 3362, 3062, 2958, 2916, 2854, 1649, 1540, 1503, 1457, 1410, 1374, 1306, 798, 756; FAB-MS (NBA) *m/z* (rel. intensity) 404 (M^+, 2), 405 ($M+H^+$, 3). Accurate mass for $C_{20}H_{22}N_2O_5{}^{35}Cl_2$: calcd. 405.1217, obsd. 405.1216. Anal. Calcd for $C_{20}H_{21}N_2O_5Cl$: C, 59.33; H, 5.23; N, 6.92. Found: C, 59.56; H, 5.33; N, 7.13.

4.8.1. An alternative synthesis of 5c. A mixture of 2-(4-benzyloxy-2-nitrophenyl)ethyl chloride (1.00 g, 3.44 mmol), platinum oxide (PtO_2) (200 mg) and chilled THF (50 mL) was degassed and purged with H_2 three times, and allowed to shake at 55 PSI for an hour. The solution was filtered over Celite, concentrated, and the residue was coevaporated twice with dry CH_2Cl_2 (5 mL each). The resulting 2-(2-amino-4-benzyloxyphenyl)ethyl chloride was dissolved in dry CH_2Cl_2 (50 mL), and added to a stirring suspension of 5,6,7-trimethoxyindole-2-carboxylic acid (961 mg, 3.83 mmol) and benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (2.00 g, 3.84 mmol) in dry CH_2Cl_2 (440 mL). The reaction mixture was flushed with N_2 and allowed to stir at room temperature for 10 min. Dry *N*,*N*-diisopropylethylamine (1.5 mL, 8.6 mmol) was added, and the solution was stirred overnight at room temperature. The solution was diluted with 100 mL of CH_2Cl_2 and washed once with water (100 mL), once with 10% HCl (1 M) (100 mL), once with saturated aqueous $NaHCO_3$ (100 mL) and once with saturated NaCl (100 mL). The organic layer was dried with anhydrous sodium sulfate, and concentrated. The product was purified using a 16:1 CH_2Cl_2/EtOAc silica gel column. The product (benzyl 4-(2-chloroethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenyl ether) was isolated as a light yellow foamy solid (1.13 g, 2.29 mmol, 66%). TLC (16:1 CH_2Cl_2/EtOAc) $R_f = 0.38$;

^{1}H NMR (500 MHz $CDCl_3$) 9.19 (s, 1H), 8.16 (s, 1H), 7.62 (d, 3.0, 1H), 7.45 (d, 7.5, 2H), 7.39 (t, 7.5, 2H), 7.33 (t, 7.5, 1H), 7.15 (d, 8.5, 1H), 6.90 (s, 1H), 6.85 (dd, 3.0, 8.5, 1H), 6.84 (s, 1H), 5.09 (s, 2H), 4.08 (s, 3H), 3.94 (s, 3H), 3.91 (s, 3H), 3.83 (t, 6.5, 2H), 3.10 (t, 6.5, 2H); IR (neat) 3296, 3073, 2970, 2924, 2850, 1751, 1709, 1644, 1579, 1537, 1499, 1467, 1411, 1304, 797, 732; EI-MS *m/z* (rel. intensity) 494 (M^+, 20), 458 (M^+-HCl, 100).

To a flask containing benzyl 4-(2-chloroethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenyl ether (2.17 g, 4.38 mmol) was added an aqueous solution of 25% NH_4HCO_2 (17.60 mL, 0.0697 mmol) and THF (100 mL). The solution was allowed to stir in an ice bath for 10 min and Pd/C 10% (450 mg) was added. The mixture was stirred under an atmosphere of H_2 at room temperature overnight. The reaction mixture was filtered over Celite and the filtrate was concentrated. The resulting yellowish oil was purified using a silica gel column, beginning with 0.5% MeOH/$CHCl_3$. The MeOH percentage was increased by 0.5% increments every 100 mL. The product was collected and concentrated on a concentrated to produce compound **5c** as a white foamy solid (880 mg, 2.17 mmol, 50%).

4.9. 4-(2-Bromoethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenol (5d)

A solution of compound **5c** (58 mg, 0.14 mmol) and lithium bromide (39 mg, 0.45 mmol) in dry DMF (2 mL) and was stirred under a N_2 atmosphere at 60 °C for 4 days. At that time, ethyl acetate (50 mL) was added to the reaction mixture. The solution was washed with water (2×50 mL) and dried (Na_2SO_4). The residue was purified by preparative TLC (0.5% MeOH/$CHCl_3$) to give the desired product **5d** as a yellowish film (10 mg, 16%). TLC (2% MeOH/$CHCl_3$) $R_f = 0.35$. 500 MHz ^{1}H NMR ($CDCl_3$) 9.42 (s, 1H), 8.23 (s, 1H), 7.66 (s, 1H), 7.64 (s br, 1H), 7.03 (d, 8.5, 1H), 6.87 (d, 2.0, 1H), 6.77 (s, 1H), 6.65 (dd, 2.0, 8.5, 1H), 4.04 (s, 3H), 3.88 (s, 3H), 3.85 (s, 3H), 3.77 (t, 6.5, 2H), 3.04 (t, 6.5, 2H); IR (Neat) 3317, 3054, 2957, 2931, 2852, 1712, 1651, 1616, 1537, 1506, 1462, 1410, 1370, 1339, 1305, 735, 699; EI-MS *m/z* (rel. intensity) 449 ($M+H^+$, <1), 368 ($M-HBr$, 65). Accurate mass for $C_{20}H_{20}N_2O_5$: calcd. 368.1372, obsd. 368.1373.

4.10. 4-(2-Iodoethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenol (5e)

A solution of compound **5c** (50 mg, 0.12 mmol) and sodium iodide (25 mg, 0.17 mmol) in dry acetone (4.0 mL, over Molecular sieves 3A) was heated to reflux under a Drierite drying tube overnight. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (50 mL), washed with water (10 mL), and then dried over anhydrous sodium sulfate. Removal of the solvent gave a brown oil, which was purified by preparative silica gel TLC (5% MeOH/$CHCl_3$). The desired product **5e** was obtained as an off-white film (3.5 mg, 6%). TLC (5% MeOH/$CHCl_3$) $R_f = 0.39$. 500 MHz ^{1}H NMR ($CDCl_3$) 9.48 (s, 1H), 7.97 (s, 1H), 7.67 (d, 2.0, 1H), 7.09 (d, 8.0, 1H), 6.96 (d, 2.0, 1H), 6.86 (s, 1H), 6.72 (dd, 2.0, 8.0, 1H), 6.28 (s br, 1H), 4.11 (s, 3H), 3.95 (s, 3H), 3.91 (s, 3H), 3.38 (t, 6.5, 2H), 3.22 (t, 6.5, 2H); IR (Neat) 3274, 3079, 2928, 2848, 1696, 1643, 1589, 1541, 1497, 1465, 1412, 1364; FAB-MS (NBA) *m/z* (rel. intensity) 497 ($M+H^+$, 2), 496 (M^+, 1). Accurate mass for $C_{20}H_{21}N_2O_5I$: calcd. 496.0495, obsd. 496.0501.

4.11. 4-(2-Chloroethyl)-3-(5-methoxyindole-2-carboxamino)phenol (5f)

The procedure was similar to that used for the synthesis of compound **5a**, except 5-methoxyindole-2 carboxylic acid (180 mg, 0.941 mmol) was used. Compound **5f** was obtained as an off-white residue (29 mg, 0.0842 mmol, 10%). Mp = 64–70 °C; TLC (1% MeOH/$CHCl_3$) $R_f = 0.42$; ^{1}H NMR (500 MHz, $CDCl_3$) 9.08 (s, 1H), 8.26 (s, 1H), 7.56 (d, 2.5, 1H), 7.36, (d, 9.0, 1H), 7.12 (d, 8.5, 1H), 7.10 (d, 2.0, 1H), 7.01 (dd, 3.0, 8.0, 1H), 6.95 (s, 1H), 6.73 (dd, 3.0, 8.0, 1H), 5.38 (s, 1H), 3.87 (s, 3H), 3.84 (t, 6.5, 2H), 3.11 (t, 6.5, 2H); IR (neat) 3290, 3050, 2957, 2916, 1653, 1618, 1534, 1508, 1472, 1451, 715; EI-MS *m/z* (rel. intensity) 344 (M^+, 20), 308 (M^+-HCl, 100); Accurate mass for $C_{18}H_{17}N_2O_3{}^{35}Cl$: calcd. 334.0928, obsd. 344.0914.

4.12. 4-(2-Chloroethyl)-3-(2-methoxycinnamoylamido)phenol (5g)

The procedure was similar to that used for the synthesis of compound **5a**, except 2-methoxycinnamic acid (168 mg, 0.942 mmol) was used. Agent **5k** was isolated as an off-white foamy solid (70.4 mg, 0.213 mmol, 25%). Mp = 70 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.61$; ^{1}H NMR (500 MHz, $CDCl_3$) 8.01 (d, 16.0, 1H), 7.53 (d, 6.5, 1H), 7.49 (s br, 1H), 7.36 (t, 7.0, 1H), 7.27 (s, 1H), 7.08 (d, 8.0, 1H), 6.98 (t, 7.5, 1H), 6.93 (d, 7.5, 1H), 6.69 (dd, 2.0, 6.5, 1H), 6.68 (d, 16.0, 1H), 5.35 (s br, 1H), 3.91 (s, 3H), 3.76 (t, 6.5, 2H), 3.05 (t, 6.5, 2H); IR (neat) 3253, 3071, 3011, 2925, 2839, 1648, 1614, 1541, 1485, 1459, 753; EI-MS *m/z* (rel. intensity) 331 (M^+, 2), 295 (M^+-HCl, 35); Accurate mass for $C_{18}H_{18}NO_3{}^{35}Cl$: calcd. 331.0975, obsd. 331.0970.

4.13. 4-(2-Chloroethyl)-3-(3-methoxycinnamoylamido)phenol (5h)

The procedure was similar to that used for the synthesis of compound **5a**, except 3-methoxycinnamic acid (168 mg, 0.942 mmol, 25%) was used. Product **5h** was obtained as a light yellow solid (45.9 mg; total yield 100.6 mg, 0.303 mmol, 35%). Mp = 220 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.64$; ^{1}H NMR (500 MHz, $CDCl_3$) 7.73 (d, 16.0, 1H), 7.48 (s br, 1H), 7.32 (t, 8.0, 1H), 7.31 (s, 1H), 7.22 (d, 8.0, 1H), 7.10 (d, 8.5, 1H), 7.08 (d, 2.0, 1H), 6.94 (dd, 2.0, 8.0, 1H), 6.70 (d, 7.0, 1H), 6.53 (d, 16.0, 1H), 4.90 (s, 1H), 3.85 (s, 3H), 3.77 (t, 6.5, 2H), 3.05 (t, 6.5, 2H); IR (nujol) 3356, 3252, 2727, 1709, 1640, 1597, 1541, 718; EI-MS *m/z* (rel. intensity) 331 (M^+, 5), 295 (M^+-HCl, 32).

4.14. 4-(2-Chloroethyl)-3-(4-methoxycinnamoylamido)phenol (5i)

The procedure was similar to that used for the synthesis of compound **5a**, except 4-methoxycinnamic acid (168 mg, 0.942 mmol) was used. The product **5i** was obtained as a light tan/yellow solid (23.3 mg; total yield 74.2 mg, 0.22 mmol, 26%). Mp = 210–213 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.60$; ^1H NMR (500 MHz, $CDCl_3$) 8.60 (s br, 1H), 8.08 (s br, 1H), 7.58 (d, 16.0, 1H), 7.48 (m, 2H), 6.97 (d, 8.0, 1H), 6.84 (m, 2H), 6.62 (d br, 7.0, 1H), 6.49 (d br, 16.0, 1H), 3.76 (s, 3H), 3.62 (t, 7.5, 2H), 2.95 (t, 7.5, 2H); IR (nujol) 3295, 3183, 3063, 1644, 1588, 1545, 1511, 696; EI-MS *m/z* (rel. intensity) 331 (M^+, 3), 295 (M^+, 29).

4.15. 4-(2-Chloroethyl)-3-(2,6-dimethoxy-5-pyridyl)-E-ethen-1-ylcarboxamidophenol (5j)

The procedure was similar to that used for the synthesis of compound **5a**, except 3-(2,6-dimethoxy-5-pyridyl)acrylic acid (245 mg, 1.09 mmol) was used. Compound **5j** was isolated as a light yellow solid (234 mg, 74%). Mp = 153–155 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.55$; ^1H NMR (500 MHz, $CDCl_3$) 7.80 (d, 15.5, 1H), 7.69 (d, 8.0, 1H), 7.53 (s br, 1H), 7.44 (s br, 1H), 7.08 (d, 8.5, 1H), 6.68 (dd, 1.0, 8.0, 1H), 6.61 (d, 15.5, 1H), 6.36 (d, 8.0, 1H), 5.91 (s br, 1H), 4.05 (s, 3H), 3.96 (s, 3H), 3.75 (t, 7.5, 2H), 3.04 (t, 7.5, 2H); IR (nujol) 3273, 3180, 1652, 1594, 1483, 665; FAB-MS *m/z* (rel. intensity) 363 ($M+H^+$, 7). Accurate mass for $C_{18}H_{20}N_2O_4Cl$: calcd. 363.1112: obsd. 363.1118.

4.16. 4-(2-Chloroethyl)-3-(6-methoxy-5-pyridyl)-E-ethen-1-ylcarboxamidophenol (5k)

The procedure was similar to that used for the synthesis of compound **5a**, except 3-(6-methoxy-5-pyridyl)acrylic acid (0.215 g, 1.10 mmol) was used. The product **5k** was obtained as an off-white solid. Mp 180 °C. TLC (10% MeOH/$CHCl_3$) $R_f = 0.50$. ^1H NMR (DMSO-d_6) 8.85 (s, 1H), 8.32 (s, 1H), 8.00 (s, 1H), 7.91 (s br, 1H), 7.88 (dd, 1.5, 8.0), 7.72 (d, 15.1, 1H), 7.00 (d, 8.0, 1H), 6.80 (d, 15.1, 1H), 6.52 (dd, 1.5, 8.0, 1H), 4.28 (br t, 2H), 3.97 (s, 3H), 3.15 (br t, 2H); IR (nujol) 3095, 1646, 1602, 1593, 1495, 747; FAB-MS (NBA) *m/z* (rel. intensity) 297 (M+H−HCl, 6). Electrospray MS *m/z* (rel. intensity) 333 ($M+H^+$, 5), 297 ($M+H^+$−HCl, 100).

4.17. 4-(2-Chloroethyl)-3-[[5-(4-bis-(2-chloroethyl)-amino)benzamido]indole-2-carboxamido]phenol (5m)

The intermediate **5l** was prepared using a procedure similar to that used for the synthesis of **5a**, except 5-nitroindole-2-carboxylic acid (372 mg, 1.81 mmol) was used. 4-(Chloroethyl)-3-(5-nitroindole-2-carboxamido)phenol **5l** was isolated as a yellow solid (70 mg, 14%). Mp 290–300 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.43$; ^1H NMR ($CDCl_3$, 500 MHz) 10.98 (s br, 1H), 8.87 (s br, 1H), 8.67 (d, 2.0, 1H), 8.59 (s, 1H), 8.17 (dd, 2.0, 9.0, 1H), 7.55 (d, 9.0, 1H), 7.20 (d, 2.5, 1H), 7.11 (d, 8.5, 1H), 6.77 (dd, 2.5, 8.5, 1H), 3.78 (t, 7.5, 2H), 3.08 (t, 7.5, 2H); IR (neat) 3391, 3261, 2929, 2858, 1658, 1616, 1545, 1462, 1379, 1326, 739; FAB-MS (NBA) *m/z* (rel. intensity) 360 ($M+H^+$, 4). Accurate mass for $C_{17}H_{15}N_3O_4{}^{35}Cl$: calcd. 360.0751, obsd. 360.0762.

A suspension of compound **5l** (250 mg, 0.69 mmol) and 10% Pd–C (187 mg) in chilled THF (20 mL) was hydrogenated at atmospheric pressure and room temperature overnight. The suspension was filtered over a pad of Celite, and the filtrate was concentrated to give a yellow solid. To this solid was added p-N,N-bis-(2-chloroethyl)aminobenzoic acid (219 mg, 0.84 mmol), EDCI (436 mg, 2.28 mmol) and freshly distilled and dry DMF (10 mL). The reaction mixture was stirred under a N_2 atmosphere and at room temperature for two days. The reaction mixture was concentrated using a Kugelrohr apparatus (0.1 mmHg, 60 °C), and the residue was partitioned in chloroform and water. The water layer was further extracted twice with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica gel column chromatography (0–10% methanol in chloroform). Compound **5m** was obtained as a white solid (96 mg, 24%). Mp 80–90 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.52$; ^1H NMR (DMSO-d_6, 500 MHz) 11.65 (d, 1.0, 1H), 9.84 (s, 1H), 9.49 (S, 1H), 8.12 (d, 2.0, 1H), 7.90 (d, 9.0, 2H), 7.51 (dd, 2.0, 8.5, 1H), 7.39 (d, 9.0, 1H), 7.30 (d, 1.0, 1H), 7.17 (d, 8.5, 1H), 6.85 (d, 9.0, 2H), 6.78 (d, 2.5, 1H), 6.68 (dd, 2.5, 8.5, 1H), 3.78 (m, 8H), 3.75 (t, 7.5, 2H), 2.97 (t, 7.5, 2H); IR (neat) 3390, 3019, 2965, 2921, 2856, 1643, 1600, 1539, 1513, 1464, 1447, 1256, 1229, 1180, 1153, 1093, 1044, 1017, 870, 799; UV–vis (ethanol) 220 ($\varepsilon = 1.1 \times 10^6\,M^{-1}cm^{-1}$), 310 ($\varepsilon = 3.8 \times 10^4\,M^{-1}cm^{-1}$); FAB-MS (NBA) *m/z* (rel. intensity) 573 ($M+H^+$, 1). Accurate mass for $C_{28}H_{28}N_4O_3{}^{35}Cl_3$: calcd. 573.1227, obsd. 573.1232.

4.18. 4-(2-Chloroethyl)-3-[2-(4-*N*,*N*-(diethyl)aminophenyl)benzimidazole-6-carboxamido]phenol (5n)

The procedure was similar to that used for the synthesis of compound **5a**, except 2-(4-(*N*,*N*-diethyl)aminophenyl)benzimidazole-6-carboxylic acid (0.350 g, 1.13 mmol) was used. Compound **5n** was isolated as a white solid (70 mg, 13% yield). Mp = 223 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.43$; ^1H NMR (500 MHz, $CDCl_3$ and 2 drops of DMSO-d_6) d 8.78 (s br, 1H), 8.65 (s br, 1H), 8.22 (s, 1H), 8.14 (d, 9.0, 2H), 7.83 (d, 8.5, 1H), 6.76 (d, 9.0, 2H), 6.73 (dd, 2.0, 8.0, 1H), 3.77 (t, 6.5, 2H), 3.45 (q, 7.5, 4H), 3.07 (t, 7.5, 2H), 1.23 (t, 7.5, 6H); IR (nujol) 3408, 1607, 1493, 1379, 1306, 798, 663; FAB-MS (NBA) *m/z* (rel. intensity) 463 ($M+H^+$, 5). Accurate mass for $C_{26}H_{28}N_4O_2{}^{35}Cl_2$: calcd. 463.1901, obsd. 463.1901.

4.19. *N*-(2-(2-Bromoethyl)-4-hydroxyphenyl)-1-methyl-4-(1-methyl-4-butanamido pyrrole-2-carboxamido)pyrrole-2-carboxamide (5o)

The procedure was similar to that used for the synthesis of compound **5b**, except *N*-methyl-4-(*N*-methyl-4-butanamidopyrrole-2-carboxamide)-pyrrole-2-carboxylic-acid (830 mg, 2.50 mmol) was used. The product **5o** was obtained as an off-white foamy solid (230 mg,

0.462 mmol, 40%). Mp 138–150 °C; TLC (10% MeOH/$CHCl_3$) $R_f = 0.13$; ^1H NMR ($CDCl_3$ in 1 drop DMSO-d_6, 500 MHz) 9.85 (s, 1H), 9.74 (s, 1H), 9.22 (s, 1H), 7.39 (br s, 1H), 7.33 (d, 2.0, 1H), 7.14 (d, 2.0, 1H), 7.02 (d, 8.0, 1H), 6.87 (d, 2.0, 1H), 6.69 (d, 2.0, 1H), 6.41 (dd, 2.5, 8.0, 1H), 4.22 (t, 8.5, 2H), 3.83 (s, 3H), 3.73 (s, 3H), 2.99 (t, 8.0, 2H), 2.21 (t, 7.0, 2H), 1.59 (sextet, 7.5, 2H), 0.895 (t, 7.5, 3H); IR (Nujol) 3368, 3270, 3125, 1632, 1600, 728; FAB-MS (NBA) *m/z* (rel. intensity) 450 ($M+H^+-HBr$, 10), 449 ($450-H^+$, 10). Accurate mass (FAB-MS) for $C_{24}H_{28}N_5O_4$: calcd. 450.2141, obsd. 450.2147, for $C_{24}H_{27}N_5O_4$: calcd. 449.2063, obsd. 449.2082.

4.20. *O*-4-(2-Chloroethyl)-3-(5,6,7-trimethoxyindole-2-carboxamino)phenyl-N-methylpiperazinyl carbamate (5p)

A solution of compound **5c** (70 mg, 0.171 mmol) in distilled dry THF (1.5 mL) and dry CH_2Cl_2 (30 mL) was chilled in an ice bath. Dry triethylamine (0.048 mL, 0.342 mmol) and *p*-nitrophenyl chloroformate (86 mg, 0.428 mmol, dissolved in 5 mL of CH_2Cl_2) were added. The solution was allowed to stir at 0 °C under a N_2 atmosphere for three hours. 4-Methylpiperazine (0.057 mL, 0.51 mmol) was added to the solution and the reaction mixture was allowed to stir under N_2 for 5 h. At that time, the solvent was removed under reduced pressure and the resulting yellow residue was dissolved in $CHCl_3$ and purified on a silica gel column using $CHCl_3$/MeOH (0–2.5%) as the solvent. Carbamate **5p** was isolated as a white solid (78.3 mg, 0.147 mmol, 86%). Mp = 84 °C; TLC (2.5% MeOH/$CHCl_3$) $R_f = 0.25$. 500 MHz ^1H NMR ($CDCl_3$) 9.09 (s, 1H), 8.12 (s, 1H), 7.65 (d, 2.5, 1H), 7.16 (d, 8.0, 1H), 6.94 (dd, 2.5, 8.0, 1H), 6.83 (d, 2.0, 1H), 6.78 (s, 1H), 4.02 (s, 3H), 3.87 (s, 3H), 3.84 (s, 3H), 3.77 (t, 6.5, 2H), 3.67 (m, 2H), 3.57 (m, 2H), 3.08 (t, 6.5, 2H), 2.45 (m, 4H), 2.32 (s, 3H); IR (Neat) 3299, 3140, 3007, 2936, 2794, 1719, 1661, 1590, 1532, 1497, 1461, 1426, 1368, 752; FAB-MS *m/z* (rel. intensity) 531 ($M+H^+$, 10). Accurate mass for $C_{26}H_{31}N_4O_6{}^{35}Cl$: 530.1932, obsd. 530.1907.

4.21. Cytotoxicity studies on human chronic myeloid leukemia K562 cells

The compounds 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} M 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 K562 human chronic myeloid leukemia cells were maintained in RPM1 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 and were incubated with a specified dose of drug continuously for 4 days at 37 °C in the dark. The incubation was terminated by centrifugation (5 min, 300 g) and the cells were washed once with drug-free medium. Following the appropriate drug treatment, the cells were transferred to 96-well microtitre plates, 10^4 cells per well, 8 wells per sample. Plates were then kept in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. The assay is based on the ability of viable cells to reduce a yellow soluble tetrazolium salt, 3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) to an insoluble purple formazan precipitate. Following incubation of the plates for 4 days (to allow control cells to increase in number by 10 fold) 20 μL of a 5 mg/mL solution of MTT in phosphate buffered saline was added to each well and the plates further incubated for 5 h. The plates were then centrifuged for 5 min at 300 g and the bulk of the medium pipetted from the cell pellet leaving 10–20 μL per well. DMSO (200 μL) was added to each well and the samples agitated to ensure complete mixing. The optical density was then read at a wavelength of 550 nm on a Titertek Multiscan ELISA plate reader, and the dose–response curve was constructed. For each curve, an IC_{50} value was read as the dose required to reduce the final optical density to 50% of the control value.

4.22. Cytotoxicity studies on human colon, prostate, and breast cancer cells

The human colon adenocarcinoma cell line, LS 174T, was obtained from the European Collection of Animal Cell Cultures, CAMR, Porton Down, UK. LS174T cells were routinely cultured in Eagle's MEM supplemented with 1% nonessential amino acids, 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin at 37 °C in a 5% CO_2, 95% air-humidified incubator. Cells were seeded in 96-well microtiter plates at densities of 1000 cells/well. The optimal seeding density was determined to ensure exponential growth in control wells throughout the experimental period and to obtain a linear relationship between absorbance at 492 nm and cell number when analyzed by the sulforhodamine B (SRB) assay. Twenty-four hours after seeding the cells were incubated continuously for 3 days with the drugs (concentration range 0.01–500 μM). The cells were washed once and then incubated in drug-free medium for 6 days. At the end of the incubation cell growth was determined by the SRB assay, which quantifies viable cells by measuring their total cellular protein content. The IC_{50} (concentration of drug giving 50% survival in vitro), was calculated from the dose–response curve obtained by plotting the percentage of inhibition versus the log molar drug concentration. Similar studies were conducted against the growth of PC3 (human prostate cancer cells) and MCF-7 (human breast cancer cells).

4.23. Cytotoxicity studies on murine L1210 leukemia and P815 mastocytoma cells

The P815 and L1210 cell lines were obtained from American Type Tissue Culture Collection (ATCC). The cell lines were grown in Delbecco's Modified Eagle Medium (DMEM, Atlanta Biologicals) 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 resuspended in fresh DMEM at a concentration of 8×10^5 cells/mL. This cell suspension (100 μL) was added to 96 well flat-bottom cell culture plates. At this concentration, 80,000 cells were seeded in each well. Drug solutions (dissolved in DMSO and diluted in DMEM) were added to each well (5 μL/well) resulting in final concentrations ranging from 1×10^{-4} to 1×10^{-12} M. 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 of this 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 acid isopropanol solution (16 μL 12.1 N HCl in 5 mL isopropanol) was added to each well. The contents of the well 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 read on a Dynatech Plate Reader, utilizing Dynex Revelation 3.2 software, with a test wavelength 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).

4.24. NCI in vitro cytotoxicity screen

Compounds **5a** and **5c** were also tested against a panel of 60 human cancer cell lines at the National Cancer Institute, Bethesda, MD.[31] The cytotoxicity studies were conducted using a 48 h exposure protocol, and a sulforhodamine Bassay. Dose–response curves were used to generate the GI_{50} (concentration of drug needed to inhibit the growth by 50%), TGI (total growth inhibition), and LC_{50} (concentration needed to kill 50% of cells).

4.25. Taq polymerase stop assay on compounds 5a, b, l, m, o using pBR322 plasmid DNA

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 vol of 95% ethanol and washed with 70% ethanol. The resulting pellet was dried by lyophilization.

Prior to drug/DNA incubation, plasmid pBR322 DNA was linearized with *Pvu*II restriction enzyme to provide a stop for the Taq downstream from the primer. The oligodeoxynucleotide primers were 5′-end labeled prior to amplification using T4 polynucleotide kinase and [γ^{32}P]-ATP (5000 Ci/mmol, Amersham, UK). The labeled primers were purified by elution through Bio-Rad spin columns. The primer 5′-GCATTGGTAACTGTCAGACC-3′ binds in the sequence 3303–3284 and was used to examine the top strand. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 50 pmol of labeled primer, 250 μM of each dNTP, 1U 'Red Hot' Taq polymerase, 20 mM $(NH_4)_2SO_4$. 75 mM Tris–HCl, pH 9.0, 0.01% Tween, 2.5 mM $MgCl_2$, and 0.01% gelatin. After an initial denaturation at 94 °C for 4 min, the cycling conditions were as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for a total of 30 cycles. After being amplified, the samples were ethanol precipitated and washed with 70% ethanol. Samples were dissolved in formamide loading dye, heated for 2 min at 90 °C, cooled on ice, and electrophoresed at 2500–3000 V for 3 h on a 80 cm × 20 cm × 0.4 mm 6% acrylamide denaturing sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham, UK). Densitometry was carried out on a Bio-Rad GS-670 imaging densitometer.

4.26. Taq polymerase stop assay and thermal cleavage analysis for compound 5a using pUC18 plasmid DNA

For the Taq DNA polymerase stop studies, the procedure was similar to that described above, except plasmid pUC18 was linearized with *Hin*dIII, which cuts at only one site in the plasmid (position 399) and provides a stop for the Taq DNA polymerase downstream from the primer. The synthetic primer 5′-CTCACTCAAAGGCGGTAATAC-3′ binds to the complementary (bottom) strand at position 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).

For the thermal cleavage analysis, 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 resuspended 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. Samples were chilled, precipitated, and dried.

4.27. Flow cytometry studies

The P815 cell line was used for this experimental procedure. The cells were incubated with drugs in a final concentration equaling 10 times their IC_{50} values. The $10 \times IC_{50}$ concentrations for cisplatin and compound **5c** were 1000 and 56 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 resuspended 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). 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 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.

4.28. Hematopoietic progenitor assay

For direct incubation of the drug solutions with bone marrow cells, the concentration of the drug solution was chosen to be 0.0084 μM (the IC_{50} value for the *seco*-CBI-TMI compound **4** 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 6-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.

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