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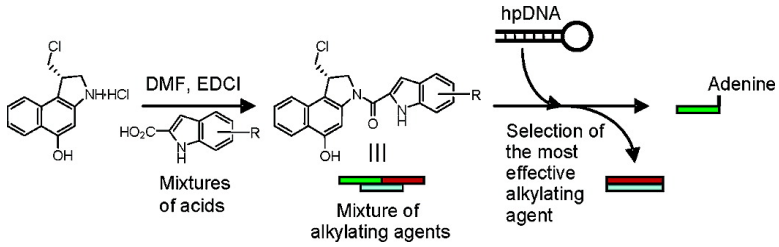

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A Powerful Selection Assay for Mixture Libraries of DNA Alkylating Agents

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CC-1065[1] and the duocarmycins (**1** and **2**)[2,3] are among the most potent antitumor antibiotics discovered to date (Figure 1). The biological activity of the natural products originates from their sequence-selective alkylation of duplex DNA,[4−8] that has been shown to proceed by adenine N3 addition to the least substituted cyclopropane carbon within the alkylation subunit. The DNA-binding subunit contributes to the delivery of the alkylation subunit to minor groove AT-rich sequences and plays an integral role in catalysis of the DNA alkylation reaction,[9] thus enhancing both the alkylation selectivity and efficiency.

A recent study on the effects of the DNA-binding subunits revealed a preference for the first attached DNA-binding subunit as tricyclic (e.g., dihydropyrroloindole) > bicyclic (e.g., indole) > monocyclic (e.g., pyrrole) heteroaromatics using the (+)-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI)[10] alkylation subunit and identified compounds more potent than the natural products (Figure 1). More significantly, a series of CBI analogues bearing a single indole (bicyclic) DNA-binding subunit have been prepared with substituent variations on the indole 4−7 positions to establish their effects, and several simplified derivatives surpassed the properties of the natural products.[12] The results of such studies provided important principles for the further development of simplified compounds with improved biological properties and were conducted enlisting the synthesis of libraries of individual compounds.

Herein, we disclose a powerful selection assay to identify the most effective DNA-alkylating agents directly from combinatorial mixture libraries amenable to a more extensive exploration of such analogues (Figure 2). It enables the direct identification of the most effective DNA alkylating agents from synthetically more accessible mixture libraries[13] by competition for a stoichiometry limiting deoxyribonucleotide containing a single alkylation site. Following a competitive mixture alkylation of the DNA (24−36 h, 0.1 M Tris buffer, pH 7.2), unreacted compound is removed by EtOAc extraction, and the buffer solution containing the alkylated DNA is warmed at 100 °C (30 min) to induce a stoichiometric thermal depurination with the release of the adenine adduct (e.g., **5**).[14] Organic extraction (EtOAc) of the buffer quantitatively[14] and cleanly partitions the released adenine adducts into the organic phase (uncontaminated by starting material or DNA) from which they can be separated (reverse phase HPLC), quantitated (ELSD), and identified (ESI-MS).

Initially, three CBI-indole derivatives (Figure 2a) having distinct alkylation rates and efficiencies[12] (5-SO_2Me (2.5) > 5,6,7-OMe (TMI) (1.0) > 5-OH) and corresponding cytotoxic potencies[12] (L1210 IC_{50} = 3, 30, and 70 pM, respectively) were examined. An equimolar mixture of the three compounds (1.0 equiv total) was treated with hpDNA (0.3 equiv for competition and 1.5 equiv for noncompetition control). The competition assay yielded a ratio of the adenylated products of 1.0:0.6:0.3 (5-SO_2Me:TMI:5-OH) favoring the more effective compound (Figure 3a), while the

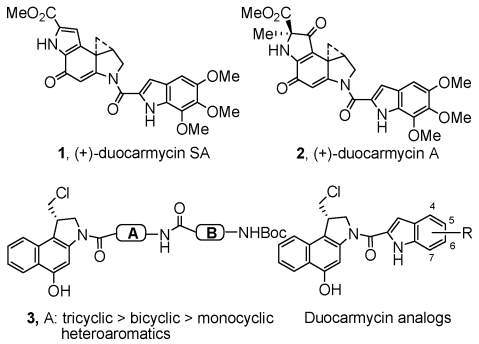

Figure 1. Structures of the duocarmycins and analogues.

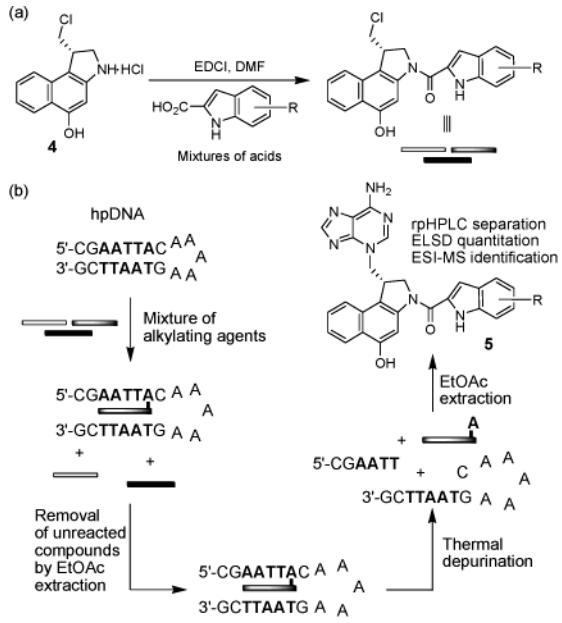

Figure 2. (a) Preparation of a mixture library of DNA-alkylating agents and (b) selection and identification of the most effective compounds from the mixture library.

noncompetitive control afforded a ratio of 1.0:0.9:0.6 after 24 h. The product ratios were obtained using an evaporative light-scattering detector (ELSD, calibrated using an authentic adduct) following HPLC separation (20−65% A over 30 min; A: CH_3-CN, B: 0.1% aqueous formic acid; C-18 rpHPLC).[15] When this analysis was conducted with the less effective 7-OMe (IC_{50} = 300 pM) or 5-H (IC_{50} = 2700 pM) derivatives in place of 5-OH, no adenine adduct was observed for 7-OMe or 5-H under the competition assay conditions, resulting in only two products in the ratio of 1.0:0.5 (5-SO_2Me:TMI) that mirror the reported relative rates of DNA alkylation (2.5:1).[12]

The size of the library was expanded to a 10-compound mixture (5-SO_2Me, 5-NHCOPr, TMI, 5-OBu, 5-SEt, 5-OCF_3, 5-vinyl, 5-N_3,

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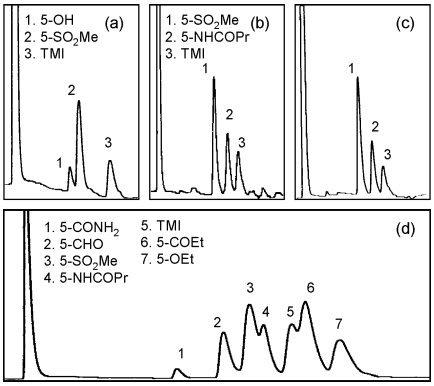

Figure 3. HPLC-ELSD traces from selection assays: (a) three compounds with 0.3 equiv of hpDNA; (b) an authentic mixture of 10 alkylating agents with 0.1 equiv hpDNA; (c) a synthetic library of 10 compounds obtained from corresponding carboxylic acid mixtures with 0.1 equiv of hpDNA; (d) a synthetic library of 45 compounds with limiting hpDNA.

5-Br, and 5-H) displaying a range of IC_{50} values (3−2700 pM).[16] This mixture was chosen such that each product had a different molecular weight and whose members displayed properties distributed over a wide range. Only the three most potent compounds (5-SO_2Me, 5-NHCOPr, and TMI) provided adenine adducts in the presence of 0.1 equiv of hpDNA, providing a product ratio of 1.0:0.7:0.6 (Figure 3b). Notably, all members of this mixture can alkylate DNA effectively, yet the assay selects and accurately rank-orders the more effective members. To fully implement this selection assay, a mixture library of the same 10 compounds was generated from an equimolar mixture of indole-2-carboxylic acid derivatives and *seco*-CBI (**4**) with purification by a simple acid−base extraction.[11,17] Thus, an equimolar mixture of the 10 indole-2-carboxylic acids (0.1 equiv each) was treated with EDCI (3.0 equiv) and excess of *seco*-CBI[18] (1.25 equiv) for an extended reaction time (8 h) to ensure complete conversion to each CBI derivative. The pure mixture library of 10 compounds was obtained after simple acid−base extractive workup to remove unreacted **4** and any residual carboxylic acid. The selection assay was performed with this synthetic mixture using 0.25 equiv of hpDNA. Consistent with expectations, only the same three adenylated products (5-SO_2Me, 5-NHCOPr, and TMI) were observed in a relative ratio of 1.0:0.7:0.6, indistinguishable from that of the authentic mixture (Figure 3c).

The selection assay was conducted on an even larger library of 45 alkylating agents whose cytotoxic properties had been established with the individual compounds.[12] The library was prepared from a mixture of the 45 indole-2-carboxylic acids as illustrated in Figure 2. A total of seven compounds[16,19] were observed to alkylate the hpDNA under the competition assay conditions with limiting DNA (1/22.5 equiv) (Figure 3d). Six of them proved to be the expected most effective alkylating derivatives (L1210 $IC_{50} \leq 30$ pM).[16,19] Only one, 5-CHO ($IC_{50} = 100$ pM), provided an unexpected competitive DNA alkylation capability, and it proved to be the only identified compound with an IC_{50} value greater than 30 pM. This was not investigated in detail since the candidate represents an electrophilic aldehyde. Although many explanations may account for this behavior, it may simply represent an instance where other features impacting the cellular activity of a derivative (e.g., metabolic stability) diminish its potency relative to its activity against the isolated target biomolecule. As such and to the extent that relative DNA alkylation rates and efficiencies correlate with functional cytotoxic activity, the assay successfully selected the expected most effective library members even in mixtures as large as 45.

Thus, a simple selection assay that permits the separation (HPLC), quantitation (ELSD), and identification (ESI-MS) of thermally released adenine adducts was developed that can establish the most effective DNA alkylating agents from a mixture screened against any target sequence of choice. The assay is fast, effective, and applicable to assessing mixture libraries of duocarmycin derivatives that can be generated in a single step from a mixture of indole-2-carboxylic acids, enlisting a simple acid−base extraction protocol for mixture purification. As such, this assay should permit the more extensive examination of unexplored structural motifs by simultaneously scanning a range of samples. Moreover, it serves as a general protocol applicable to other classes of DNA alkylating agents that provide thermally labile adducts (adenine-N3, guanine-N3, adenine-N6, and guanine-N6 alkylation).

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Supporting Information Available: Preparation of Ad-CBI-TMI and Ad-CBI-indole-5-SO_2Me, ELSD calibration curves, preparation of the libraries, assay conditions and procedures, and the indole-2-carboxylic acids used in the 45-compound library assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Experimental factors influencing and conditions for ensuring a stoichiometric depurination reaction and quantitative isolation of the resulting adenine adducts have been detailed, see: Boger, D. L.; Johnson, D. S.; Yun, W. *J. Am. Chem. Soc.* **1994**, *116*, 1635.
(15) The adenine adduct of CBI-TMI was used as the calibration standard for quantification of the product ratio. Each data point of the calibration curve demonstrated deviations of 4−11% from the calibration line. Ad-CBI-indole-5-SO_2Me was compared to the Ad-CBI-TMI calibration curve at three different concentrations with percent error ≤10%. Details are described in Supporting Information.
(16) Typically, the in vitro cytotoxic potencies follow the relative efficiencies of DNA alkylation. In this series, the cytotoxic activity was established for each individual compound, whereas only 5-SO_2Me, TMI, and 5-H were previously examined in DNA alkylation assays.[12]
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(19) The identity of compounds with the same molecular weight but with different structures was determined by HPLC correlation of retention times with the products obtained by treating the individual candidate compounds with hpDNA.

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