Notes

Use of COMPARE Analysis to Discover New Natural Product Drugs: Isolation of Camptothecin and 9-Methoxycamptothecin from a New Source

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Analysis of cytotoxicity data of extracts from the National Cancer Institute's Active Repository by the COMPARE protocol was carried out using camptothecin as a reference point. Extracts identified by this process were further characterized by a selective yeast bioassay for inhibitors of topoisomerase I and by a biochemical assay for compounds that stabilize the topoisomerase I−DNA covalent binary complex. Five of the extracts were positive in the yeast bioassay, and eight extracts showed activity on the assay that monitors stabilization of the topoisomerase I−DNA complex. Four of the latter extracts were inactive in the yeast bioassay, and thus would not have been identified as hits without the COMPARE preselection process. One of the extracts, from *Pyrenacantha klaineana*, was selected for detailed investigation, and fractionation of this extract yielded camptothecin and 9-methoxycamptothecin as the bioactive constituents.

The search for new anticancer drugs from nature continues to be a fruitful activity, as evidenced by the recent successes of natural products as pharmaceutical agents. Thus, Shu lists 32 natural products or natural product analogues that have entered clinical use or clinical trials over the past few years,[1] while the presentations of Cragg and his colleagues[2] and of Nisbet and his colleagues[3] also testify to the value of natural products in the discovery of new drugs. If this approach is to remain competitive with other approaches, such as combinatorial chemistry, however, it is essential that it be conducted as efficiently as possible. In particular, the mere isolation of a new cytotoxic agent is unlikely to lead to a drug unless and until its mechanism of action is known, because this is an essential piece of information for drug development. As a historical example of this, the major anticancer drug paclitaxel (Taxol) did not arouse much enthusiasm until its novel mechanism of action was discovered,[4] even though it was already in preclinical development at the National Cancer Institute. Because of this need to know the mechanism of action of a newly isolated agent, the collaborative natural product drug discovery program at the University of Virginia, Virginia Polytechnic Institute and State University, and SmithKline Beecham Pharmaceuticals has used a mechanism-based approach from its inception. A new approach to drug discovery has recently been developed that combines mechanism-based assays with database mining to discover new inhibitors of topoisomerase I.

The assays used for drug discovery target agents that cause damage to DNA, either directly or indirectly, by inhibiting religation of enzyme−DNA covalent binary complexes formed with topoisomerases I or II. This mechanism was selected because DNA-interactive agents form by far the largest single class of clinically used anticancer drugs, with 27 of the 64 clinically used drugs listed in a recent review being classified as DNA-interactive agents.[5] Because DNA exists in the cell primarily in a supercoiled form and because most of its functions require unwinding, these functions also require the services of enzymes to allow this unwinding. The two primary enzymes for this task are known as topoisomerase I and topoisomerase II, which cleave and religate one and two strands of DNA, respectively. Inhibition or interference of the religation steps of either of these enzymes in mammalian cells by the so-called DNA topoisomerase poisons[6] leads to cell death, and thus topoisomerase-interfering compounds (poisons) can serve as anticancer agents. At present the only clinically used drugs that interfere with topoisomerase I are derivatives of the natural product camptothecin[7] (**1**), which is known to be an inhibitor of topoisomerase I.[8] Although camptothecin is too insoluble to be used directly, its analogues, topotecan and irinotecan, are in clinical use, and scientists are actively searching for additional inhibitors of this enzyme.[9] Because inhibitors of topoisomerase I do not occur abundantly in nature, the discovery of new inhibitors of this type is an important research goal.

The recent initiative by the NCI to open the NCI Repository of natural product extracts to qualified outside investigators has allowed the pursuit of a novel approach to the identification of potential inhibitors of topoisomerase I. The NCI Active Repository consists of extracts that showed significant activity in the NCI 60-cell line screen,[10] and the cytotoxicity data for these extracts is thus available. This has opened up the possibility of using these data in a novel way, by mining the database for extracts with

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Table 1. Extracts That Selectively Inhibit the Growth of Topoisomerase I-Containing Yeasts or That Stabilize the Topoisomerase I−DNA Covalent Binary Complex

extract no.	vector (IC_{50} μg/mL)	RS321N phTop1 Gal (IC_{50} μg/mL)	RS321N phTop1 Glu (IC_{50} μg/mL)	pRAD52 Gal (IC_{50} μg/mL)	pRAD52 Glu (IC_{50} μg/mL)	WT erg6 Glu (IC_{50} μg/mL)	top1 rad52 Glu (IC_{50} μg/mL)	rad52 Glu (IC_{50} μg/mL)	stabilization of the DNA complex
1	<0.78	<0.78	<0.78	<0.78	<0.78	<0.78	<0.78	0.90	weak
2	>100	13.5	>100	>100	>100	>100	>100	63.1	strong
3	1.9	1.1	2.3	1.1	1.8	2.3	1.6	6.5	strong
4	1.0	0.92	2.2	1.1	1.1	1.3	1.1	2.5	strong
5	>100	2.2	>100	39.1	>100	>100	>100	21.1	strong
6	>100	22.0	>100	>100	>100	>100	47.6	70.4	strong
7	>100	70.2	>100	>100	67.9	73.0	73.0	>100	none
8	>100	3.3	>100	>100	>100	>100	>100	10.1	strong
9	>100	>100	>100	>100	>100	>100	>100	>100	weak
N080725	>100	0.70	>100	>100	>100	32.7	>100	5.4	NT[a]
camptothecin	0.16	0.014	>100	0.18	>100	>100	>100	0.06	strong

[a] Not tested.

similar cytotoxicity profiles to known chemotherapeutic agents. Herein is reported the use of the NCI database and the COMPARE algorithm to discover extracts with cytotoxicity profiles similar to known topoisomerase I inhibitors.

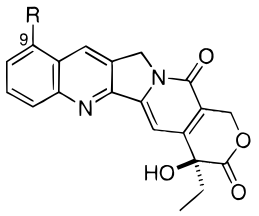
The COMPARE algorithm was developed at the NCI,[11] and its application to drug discovery has been reviewed.[12] In brief, it correlates the similarity of the mean graph cytotoxicity profile of a known anticancer agent with that of a compound or extract with an unknown mechanism of action. Compounds (or extracts containing compounds) with the same mechanism of action as the "seed" compound will have mean graphs similar to that of the "seed" and will thus show up as "hits" in the COMPARE analysis. The approach has been used productively at NCI to detect novel anti-tubulin agents[13] and to discover novel topoisomerase II agents in a database of synthetic compounds.[12] It has not, however, been used to detect extracts showing COMPARE profiles characteristic of topoisomerase I inhibitors.

The COMPARE program was run against the NCI Active Repository Database, using camptothecin as a "seed" against which the profiles of all the other extracts were compared. This approach identified 25 extracts that had COMPARE profiles similar to that of camptothecin, and these extracts are thus potential leads for the discovery of new topoisomerase I inhibitors. The extracts identified by the COMPARE algorithm were then further investigated in two different assay systems. One assay system was a yeast-based assay for topoisomerase inhibitors, while the other was a direct biochemical assay for agents that stabilize the covalent binary complex formed between DNA and human topoisomerase I. The yeast assay was carried out with a mutant strain of *Saccharomyces cerevisiae* deficient in RAD52 and yeast topoisomerase I, but harboring a plasmid containing the gene for human topoisomerase I under the control of the galactose promoter. The RAD52 repair pathway is associated with repair of double-strand DNA breaks and also of meiotic recombination.[14] When this yeast strain, designated RS321Nph-TOP 1, is grown on glucose in the absence of galactose, expression of the topo 1 gene is strongly suppressed, and, thus, compounds that produce DNA damage through interference of the topo 1 religation (topo 1 poisons) will have little effect on cell growth. When the same strain is grown in the presence of galactose, however, the gene for human topo 1 is expressed, and agents that interfere with the action of this enzyme will generate DNA damage and consequently cell growth inhibition as the RAD52 deficiency prevents the proper repair of such damage.[15] The assay has been validated with known topo 1 inhibitors such as camptothecin and topotecan; the latter has an IC_{50} value of 9.9 μg/mL against RS321Nph-TOP 1 grown on galactose, but >100 μg/mL against the same strain grown on glucose. Because there is a possibility that growth inhibition on galactose is due to inhibition of enzymes involved in galactose metabolism, testing is also carried out on strain RS321NYCp50 grown in galactose. This strain is Δrad52, Δtop1 and carries vector plasmid; an extract must not significantly inhibit the growth of this strain to be considered a true positive hit. A second yeast assay is also used in which yeast strains lacking the gene for the rad52 DNA repair pathway (designated RS322 or *rad52*) and strains that additionally lack the gene for yeast topoisomerase I (designated RS321 or *rad52.top1*) are used. A compound that selectively inhibits the RS322 strain but not the RS321 strain is also a putative inhibitor of yeast topoisomerase I. Fifteen of the extracts were found to be active in one or more of the relevant yeast strains, with five extracts showing selective activity against RS321Nph-TOP 1 grown on galactose (Table 1).

The 25 extracts were then examined in an assay that measures stabilization of the DNA-topoisomerase I covalent binary complex, which is an intermediate in the DNA unwinding process. Six of the extracts were strongly active in stabilizing the covalent binary complex between topoisomerase I and DNA, and two additional extracts were weakly active (Table 1). Interestingly, four of these active extracts (two strongly active and two weakly active) were inactive (or not selectively active) in the yeast assays, indicating that they would have been missed by a simple screening approach. This result, thus, indicates the value of the COMPARE analysis in preselecting extracts for detailed biochemical analysis.

To validate the use of the yeast assay, one of the extracts was selected for initial detailed investigation. Extract N080725 was identified as a yeast-selective agent prior to our COMPARE analysis; it was prepared from stems of *Pyrenacantha klaineana* Pierre ex Exell & Mendoca (Icacinaceae). Fractionation of this extract as described in the Experimental Section, using the RS322 (*rad52*) and RS321 (*rad52.top1*) strains of *S. cerevisiae* as the fractionation assay, led to the isolation of two bioactive compounds identified as camptothecin (**1**) and 9-methoxycamptothecin (**2**)[16] by NMR and MS methods. Because complete NMR spectral data were not available in the literature for compound **2**, all of its signals were assigned by ^1H and ^{13}C NMR, HMQC, and HMBC spectra.

The isolation of camptothecin and 9-methoxycamptothecin from *P. klaineana* is not unexpected, because, although this plant belongs to a different family (Icacinaceae) from that of *Camptotheca acuminata* (Nyssaceae), various camptothecin analogues have been isolated from *Nothapodytes foetida* (formerly *Mappia foetida*) (Icacinaceae).[17]



1 R = H
2 R = OCH_3

These results confirm the validity and usefulness of this approach, which complements the random screening approach to drug discovery. Another example of its value is its use to discover functional analogues of bleomycin.[18] Studies are currently in hand on the isolation of additional topoisomerase inhibitors from the other extracts identified as having the same COMPARE pattern as camptothecin.

Experimental Section

General Experimental Procedures. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded in $CDCl_3$ on a Varian Unity 400 NMR instrument at 399.951 MHz for ^1H and 100.578 MHz for ^{13}C, using standard Varian pulse sequences programs. UV spectra were measured on a Shimadzu UV 1201 UV spectrophotometer.

Yeast Bioassay. The yeast topoisomerase I mutation used in the assay strains is as previously described.[15] For assaying inhibitors of human topoisomerase I, strain RS321N (*MAT*a *ada2*-1 *can1*-100 *erg6*-1 *his3*-11,15 *leu2*-3,112 *trp1*-1 *ura3*-1 *rad52*-8::TRP1 *top1*-8::LEU2), which carried a defective TOP1 allel in the chromosome, was transformed to URA+ with a single copy plasmid containing the gene for human DNA topoisomerase I driven by the yeast GAL1 promoter.[19] As a control, the same yeast strain was transformed with the Ycp50-based vector plasmid[20] to result in strain RS321NYCp50. For assaying compounds that affect the yeast topoisomerase I, an additional strain RS322N (*MAT*a *ada2*-1 *can1*-100 erg6-1 *his3*-11,15 *leu2*-3,112 *trp1*-1 *ura3*-1 *rad52*-8::TRP1) was used to pair with RS321N.

The assay was carried out in 96-well microtiter plates. Yeast cells were diluted to an OD_{600} of 0.01 in the synthetic minimal medium, either with glucose or galactose as carbon sources.[21] A volume of 100 μL of the diluted culture was applied to wells of a sterile microtiter plate, testing compounds were dissolved in DMSO and then added to the microtiter wells, with the final concentration of DMSO to be less than 1%. The microtiter plate was placed in a humidity-balanced chamber and the cells incubated at 30 °C for 48 h. The plates containing the cells were shaken on a plate shaker for 1 min at 500 rpm. The OD_{595} of the wells was measured with a UV_{max} microtiter plate reader (Molecular Device, Sunnyvale, CA), and the background subtracted OD_{595} was used as a measurement of cell growth. The percentage of decrease in cell growth in compound-containing wells relative to the drug-free control wells was used as a measurement of growth inhibition in concentration response and for IC_{50} derivation.

Stabilization of the DNA−Topoisomerase I Covalent Binary Complex. Supercoiled pB*R*322 plasmid DNA (250 ng) was incubated at 37 °C with 250 ng of human topoisomerase I in an incubation mixture (final volume 20 μL) containing 40 mM Tris−HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 50 μg/mL bovine serum albumin and crude extracts (dissolved in 10% aqueous DMSO to 1.0 mg/mL, final concentration 100 μg/mL). Each set of experiments included two negative controls (DNA alone, DNA + 20 μM camptothecin), one enzyme control (DNA + enzyme), and one positive control (DNA + enzyme + 20 μM camptothecin). After 60 min at 37 °C, the reaction was stopped by the addition of SDS and proteinase K at final concentrations of 1% and 1.0 mg/mL, respectively, and incubated for an additional 60 min. Aliquots (25 μL) were mixed with 5.0 μL of 30% glycerol−0.01% bromophenol blue and analyzed by electrophoresis on a 1% agarose gel containing 0.7 μg/mL ethidium bromide. The electrophoresis was carried out in tris-borate buffer, pH 8.3, containing 2 mM EDTA, at 50 V for 15 min, then at 110−120 V for 3 h. Following electrophoresis, the gel was photographed under ultraviolet light.

Plant Material. Stems of *P. klaineana* were collected by C. C. H. Jongkind of the Missouri Botanical Garden in the Ankasa Game Reserve in Ghana in March 1995, and taxonomically identified by D. K. Abbiw working with the Missouri Botanical Garden. Herbarium vouchers were taken in Ghana, with one being given to the Ghanaian authorities, one being maintained at the Missouri Botanical Garden, and one being held for scientific access at the Botany Department of the Natural History Museum under the accession number 0EQJ0958. The plant was air-dried and transported to the USA by air; dried material was stored at −20 °C until it was processed.

Extraction. Plant material (1 kg) was ground to a fine powder using a ball mill and then sequentially extracted. The complete powdered material was suspended in a gravity percolator using an equal volume of 50:50 (v/v) $CH_3OH-CH_2Cl_2$ and allowed to stand overnight at 24 °C. The organic liquid was allowed to run into a flask, and the wet plant debris was washed with one-tenth volume of methanol, collecting all organic solvents in the same flask. The contents were evaporated to dryness under vacuum at less than 40 °C and finally dried in a tared bottle under high vacuum at room temperature overnight to give 25 g of an oily solid. The plant debris following the organic extraction was dried by pulling air through the powder in the percolator, an equal volume of deionized water was added, and the extraction was allowed to proceed overnight at 24 °C. The aqueous solution was removed, lyophilized using a Virtis commercial lyophilizer, beginning at −50 °C and raising the temperature over 5 days to ambient. The pellet (35 g) was removed to a tared bottle. Both fractions were maintained at −20 °C until used for either testing or for other purposes.

Isolation. The crude extract N080725 (6.6 g, IC_{12} 250 μg/mL in the RS322 assay, 8000 μg/mL in the RS321 assay, and >8000 μg/mL in the RJ03 assay) was partitioned between *n*-hexane and 60% aqueous MeOH. The aqueous MeOH fraction was then diluted to 50% aqueous MeOH and partitioned with CH_2Cl_2 to give a bioactive CH_2Cl_2 fraction (0.77 g 10.9%, IC_{12} 26.4 μg/mL against RS322, 4000 μg/mL against RS321, and >4400 μg/mL against RJ03). The CH_2Cl_2 fraction was then subjected to column chromatography on Si gel with elution by CH_2Cl_2−acetone, 8:2, to give eight fractions, after combination of similar fractions as judged by TLC. Bioactivity was detected in fraction 4 (28.8 mg, IC_{12} 4.8 μg/mL against RS322). Fraction 4 was subjected to chromatography on Si gel again with elution by CH_2Cl_2−acetone (8:2), and two UV active zones (14.7 and 9.3 mg) were collected. The fraction (9.3 mg) corresponding to the more polar zone on TLC plate of Si gel (CH_2Cl_2−acetone, 9:1) yielded compound **1** (4.8 mg), and compound **2** (1.6 mg) was isolated from the less polar zone, 14.7 mg.

Compound 1: amorphous powder; $[\alpha]^{25}_D$ −70.8° (*c* 0.1, pyridine); UV λ_{max} (MeOH, log ϵ) 219.5 (4.43), 263.5 (4.26), 306 (3.74), 319.5 (3.89), 358 (4.13), and 370 (sh, 4.11); ^1H NMR (DMSO-d_6) δ_H 5.29 (2H, s, H-5), 8.69 (1H, s, H-7), 8.12 (1H, dd, H-9), 7.71 (1H, ddd, H-10), 7.86 (1H, ddd, H-11), 8.17 (1H, dd, H-12), 7.35 (1H, s, H-14), 5.42 (2H, s, H-17), 0.87 (3H, t, H-18), 1.86 (1H, q, H-19), 6.53 (1H, s, −OH); ^{13}C NMR (DMSO-d_6) δ_C 152.73 (C-2), 145.50 (C-3), 50.26 (C-5), 129.86 (C-6), 131.58 (C-7), 127.97 (C-8), 128.52 (C-9), 127.68 (C-10), 130.41 (C-11), 129.04 (C-12), 147.93 (C-13), 96.72 (C-14), 150.01 (C-15), 119.07 (C-16), 156.84 (C-16a), 65.25 (C-17), 7.78 (C-18), 30.28 (C-19), 72.38 (C-20), 172.49 (C-21); all signals assigned by ^1H and ^{13}C NMR, HMQC, and HMBC; their chemical shifts were the same as the data for camptothecin in the literature;[16] HREIMS *m*/*z* 348.1112 (calcd for $C_{20}H_{16}N_2O_4$ 348.1110).

Compound 2: amorphous powder; $[\alpha]^{25}_D$ +33.3° (*c* 0.05, $CHCl_3$−MeOH, 4:1); UV λ_{max} (MeOH, log ϵ) 218 (4.37), 253

(4.22), 288 (3.56), 359.5 (4.06), 369 (4.05); ^{1}H NMR (DMSO-d_6) δ 5.29 (1H, s, H-5), 8.84 (1H, s, H-7), 6.97 (1H, dd, H-10), 7.72 (1H, ddd, H-11), 7.82 (1H, dd, H-12), 7.70 (1H, s, H-14), 5.75 (1H, d, J = 16.18 Hz, H-17a), 5.31 (1H, d, J = 16.18 Hz, H-17b), 1.04 (3H, t, J = 7.40, H-18), 1.89 (2H, q, H-19), 4.06 (3H, s, $-OCH_3$), 6.53 (1H, s, $-OH$); ^{13}C NMR ($CDCl_3$) δ 152.73 (C-2), 146.58 (C-3), 50.30 (C-5), 127.64 (C-6), 126.20 (C-7), 120.77 (C-8), 155.20 (C-9), 105.49 (C-10), 130.61 (C-11), 121.78 (C-12), 149.73 (C-13), 98.09 (C-14), 150.10 (C-15), 118.57 (C-16), 157.69 (C-16a), 66.39 (C-17), 7.82 (C-18), 30.93 (C-19), 72.75 (C-20), 173.98 (C-21), 55.96 ($-OCH_3$); HREIMS m/z 378.1223 (calcd for $C_{21}H_{18}N_2O_5$ 378.1216).

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