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Evaluation of indenoisoquinoline topoisomerase I inhibitors using a hollow fiber assay

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Abstract—The indenoisoquinolines are a novel class of non-camptothecin topoisomerase I (Top1) inhibitors whose mechanism of action involves trapping the covalent complex formed between DNA and Top1 during cellular processes. As an ongoing evaluation of the indenoisoquinolines for Top1 inhibition and anticancer activity, indenoisoquinoline analogs have been screened in the National Cancer Institute's hollow fiber assay (HFA). Some of the derivatives demonstrated significant activity at intraperitoneal and subcutaneous fiber placement sites, along with net cancer cell kill in one or more cell lines.

The development of a therapeutic agent is costly and time consuming with thousands of potential agents needing to be evaluated every year. For the past 20 years, the National Cancer Institute (NCI) has carried out a rational evaluation of anticancer agents with a goal of streamlining discovery and development from in vitro cellular assays to clinical trials.[1–6] Initial steps in the drug discovery process involve solicitation of samples for screening against 60 human tumor cell lines, representing nine histological types.[1] Efficacy in this model is used as a prerequisite for further testing and also provides opportunities for guiding the elucidation of molecular targets using the COMPARE analysis[7] (which was instrumental for determining the role of indenoisoquinolines as Top1 inhibitors).[1–6] Recognizing that the bottleneck in the evaluation process involved in vivo xenograft analyses, the NCI instituted a novel assay in the mid-1990s to allow the rapid screening of anticancer agents in an in vivo process prior to xenograft analysis, thereby minimizing time and cost by eliminating poorly performing molecules.[1,2] This new assay involved the implantation of polyvinylidene fluoride (PVDF) 'hollow fibers' containing tumor cells into immunologically compromised mice to provide an assay that mimicked xenograft implantation.[2] This would allow researchers to pre-screen molecules by dosing the mice at a set interval and evaluating cytotoxicity by simply removing the implanted fibers containing the tumor cells and performing a standard cellular viability assay.[2] This hollow fiber assay (HFA) has been adopted as standard practice by the NCI's Developmental Therapeutics Program for the evaluation of therapeutic agents since its implementation.[1–6] Furthermore, the HFA has been modified beyond its initial scope to provide a method to evaluate angiogenesis, microtubule and cell cycle disruption.[8–13]

Several years ago, NSC 314622 (**1**) was implicated as a topoisomerase I (Top1) inhibitor after a COMPARE analysis[7] of its cytotoxicity profile revealed a strong resemblance to that of other known Top1 inhibitors, including camptothecin (**2**) and the clinically useful anticancer drugs irinotecan (**3**) and topotecan (**4**) (Fig. 1).[14] Follow-up in vitro testing confirmed this correlation,

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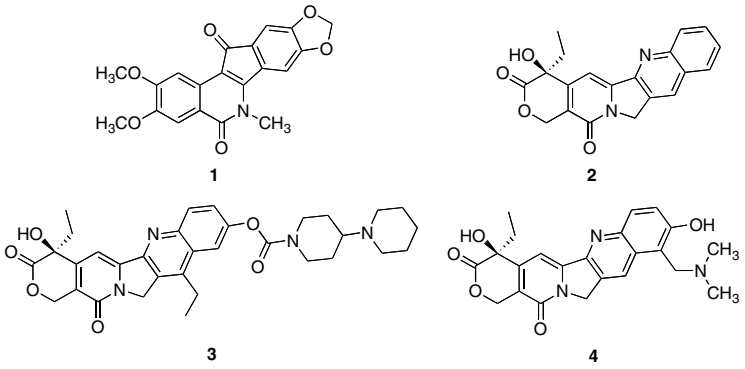

Figure 1. Camptothecin and non-camptothecin topoisomerase I inhibitors.

but indicated distinct differences in the biological activities of NSC 314622 (**1**) in comparison with the clinically useful camptothecin derivatives. This finding warranted further development of the indenoisoquinolines. Notable distinctions included DNA cleavage site specificity, cleavage complex stability, and chemical stability of the indenoisoquinolines.[14] Development of NSC 314622 (**1**) as an anticancer agent was limited by its moderate potency, both as a cytotoxic agent in cancer cells and as a Top1 inhibitor. Thus, a number of additional indenoisoquinolines related to **1** have been synthesized and evaluated for cytotoxicity, Top1 inhibition, and activity in the National Cancer Institute's hollow fiber assay, which is the focus of this communication.

Indenoisoquinoline analogs **1** and **5**–**25** (Fig. 2) were synthesized according to previously published methods and were supplied to the Biological Testing Branch of the Developmental Therapeutics Program of the NCI as 450 mg samples for evaluation in the HFA.[15–23]

A number of indenoisoquinoline derivatives have been evaluated as anticancer agents in an in vivo hollow fiber animal model, in which polyvinylidene fluoride hollow fibers containing various cancer cell cultures are implanted intraperitoneally (IP) and subcutaneously (SC) into mice and the derivatives were administered by the IP route.[24] The effects of the compounds on the reduction of viable cancer cell mass compared to those of controls were determined. Each compound was tested in the hollow fiber assay against a panel of 12 human tumor cell lines as described previously.[2] The compounds were solubilized in 10% DMSO in saline/Tween 80R and administered intraperitoneally once daily for a total of four doses at each of two dose levels. The two

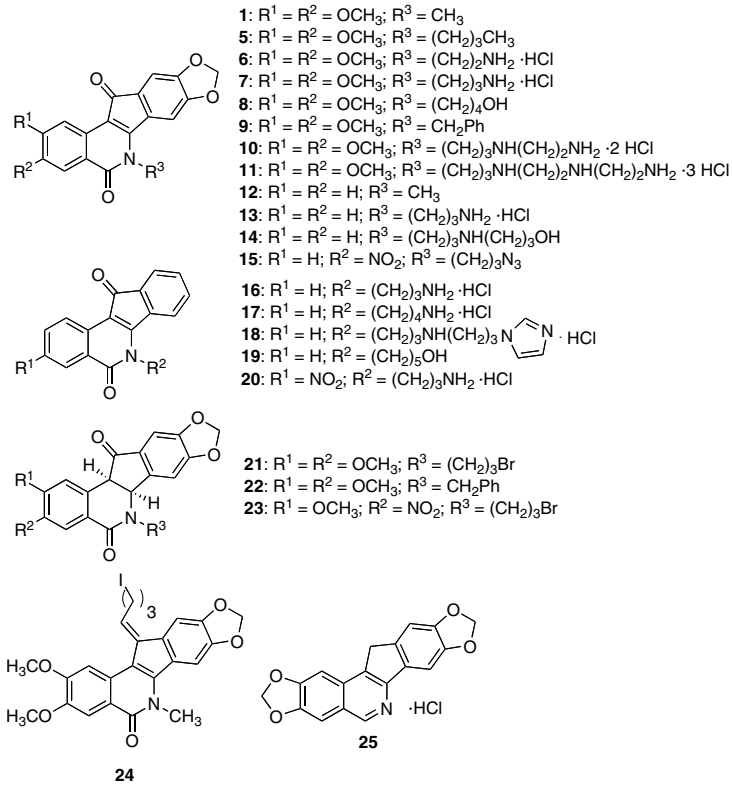

Figure 2. Indenoisoquinoline analogs evaluated in the hollow fiber assay.

doses were selected based on single dose toxicity studies for each derivative. The highest single dose that did not produce toxicity was determined and multiplied by 0.375 and 0.25 to provide the highest and lowest administered dose level, respectively.[2] The day after the last dose, the fibers were collected and assessed for viable cell mass using an MTT dye conversion assay.[2] A score of 2 was assigned each time the compound produced a 50% or greater reduction in viable cell mass compared to vehicle-treated controls. The score for each compound was summed for the intraperitoneal fibers and the subcutaneous fibers to provide the total score for each derivative. Using this scoring system, a maximum total score of 96 can be achieved (12 cell lines × 2 sites × 2 doses × 2 points per positive result).[2] Generally speaking, compounds with a combined IP + SC score of 20, an SC score of 8, or a net cell kill of one or more of the cell lines are referred for further studies.[2] The results of the HFA are summarized in Table 1, along with the ability of the derivatives to inhibit Top1 and their mean graph midpoints (MGM) from their evaluation in the NCI's 60 cell screen. For comparison purposes, the activities of lead compounds **1**,[21] **22**,[21] taxol,[25] vinblastine sulfate,[25] and cisplatin[25] are also provided.

The indenoisoquinolines had various biological activities with regard to Top1 inhibition and the NCI's 60 cell screen ranging from weak to excellent activity in both assays. Table 1 indicates that in general, correlations exist between MGM values and Top1 inhibition. Assuming potent Top1 inhibition to be a score of +++ or better, then compounds **6**, **7**, **10**, **11**, **14**, **15**, **16**, **17**, and **20** should have potent MGM values. Compounds **21** and **23** should be disregarded due to a prodrug activation hypothesis[27] (explained below). Therefore, of the nine remaining compounds, only compounds **11** and **14** are outliers, which indicates a high degree of correlation. The data in Table 1 also support a correlation between MGM values and performance in the HFA. As noted above, the NCI uses a combined IP + SC score of 20, an SC score of 8 or better, or a net cell kill to merit advancement. If one uses this as the standard for validating a correlation, then of the thirteen compounds reported in Table 1 that met criteria for advancement, nine of the compounds had submicromolar MGM values. A more modest correlation existed between Top1 inhibition and performance in the HFA. Of the nine compounds that had Top1 inhibition values of +++ or better, only five compounds (**6**, **7**, and **14**–**16**) met the criteria for advancement. Although this correlation was not great, one must realize the inherent flaws in comparing in vitro and in vivo assays. Metabolism, clearance, and excretion all complicate matters so it was not surprising that the correlation was less between Top1 inhibition and performance in the HFA. A more

Table 1. Cytotoxicities, topoisomerase I inhibitory activities, and hollow fiber activities of indenoisoquinoline analogs

Compound	MGM[a] (μM)	Top1 inhibition[b]	IP score[c]	SC score[c]	Total score	Cell kill[d]
1	20.0	++	2	6	8	Y
2	0.0405 ± 0.0187	++++	NT	NT	NT	NT
5	41.6	+	2	6	8	N
6	0.338 ± 0.154	++++	14	4	18	Y
7	0.160 ± 0.02	+++	16	4	20	N
8	3.20	+	4	12	16	N
9	41.6	+	0	4	4	N
10	0.347	+++	8	0	8	N
11	2.40 ± 0.877	++++	6	2	8	N
12	45.0	+	0	0	0	N
13	0.251 ± 0.072	+	4	4	8	N
14	15.5	++++	4	8	12	N
15	0.098 ± 0.081	++++	20	8	28	Y
16	0.315 ± 0.293	+++	18	4	22	Y
17	0.156 ± 0.015	+++	4	4	8	N
18	1.01 ± 0.444	++	0	0	0	N
19	14.4	+	2	6	8	N
20	0.146 ± 0.141	++++	10	8	18	N
21	0.690 ± 0.00	+	6	2	8	Y
22	1.18	NT	0	8	8	Y
23	0.016 ± 0.004	+	14	2	16	N
24	8.49 ± 8.11	0	0	2	2	N
25	0.271 ± 0.259	++	2	4	6	N
Taxol[25]	0.0253	NT	24	8	32	Y
Vinblastine sulfate[25]	25 pM	NT	18	10	28	Y
Cisplatin[25]	0.935	NT	14	8	22	Y

[a] Mean graph midpoint for growth inhibition of all human cancer cell lines successfully tested.

[b] The compounds were tested at concentrations ranging up to 10 μM. The activity of the compounds to produce Top1-mediated DNA cleavage was expressed semi-quantitatively as follows: +, weak activity; ++, similar activity as the parent compound **1**; +++ & ++++, greater activity than the parent compound **1**; ++++, similar activity as 1 μM camptothecin; NT, Not tested.

[c] The IP and SC scores listed are the sums of all the IP and SC scores for each compound.

[d] A net cell kill at one or more implant sites is indicated with a Y.

logical comparison would be between the cell screen and HFA since cellular penetration is required in both assays.[3]

Interestingly, there are several trends in performance at the IP and SC sites and the MGM values of the tested derivatives. In general, compounds with sub-micromolar MGM's performed equal to or better at IP implant sites (the lone exception being compound **25**) and compounds with greater than 1 μM MGM's always performed equal to or better at the SC implant site. The reason for this trend is not entirely clear at this time, but it may involve 'activation' of the molecules by the animal prior to cellular uptake as a prerequisite for activity at the SC implant site, and this 'activation' may not occur during the in vitro 60 cell screen. On the other hand, compounds with a total score of 18 or better (compounds **6**, **7**, **15**, **16**, and **20**) always had superior activity at the IP site compared to the SC site. This could imply efficient clearance or metabolism by the animal, thereby attenuating activity at the SC site. Lastly, sub-micromolar MGM values tend to indicate an increased tendency for net cell kill, but there are exceptions (such as compound **1**).

Nine of the twenty-two evaluated compounds (**1**, **5**, **8**, **9**, **14**, **19**, **22**, **24**, and **25**) performed better at the SC implant site than at the IP implant site. As noted in the requirements for advanced xenograft testing, an SC score of 8 automatically satisfies the criteria for further xenograft study. This is due, in part, to the physiological requirement of uptake and distribution for a molecule to exert activity at the SC implant site when it is administered intraperitoneally.[2] Focusing on the SC site, the largest gain in activity (with respect to the SC site) was an increase of 8 relative to the IP site accomplished by both compounds **8** and **22**. However, both of these molecules possess functionalities that may be oxidized within the animal (such as the pendent hydroxyl group for **8** and the dihydro aromatic skeleton for **22**), thereby making these compounds prodrugs with the oxidation product being responsible for the increased subcutaneous activity. Further supporting this rationale, compound **19** also possessed a pendent hydroxyl group and demonstrated increased activity at the SC site (6) compared to the IP site (2). With regard to net cell kill, the dihydroindenoisoquinoline subclass (**21**–**23**) were more effective than the other subclasses, with two of the three molecules being promoted to xenograft studies as a result of net cell kill.

It is likely that the indenoisoquinoline derivatives are not producing their anticancer effects in exactly the same way. For example, dihydro compounds **21**–**23** are generally weak inhibitors of Top1. In spite of this, both compounds **21** and **22** had sufficient activity in the HFA (net cell kill in one or more cell lines) to qualify them for advanced xenograft study. Interestingly, the crystal structures of two indenoisoquinolines in complex with DNA and Top1 have been solved (PDB ID: 1SC7 and 1TL8)[23,26] and indicate that for steric reasons, the dihydroindenoisoquinolines (which are not planar) could not possibly be intercalating with the DNA at the break site.[23,26] Thus, our hypothesis has been that the dihydro compounds are metabolized in vivo by a 2-electron oxidation, thereby providing the corresponding planar indenoisoquinolines that are sterically able to intercalate between the DNA base pairs.[27] This mechanism of action is also supported, in part, by a COMPARE analysis.[7,27] Furthermore, we know that chemically, the dihydro compounds are very prone to dehydrogenation, which reflects the fact that the double bond formed upon oxidation is tetrasubstituted and highly conjugated. Consistent with this hypothesis, dihydro compound **22** was more active at the SC implant site after IP administration than it was at the IP implant site. In other words, the compound appears to have to 'travel through' the animal to be activated. This would be consistent with it being a prodrug that is converted into the active species. However, the presumed oxidation product of **22** would be **9**, and this compound showed poor activity as a Top1 inhibitor (Top1 +) as well. Additionally, compounds **21** and **23** were less active at the SC implant site than at the IP implant site, suggesting that the pro-drug hypothesis is not definitively supported by the HFA, but subtle differences in the pharmacokinetic properties of the individual dihydro compounds and their metabolism may significantly affect their ability to function as prodrugs, and this may explain the discrepancies among the molecules studied in the HFA.

Table 1 also provides for comparisons between the indenoisoquinoline derivatives studied in the HFA and the clinically useful drugs taxol, vinblastine sulfate, and cisplatin.[25] Regarding the mean graph midpoint (MGM) values derived from the NCI 60 cell screen, many of the indenoisoquinoline derivatives are more cytotoxic than cisplatin and compounds **7**, **13**, **15**, **17**, **20**, and **23** have comparable cytotoxicities to that of taxol (one order of magnitude difference or less). However, of the twenty-two tested indenoisoquinoline analogs, none were more potent in the hollow fiber assay than taxol, but compound **15** was equal in potency to vinblastine sulfate and more potent than cisplatin.

The nitro substituent present in compound **15** was previously reported[18] to improve in vitro biological activity of the indenoisoquinolines through improved π-stacking interactions with DNA and second generation analogs further exploring the effect of the nitro substituent are currently being evaluated. The potency of **16** was equal to that of cisplatin in the HFA. In general, indenoisoquinolines possessing an amino-substituted lactam sidechain display potent activity in cytotoxicity assays.[16–18,20–23] Consequently, it would be expected that these compounds would show significant activity in the HFA. However, only half of the amino-substituted compounds met the HFA criteria for advanced study. Thus, subtle differences between amino-substituted compounds must play a significant role in their in vivo activity and further studies are warranted.

In summary, a variety of cytotoxic indenoisoquinoline Top1 inhibitors were screened in the HFA, with nearly half of the molecules satisfying the requirements for fur-

ther in vivo testing. In general, highly cytotoxic molecules demonstrated better activity in the HFA at the IP implant site, while less cytotoxic indenoisoquinolines exerted more activity at the SC implant site. This may imply that individual molecules require 'activation' prior to eliciting activity (further supported by hydroxyl-substituted and dihydro compounds). Furthermore, several indenoisoquinolines performed equally as well as clinically useful agents in the HFA, helping to validate the indenoisoquinolines as potential therapeutic agents and supporting their further development as anticancer agents.

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