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Structure–activity studies of phenanthroindolizidine alkaloids as potential antitumor agents

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Abstract—Five phenanthroindolizidine alkaloids (PA) were chemically synthesized and seven were isolated from *Tylophora atrofoliculata*. To facilitate future drug design of phenanthroindolizidine alkaloids as potential antitumor agents, we have explored the structure–activity relationships (SAR) of this class of compounds. We demonstrated that DCB-3503 and tylophorinidine (PA-7) were among the most active compounds against tumor growth both in vitro and in vivo. In the hepatocellular carcinoma cell line HepG2, the GI_{50}s of DCB-3503 and PA-7 were 35 ± 5 nM and 11 ± 5 nM, respectively. DCB-3503 and PA-7 significantly inhibited HepG2 tumor growth in nude mice at a dose of 9 mg/kg given by intraperitoneal (ip) injections twice a day every third day for a total of four cycles ($P < 0.05$ for DCB-3503 and $P < 0.01$ for PA-7). Their potent antitumor activities correlated with their potent NF-κB-inhibitory effects and their cyclin D1 down-regulatory effects.

Phenanthroindolizidine alkaloids (PA) have been a focus of our research due to their potent antitumor,[1–5] anti-inflammatory,[6,7] and anti-autoimmune disease properties.[8] Five tylophorine analogs that belong to the group of phenanthroindolizidine alkaloids, DCB-3500, DCB-3501, DCB-3502, DCB-3503, and DCB-3506 are synthetic compounds (Fig. 1). Among these analogs, DCB-3503 has been shown to exert potent growth-inhibitory effects against HepG2, a human hepatocellular carcinoma cell line, both in vitro and in vivo.[5] DCB-3503 was also active in vitro against PANC-1, a human pancreatic ductal carcinoma cell line.[9] In addition, DCB-3500, DCB-3501, DCB-3502, and DCB-3503 were equally effective against both parental human nasopharyngeal carcinoma KB cells and KB variants, which are resistant to etoposide, hydroxyurea or camptothecin.[5] During our investigation of the mechanisms of action of the tylophorine analogs, we discovered that this class of compounds has potent inhibitory effects against NF-κB-mediated transcription.[5] NF-κB is a family of transcription factors that play critical roles in the process of inflammation,[10] cell survival,[11] and chemoresistance.[12] Overexpression of NF-κB may contribute to chemoresistance of cancers,[12] making NF-κB an attractive target for antitumor drug development.[13] The potent inhibitory effects of phenanthroindolizidine alkaloids on NF-κB-mediated transcription may be one of the mechanisms of their antitumor activity. We previously found that DCB-3503 down-regulated the expression of cyclin D1.[9] Cyclin D1 plays a critical role during cell cycle progression from G_1 to S phase.[14] Cyclin D1 transcription and translation increase through Ras-dependent pathways during growth factor signaling.[14] Cyclin D1 overexpression has been observed in a number of human tumors[15,16] and is associated with poor prognosis and chemoresistance.[17] Therefore, the down-regulatory effect of cyclin D1 also contributes to the antitumor activity of these phenanthroindolizidine alkaloids.

Keywords: Phenanthroindolizidine alkaloids; Structure–activity relationships; Antitumor activity.
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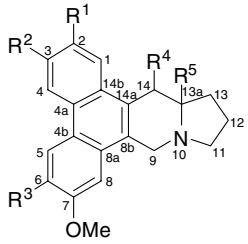
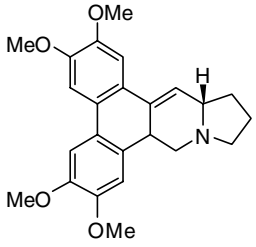


Compounds	R^1	R^2	R^3	R^4	R^5	N^{10}
DCB-3500	OMe	OMe	OMe	H	H (*S*)	–
DCB-3501	OMe	OMe	OMe	OH (*R*)	H (*S*)	–
DCB-3503	OMe	OMe	OMe	OH (*S*)	H (*S*)	–
DCB-3506	OMe	OH	OMe	OH (*S*)	H (*S*)	–
PA-2 (Tylophoridicine E)	H	OH	OMe	OH (*S*)	H (*S*)	–
PA-3 (Tylophoridicine C)	H	OMe	OH	OH (*S*)	H (*S*)	N^+–O^-
PA-4 (Tylophoridicine F)	H	OMe	OMe	OH (*R*)	H (*S*)	N^+–O^-
PA-5 [(*R*)-(+)-Deoxytylophorinidine][a]	H	OMe	OH	H	H (*R*)	–
PA-6 (Tylophorinine)[b]	H	OMe	OMe	OH (*R*)	H (*S*)	–
PA-7 (Tylophorinidine)	H	OMe	OH	OH (*S*)	H (*S*)	–

[a] Compound **5** in ref. 18.
[b] Compound **6** in ref. 18.



DCB-3502

Figure 1. Chemical structures of phenanthroindolizidine alkaloids.

Seven phenanthroindolizidine alkaloids, tylophoridicine D (PA-1), tylophoridicine E (PA-2), tylophoridicine C (PA-3), tylophoridicine F (PA-4), *R*-(+)-deoxytylophorinidine (PA-5), tylophorinine (PA-6), and tylophorinidine (PA-7) (Fig. 1), were isolated from the roots of *Tylophora atrofolliculata* (a medicinal plant widely distributed in Southwestern China) and found to have different degrees of growth-inhibitory activities in cell culture.[18] In this study, we explored the structure–activity relationships of these phenanthroindolizidine alkaloids through comparison of their cytotoxicities, antitumor activities in vivo, inhibitory effects against NF-κB, cAMP response element (CRE), and activator protein-1 (AP-1) mediated transcription, and their down-regulatory effects on the key cell-cycle control protein, cyclin D1. The main objectives were to study the structure–activity relationships that will aid in the future design of anti-cancer drugs from phenanthroindolizidine alkaloids and to obtain candidate compounds for clinical trials in cancer patients, especially for those who are failing current chemotherapy protocols.

The growth-inhibitory effect of the PA analogs (PA-1 was not included in this study because of the shortage of this compound) on HepG2 and PANC-1 cells were tested using the previously described cytotoxicity assay.[5] As shown in Table 1, PA-2 and DCB-3506 were the least active among the compounds tested, with GI_{50}'s (concentration of drug that inhibits cell growth by 50% after continuous drug exposure for three doubling times) against HepG2 > 5000 nM and >600 nM, respectively. PA-2 and DCB-3506 differ structurally from the rest of the PA analogs by the presence of an OH group at the R^2 position (OH replaces OMe). Interestingly, DCB-3503 with an OMe group at R^2 is more active than DCB-3506 with an OH group at R^2. This suggests that the OMe group at R^2 is required for the cytotoxic potency of a PA analog. Therefore, substituting an *O*-alkyl or an *O*-aryl group at the R^2 position may be a future direction for synthesis in this class of compounds. The major structural differences between DCB-3501 and -3503 and tylophorinine (PA-6) is at the R^1 position where DCB-3501 and -3503 have an OMe group and PA-6 has a hydrogen atom. These also differ in the configuration of the 14-OH group. The GI_{50} value for PA-6 was 20 ± 6 nM and 11 ± 4 nM in HepG2 cells and PANC-1 cells, respectively. These values were less than the GI_{50} values of DCB-3501, suggesting that the addition of the OMe group at R^1 decreases cytotoxicity. Also, PA-7 with an OH substitution at the R^3 position

Table 1. Cytotoxicity of PA analogs in HepG2 and PANC-1 cells

Compound	HepG2 GI_{50}[a] (nM)	PANC-1 GI_{50}[a] (nM)
DCB-3500	11 ± 4[b]	nd
DCB-3501	110 ± 45[b]	nd
DCB-3502	264 ± 115[b]	nd
DCB-3503	35 ± 5[b]	51 ± 3[b]
DCB-3506	>600	nd
PA-2	>5000	>5000
PA-3	98 ± 38	67 ± 9
PA-4	71 ± 9	63 ± 7
PA-5	13 ± 3	11 ± 3
PA-6	20 ± 6	11 ± 4
PA-7	11 ± 5	7 ± 0

[a] Values are means ± SD of three experiments, with each data point done in triplicate (nd, not determined).
[b] Published.[5,7]

had similar potency to PA-6 with an OMe group at the R^3 position (Table 1). This suggests that the substitution of an OH group for an OMe group at R^3 is not critical for maintaining potent cytotoxicity.

DCB-3501 and DCB-3503 are modified from DCB-3500 by addition of an (*R*)-OH and an (*S*)-OH group at the R^4 position, respectively. In HepG2 cells, the GI_{50}s of DCB-3500, DCB-3501, and DCB-3503 were determined to be 11 ± 4 nM, 110 ± 45 nM, and 35 ± 5 nM, respectively. The addition of either an OH group at R^4 did not increase the cytotoxicity in cell culture, although the addition of an (*S*)-OH group at R^4 affected the cytotoxic activity less than the addition of an (*R*)-OH group at R^4; thus a 14-(*S*)-OH group seems to favor activity over an epimeric 14-(*R*)-OH group. We previously observed that DCB-3503 was more potent against tumor growth in vivo than DCB-3500.[5] The antitumor activity of DCB-3503 was confirmed in this study (as shown in Fig. 2) using nude mice bearing HepG2 tumor xenografts by ip injections at 9 mg/kg twice per day every third day for four cycles (DCB-3503 vs control: $P < 0.05$). While PA-5 did not show activity against tumor growth in vivo (Fig. 2a), PA-7 treatment at 9 mg/kg twice per day every third day for four cycles showed a significant tumor-suppressive effect (PA-7 vs control: $P < 0.01$), as shown in Fig. 2a. The lack of an in vivo effect for PA-5 could be due to its structural differences from PA-7. The stereocenter at C-13 is of the (*R*)-configuration in PA-5, while it is of the (*S*)-configuration in PA-7. Moreover, there is an addition of an (*S*)-OH group at R^4 in PA-7. DCB-3503 is more active against tumor growth than DCB-3500, and PA-7 is more active than PA-5 in vivo. Interestingly, both DCB-3503 and PA-7 have (*S*)-OH groups at position R^4. It is our hypothesis that the addition of an OH group at R^4 may favor the pharmacokinetics of these compounds in vivo. This point requires further investigation. There was no significant difference in body weight loss comparing the groups treated with PA-7 and PA-5 to that of the vehicle control group (Fig. 2b). With the group treated with DCB-3503, there was a 15% body weight loss by the end of treatment days 9 and 10. We previously observed that on discontinuation of treatment there was recovery of the loss in body weight.[5]

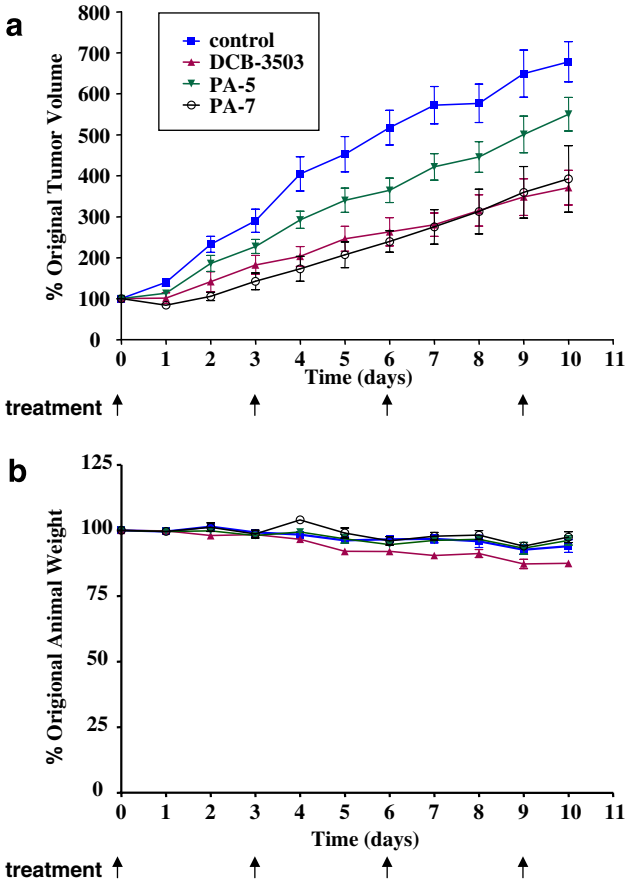


Figure 2. Antitumor activity of phenanthroindolizidine alkaloids. Four-week-old male NCr-nude mice were injected subcutaneously with 8×10^6 HepG2 cells. Treatment was initiated when the tumors were 200–300 mm^3, which is around 8 days after injection of HepG2 cells. DCB-3503, PA-5, and PA-7 were dissolved in 0.5% Tween 80 in PBS, and solvent alone served as control. Drugs were administered by ip injections. DCB-3503, PA-5, and PA-7 were administered at 9 mg/kg, every 12 h on days 0, 3, 6, and 9. HepG2 tumors were measured daily using a caliper, and the body weights of the mice were monitored for toxicity. Tumor volume was estimated by using the formula length × $width^2 \times \pi/6$. (a) Effect of DCB-3503, PA-5, and PA-7 on HepG2 tumor growth in nude mice. (b) Effect of DCB-3503, PA-5, and PA-7 on the body weight of nude mice. There were five mice in each group. Data points represent means of the groups ±SD. Statistical analysis was performed using one-way ANOVA (Dunnett's) to compare the treatment groups and the vehicle control.

In comparing the cytotoxicity of PA-4 and PA-6 (Table 1), we observed that PA-6 is ~3- to 5-fold more potent than PA-4. The structural difference between PA-4 and PA-6 is the presence of an *N*-oxide in PA-4 (Fig. 1), indicating that the addition of an *N*-oxide leads to a decrease in cytotoxicity. This is further confirmed by PA-3's decreased cytotoxicity as compared with that of PA-7. The presence of the *N*-oxide in PA-3 is the only structural difference that appears to contribute to its decrease of cytotoxicity, as the loss of R^1 or the substitution of an OH group at R^3 in PA-3 did not lead to a decrease in cytotoxicity as indicated above. The structural difference between DCB-3500 and DCB-3502 is the double bond at position 14–14a, and there was about a 20-fold difference in cytotoxicity. The GI_{50} for

DCB-3502 and DCB-3500 was 264 ± 115 nM and 11 ± 4 nM, respectively. We predict, based on the cytotoxicity and the antitumor activity in vivo, that the ideal compound would be a compound modified using the DCB-3503 or PA-7 scaffold. The removal of R^1 (replace R^1 = OMe with R^1 = H) in DCB-3503 could be beneficial to activity.

We then analyzed the effects of the different phenanthroindolizidine alkaloids on NF-κB-, CRE-, and AP-1-mediated transcription. HepG2 cells stably transfected with firefly luciferase reporter vector pBIIX-luc (containing two tandemly repeated NF-κB binding sites) were generated as previously described and referred to as HepG2-NF-κB-luc.[5] Similarly, HepG2-CRE-luc and HepG2-AP-1-luc stable cell lines were generated by transfection of HepG2 cells with pCRE-luc and pAP-1-luc (Clontech), respectively. Tumor necrosis factor α (TNFα), forskolin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were used to activate NF-κB-, CRE-, and AP-1-mediated transcription, respectively, as previously described.[5] Stable cell lines were pretreated with 3-fold serial dilutions of PA analogs for 1 h, then the cells were stimulated with their corresponding activators for 4 h. The luciferase activities were measured using Promega's luciferase assay kit. The IC_{50} was determined as the concentration of the compound at which the luciferase activity is inhibited by 50%. In Table 2, the inhibitory effects of PA analogs against NF-κB-, CRE-, and AP-1-mediated transcription are shown. The results indicate that this class of compounds preferentially inhibits NF-κB-mediated transcription, as the IC_{50} values in terms of NF-κB inhibition are significantly lower than those of CRE- and AP-1-mediated transcription. For example, the IC_{50} of DCB-3503 with regards to NF-κB-, CRE-, and AP-1-inhibition was 87 ± 6 nM, 1067 ± 231 nM, and 1067 ± 116 nM, respectively. Consistent with the cytotoxicity data, DCB-3502, DCB-3506, and PA-2 are the least active compounds, whereas PA-5, PA-6, and PA-7 are the most active compounds in terms of their NF-κB inhibition. Thus, the potency of NF-κB inhibition corresponds directly with the degree of cytotoxicity.

Table 2. IC_{50}'s of the inhibitory effect of PA analogs against NF-κB-, CRE-, and AP-1-mediated transcription in HepG2 cells

Compound	NF-κB IC_{50}[a] (nM)	CRE IC_{50}[a] (nM)	AP-1 IC_{50}[a] (nM)
DCB-3500	41 ± 20[c]	>300[b,c]	>300[b,c]
DCB-3501	315 ± 64	>300	>300[b]
DCB-3502	>300	>300	>300[b]
DCB-3503	87 ± 6[c]	1067 ± 231	1067 ± 116
DCB-3506	322 ± 19	>300	>1000
PA-2	>1000	>1000	>3000
PA-3	600 ± 173	>1000	>3000
PA-4	367 ± 116	>1000	>3000
PA-5	11 ± 6	135 ± 21	68 ± 46
PA-6	12 ± 9	80 ± 28	110 ± 14
PA-7	10 ± 4	95 ± 92	65 ± 35

[a] Values are means ± SD of three experiments, with each data point done in triplicate.
[b] Data generated from transient transfections.
[c] Graphical version was published.[5]

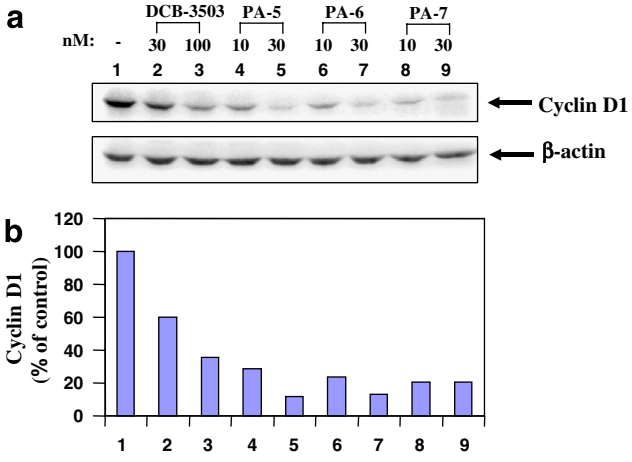


Figure 3. Effects of phenanthroindolizidine alkaloids on cyclin D1 expression. HepG2 cells were treated with DCB-3503, PA-5, PA-6, and PA-7 as indicated for 2 h. (a) cyclin D1 expressions were analyzed by Western blot using a specific monoclonal antibody; β-actin was used as the internal control. (b) Quantitation of cyclin D1 protein levels normalized with β-actin expression by densitometer scanning.

Previously we observed that cyclin D1 could be down-regulated by DCB-3503.[9] We then analyzed whether other PA analogs could also down-regulate this critical cell-cycle control protein. As shown in Figure 3, all the PA analogs studied show a dose-dependent down-regulation of cyclin D1. The cyclin D1 protein level with 10 nM PA-5 treatment is similar to that for the 100 nM DCB-3503 treatment. Similar down-regulatory effects against cyclin D1 were observed with PA-6 and PA-7 treatment. Their potency, in terms of cyclin D1 down-regulation, correlates with the degree of their cytotoxicity, as PA-5, PA-6, and PA-7 are the most active against both tumor cell growth and cyclin D1 down-regulation.

The structure–activity relationships of phenanthroindolizidine alkaloids have been studied by other investigators. Wu et al. observed that the introduction of a methoxyl functionality on the phenanthrene ring at positions-2, -3, -6, and -7 increased the cytotoxicity, and lack of the indolizidine ring led to the loss of cytotoxicity.[19] Lee et al. synthesized a novel class of phenanthrene-based tylophorine analogs with greatly enhanced yield and polarity by opening the indolizidine ring. However, the GI_{50}s of the most potent compounds synthesized were found to be in the sub-micromolar range.[20] We evaluated some compounds from Dr. Lee in our laboratory. Their mechanism of action appears to be different from the phenanthroindolizidine alkaloids we studied (our unpublished results). Although these compounds were derived from phenanthrene-based tylophorine, they may constitute a different class of cytotoxic compounds that are different from the tylophorine analogs we studied. In addition, Kim et al. reported that a rigid phenanthrene structure is required to maintain potent cytotoxicity. The unshared electron pair on nitrogen is important for a high activity, and cytotoxicity is dependent on the type and pattern of sub-

stitution on the phenanthrene ring.[21] The SAR and mechanisms of action of phenanthroindolizidine alkaloids have been studied by various investigators; however, their molecular targets are still unknown. The tylophorine analogs we studied have an impact on both NF-κB-mediated transcription and cyclin D1 expression, which is due to a decrease in its synthesis. The underlying mechanisms could be the same, a point that is currently being investigated. Based on our studies, the molecular target of tylophorine analogs may be novel and different from the targets of known anti-cancer drugs. Given the novel mechanism of action and activity against cancer and autoimmune diseases in vivo, the molecular mechanisms of this class of compounds must be further explored.

Chemical synthesis of DCB-3500 was carried out by the procedure reported by Nylander and Njoroge.[22] Both DCB-3501 and -3503 were synthesized by a procedure modified from that reported by Buckley and Rapoport.[23] DCB-3502 and -3506 are new compounds, and details of their syntheses and characterization are provided in Supplementary data to this paper.

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Supplementary data

Supplementary data associated with this article (details of the syntheses and characterization of DCB-3502 and -3506) can be found, in the online version, at doi:10.1016/j.bmcl.2007.05.021.

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