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Structural analogs of tylophora alkaloids may not be functional analogs

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Abstract—Phenanthroindolizidine-based tylophora alkaloids have been reported to have potential antitumor, anti-immuno and, anti-inflammatory activity. The structure–activity relationships of a series of tylophora alkaloids were studied to guide future drug design. Our results indicate that although these compounds are structural analogs, their potency of cytotoxicity, selectivity against NF-κB signaling pathway, and their inhibitory effects against protein and nucleic acid synthesis are different. Because they do not have an identical spectrum of targets, the studied compounds are structural, but may not be functional analogs.

Tylophora alkaloids originate from various plants of the Asclepiadaceae family, such as Tylophora, that are native of India and Southeast Asia.[1] They have antitumor,[2–6] anti-inflammatory,[7] anti-arthritis,[8] and anti-lupus activity in vivo.[9] Due to their diverse and potent pharmacological actions, they continue to be targets for synthesis, modification, and structure–activity relationship (SAR) studies since their first isolation in 1935.[10] Their molecular mechanisms of action of antitumor and anti-inflammatory activity include: inhibitory effect on protein synthesis[2] and nucleic acid synthesis,[2,11] inhibitory effect on RNA transcription which are controlled by cyclic AMP response elements (CREs), activator protein-1 (AP-1) sites, and NF-κB binding sites,[6] and ability to suppress the expression of a subset of proteins, such as cyclin D1, cyclin B1, and CDK4.[12] Tylocrebrine, a positional isomer of tylophorine, was found in clinical trials to have intolerable central nervous system (CNS) side effects. To offset such CNS side effects, a series of phenanthrene-based tylophorine derivatives (PBTs) with increased polarity was synthesized to limit the crossing of the blood–brain barrier.[13] It was assumed that these analogs would have the same mechanism of action and behave as functional analogs of the tylophora alkaloids.

Previous structure–activity relationships of phenanthroindolizidine alkaloids have concluded that the rigid phenanthrene structure (Fig. 1) on the phenanthrene ring is required to maintain potent cytotoxicity, and that the lack of an indolizidine ring or the presence of OMe ether at position 2 leads to the loss of cytotoxicity.[14–16] To continue the structure–activity relationship studies using the tylophora alkaloids rac-cryptopleurine, (−)-antofine, (−)-tylophorine, and (−)-ficuseptine C recently obtained by a concise and modular total synthesis approach,[17] and to verify whether the synthetic PBT compounds are functional analogs of the tylophora alkaloids, we compared their cytotoxicity, inhibitory effects on NF-κB, AP-1, CRE, and glucocorticoid response element (GRE), and some of their impact on protein, DNA, and RNA synthesis. We hypothesized that opening of the D-ring would have affected the potency; if the PBT compounds are functional tylophora alkaloid analogs, they might have comparable effects on the different intracellular pathways. Phenanthrene-based derivatives PBTs #28 and #31–34 were synthesized by Dr. K. H. Lee's group.[13] The tested tylophora alkaloids, rac-cryptopleurine, (−)-antofine, (−)-tylophorine, (−)-ficuseptine C, precursor #1 and precursor #2 were provided by Dr. Alois Fürstner.[17] (+)-*S*-Tylopho-

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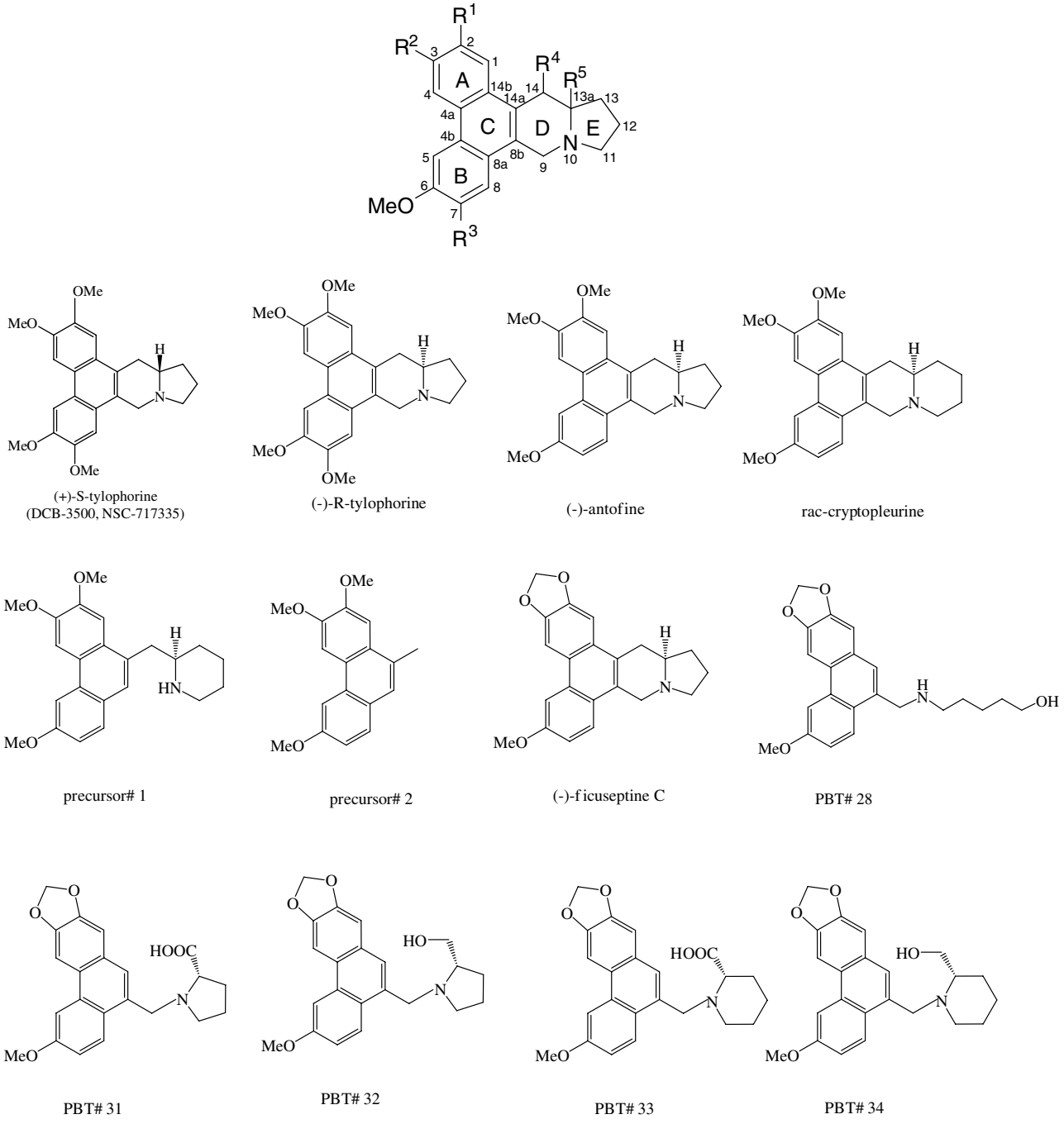

Figure 1. The chemical structures of tylophora alkaloids and phenanthrene-based tylophorine derivatives.

rine (DCB-3500) was synthesized by Dr. David C. Baker's laboratory.[16]

To analyze the structure–activity relationships of tylophora alkaloids, we first compared their cytotoxicity in HepG2, PANC-1, and CEM cells, followed by the procedures described previously.[6,16] (+)-(*S*)-tylophorine and (−)-(*R*)-tylophorine differ only in the absolute configuration of the chiral center at the 13a position, the (*R*)-configured compound led to an approximately 3- to 4-fold decrease in cytotoxicity (Table 1). The difference in the structures of (−)-antofine and (−)-(*R*)-tylophorine is at the R^3 position (Fig. 1). The absence of an OMe substituent at R^3 led to a 6-fold increase in cytotoxicity in HepG2 cells, and more than 10-fold increase in cytotoxicity in PANC-1, and CEM cells (Table 1). The structural difference between (−)-antofine and rac-cryptopleurine lies in the size of the E-ring. The presence of the six-membered E-ring in the phenanthroquinolizidine skeleton of rac-cryptopleurine led to a 2- to 4-fold increase in cytotoxicity in comparison to (−)-antofine which has a five-membered E-ring (1.5 ± 0.2 nM vs. 4.9 ± 0.4 nM in HepG2; 0.5 ± 0.1 nM vs. 2.2 ± 0.3 nM in PANC-1; and 2 ± 0.5 nM vs. 5.2 ± 0.5 nM in CEM as shown in Table 1). In contrast, precursor #1 and #2 were the least potent compounds among the tylopho-

Table 1. The GI_{50} of tylophora alkaloids and PBTs on the growth inhibition of HepG2, PANC-1, and CEM cells

	HepG2 GI_{50}[a] (nM)	PANC-1GI_{50}[a] (nM)	CEM GI_{50}[a] (nM)
(+)-(*S*)-tylophorine	11 ± 4[b]	12 ± 2	15 ± 3
(−)-(*R*)-tylophorine	33 ± 2	29 ± 6	67 ± 6
(−)-antofine	4.9 ± 0.4	2.2 ± 0.3	5.2 ± 0.5
Rac-cryptopleurine	1.5 ± 0.2	0.5 ± 0.1	2 ± 0.5
Precursor #1	1723 ± 417	904 ± 297	567 ± 58
Precursor #2	>10,000	>10,000	>2000
(−)-Ficuseptine C	371 ± 27	156 ± 26	323 ± 13
	HepG2 GI_{50}[a] (μM)	PANC-1 GI_{50}[a] (μM)	CEM GI_{50}[a] (μM)
PBT #28	2.3 ± 0.3	2.2 ± 0.3	2.0 ± 0.3
PBT #31	8.6 ± 3.6	7.4 ± 0.3	6.2 ± 0.2
PBT #32	5.4 ± 1.4	6.2 ± 0.7	2.7 ± 0.1
PBT #33	3 ± 0.7	2.3 ± 0.2	1.7 ± 0.3
PBT #34	3.1 ± 1.0	2.3 ± 0.2	1.6 ± 0.5

[a] Values are means ± SD of three experiments, with each data point done in triplicate.
[b] Published.[6]

ra-related alkaloids listed in Table 1. The structural difference between rac-cryptopleurine and precursor #1 is that the D-ring is formally opened in precursor #1, which leads to a dramatic decrease of cytotoxicity. For instance, in HepG2 cells, the GI_{50} value of rac-cryptopleurine is 1.5 ± 0.2 nM, whereas the GI_{50} value of precursor #1 is 1723 ± 417 nM. This represents a more than 1000-fold decrease of cytotoxicity. Precursor #2 represents the bare phenanthrene backbone of the tylophora alkaloids lacking the fused aliphatic heterocyclic domain. This structural change is not tolerated and results in very poor cytotoxicity, with a GI_{50} value of over 10 μM in HepG2 and PANC-1 cells (Table 1). The difference in the structures of (−)-ficuseptine C and (−)-antofine consists in the replacement of the OMe groups at R^1 and R^2 in (−)-antofine by a cyclic methylenedioxy unit in (−)-ficuseptine C. This structural change resulted in a more than 60-fold decrease of cytotoxicity in HepG2, PANC-1, and CEM cells (Table 1, compare 371 ± 27 nM vs. 4.9 ± 0.4 nM in HepG2; 156 ± 26 nM vs. 2.2 ± 0.3 nM in PANC-1; and 323 ± 13 nM vs. 5.2 ± 0.5 nM in CEM). Other phenanthrene-based tylophorine derivatives were designed to increase the polarity as mentioned previously which was achieved by formal opening of the indolizidine.[13] However, this structural modification dramatically impaired the cytotoxicity. As shown in Table 1 (lower panel), the GI_{50}s of all PBTs tested are between 1.6 and 8.6 μM in HepG2, PANC-1, and CEM cells.

Previously, we had shown that DCB-3503 and its analogs could inhibit NF-κB, CRE, and AP-1 mediated transcription, more selectively against NF-κB signaling pathway.[6] NF-κB is a family of transcription factors that play pivotal roles in chronic and acute inflammatory diseases, autoimmune diseases, and different types of cancer.[18] Recently, NF-κB and the signaling pathways that regulate its activity have become a focus of intense drug discovery and development efforts.[18] The potent inhibitory effects of DCB-3503 on NF-κB-mediated transcription may be one of the mechanisms of its antitumor activity. Here we compared the effects of the tylophora alkaloids (Fig. 1) against NF-κB, CRE, AP-1, and GRE mediated transcription. HepG2-NF-κB-luc, HepG2-CRE-luc, and HepG2-AP-1-luc stable cell lines were generated as previously described.[16] GRE mediated transcription was analyzed by transient transfection of HepG2 cells with pGRE-luc (Clontech). The IC_{50} of the inhibitory effect of the tylophora alkaloids against stimulator induced NF-κB, CRE, AP-1, GRE activity is listed in Table 2. The IC_{50} of the inhibitory effect of tylophora alkaloids against endogenous NF-κB, CRE, AP-1, and GRE mediated transcription is shown in Supplementary data Table S1. Among the four signaling pathways, NF-κB is the most sensitive signaling pathway inhibited by all the tylophora alkaloids (listed in Table 2, upper panel) except precursor #2. For instance, the IC_{50}'s of (−)-antofine for NF-κB, CRE, AP-1, and GRE are 7.3 ± 1.9 nM, 167 ± 42 nM, 135 ± 16 nM, and 24 ± 0.5 nM, respectively. NF-κB is about 20-fold more sensitive in terms of inhibition than CRE and AP-1 pathways. In addition, the rank order of their potency in terms of NF-κB inhibition was rac-cryptopleurine>(−)-antofine>(+)-(*S*)-tylophorine>(−)-(*R*)-tylophorine>(−)-ficuseptine C>precursor #1 > precursor #2. Similar rank order was observed in regards to the potency of cytotoxicity (Table 1). The rank order of potency and selectivity against endogenous NF-κB mediated transcription also had the same trend (Supplementary data Table 1). In terms of relative activity against different signaling pathways, the tylophora alkaloids have demonstrated some degree of selectivity. However, the activity of all the PBTs tested against all the four signaling pathways is very poor, with IC_{50} of more than 6 μM (Table 2, lower panel). If we calculate the relative ratio of IC_{50} (Table2) and GI_{50} (Table 1, Column HepG2) for (−)-antofine, the value would be 1.5, 34, 28, and 4.9 for NF-κB, CRE, AP-1, and GRE respectively. If we apply the same calculation for precursor #1, the value would be 1.3, 7, 5.7, and 2.6 for NF-κB, CRE, AP-1, and GRE respectively. This indicated that (−)-antofine selectively inhibited NF-κB, whereas precursor #1 showed less selectivity against the four signaling pathways. These data suggested that the different analogs might act on multiple target sites and affect different pathway through different mechanisms.

Early studies in the 1970s demonstrated that the phenanthrene alkaloid tylocrebrine inhibited protein and nu-

Table 2. The IC_{50} of the inhibitory effect of tylophora alkaloids and PBTs against stimulated NF-κB, CRE, AP-1, and GRE pathways in HepG2 cells

	NF-κB IC_{50}[a] (nM)	CRE IC_{50}[a] (nM)	AP-1IC_{50}[a] (nM)	GRE IC_{50}[a] (nM)
(+)-*S*-tylophorine	41 ± 20[b]	>300[b]	>300[b]	229 ± 33
(−)-*R*-tylophorine	317 ± 51	7910 ± 344	2257 ± 123	534 ± 113
(−)-antofine	7.3 ± 1.9	167 ± 42	135 ± 16	24 ± 0.5
Rac-cryptopleurine	1.4 ± 0.6	25 ± 0.5	18.6 ± 0.8	10 ± 0.2
Precursor #1	2171 ± 351	12,025 ± 830	9776 ± 625	4500 ± 312
Precursor #2	>10,000	>15,000	>15,000	>15,000
(−)-Ficuseptine C	1244 ± 93	2566 ± 352	3383 ± 158	2440 ± 651
	NF-κB IC_{50}[a] (μM)	CRE IC_{50}[a] (μM)	AP-1IC_{50}[a] (μM)	GRE IC_{50}[a] (μM)
PBT #28	7.9 ± 1.1	8.6 ± 0.6	8.7 ± 2.1	9.2 ± 1
PBT #31	23.5 ± 2.2	25.0 ± 6	27 ± 4.4	>30
PBT #32	21 ± 0.5	24 ± 0.8	>30	19 ± 4.2
PBT #33	7 ± 0.2	12.1 ± 0.4	6.2 ± 0.3	7.2 ± 1.9
PBT #34	10.2 ± 1.1	18.5 ± 2.1	10 ± 1.8	20.7 ± 2.8

[a] Values are means ± SD of three experiments, with each data point done in triplicate.
[b] Published.[16]

cleic acid synthesis.[11] During our studies of the mechanism of action of the tylophorine analog DCB-3503, we also observed that this compound could inhibit amino acid and thymidine, but not uridine incorporation in a time-dependent and dose-dependent manner (Gao et al., unpublished data). We observed that (−)-ficuseptine C, precursor #1, and PBTs differ significantly in terms of their cytotoxicities and their potency and selectivity against the NF-κB signaling pathway (Tables 1 and 2); we decided to investigate their effects against protein, DNA and RNA synthesis, by comparing their effects against L-[^{35}S]-methionine incorporation into protein, [^{14}C]-thymidine incorporation into DNA, and [^{14}C]-uridine incorporation into RNA. The experimental procedures are available in Supplementary data. The concentrations tested were based on each analog's specific cytotoxicity (1/3, 1, 3, and 10 EC_{50}).

As shown in Figure 2A and B, both (−)-antofine and (−)-ficuseptine C inhibited DNA and protein synthesis in a dose-dependent manner. In contrast, (−)-antofine inhibited RNA synthesis only slightly, whereas (−)-ficuseptine C also inhibited RNA synthesis in a dose-dependent manner. There are two major structural differences between PBTs and the rest of the tylophora alkaloids listed in Figure 1: (1) the OMe groups at R^1 and R^2 are replaced by a cyclic methylenedioxy moiety; (2) the indolizidine ring of the natural products has been formally opened. By comparing the effects of (−)-antofine and (−)-ficuseptine C, the role of the replacement of the OMe groups at R^1 and R^2 by a methylenedioxy bridge was clarified. Therefore, our results suggest that the replacement of the OMe ethers at R^1 and R^2 by a methylenedioxy moiety significantly changes the compound's mechanism of action. The major difference between (−)-antofine and precursor #1 is the opening of D-ring. (−)-ficuseptine C shares the methylenedioxy motif with the PBTs and has a similar pyrrolidine ring as compound PBT #32. Therefore, by comparing the effects of (−)-antofine and precursor #1, and the effects of (−)-ficuseptine C with those of PBT #32, the effect of opening the indolizidine ring was clarified. As shown in Figure 2C and D, both precursor #1 and PBT #32 inhibited DNA and RNA synthesis in a dose-dependent manner. In contrast, PBT #32 only slightly inhibited protein synthesis, whereas precursor #1 also inhibited protein synthesis in a dose-dependent manner. These results indicate that the formal opening of the D-ring or the indolizidine ring significantly changes the mechanism of action of these compounds (compare the profile changes between Fig. 2A/C and B/D). In addition, the distinct effects between (−)-antofine and PBT #32 [(−)-antofine more selectively inhibited protein synthesis, but not RNA synthesis (Fig. 2A), whereas PBT #32 more selectively inhibited RNA synthesis, but not protein synthesis (Fig. 2D)] demonstrate that replacement of the OMe groups at R^1 and R^2 by a methylenedioxy unit and the opening of the indolizidine ring totally changes their mechanism of action. Due to the significantly divergent effects of (−)-antofine, PBT #32, (−)-ficuseptine C, and precursor #1 against protein and nucleic acid synthesis, we conclude that these compounds, although being structurally fairly close, may not constitute the same class of compounds in functional terms. The concept of structural analogs with different mechanisms of action has been reported previously.[19,20] Kimball et al. recently described a series of tylophora analogs derived from Tyloindicine I, operated through an unknown mechanism of action different from the parental compound.[19] Another example is the comparison of podophyllotoxin and etoposide (VP-16), podophyllotoxin is a potent inhibitor of microtubule assembly, whereas its derivative etoposide, currently used in clinical treatment of many cancers such as small cell lung carcinoma and testicular cancer, does not inhibit tubulin polymerization, but rather acts as an inhibitor of DNA topo-isomerase II, which causes double strand breaks in DNA.[20]

In summary, phenanthrene-based compounds exhibit pronounced structure–activity relationships; both the replacement of the OMe ethers at R^1 and R^2 by a methylenedioxy bridge (Fig. 1) and the opening of the indolizidine ring result in dramatically reduced cytotoxic potency, whereas the loss of the OMe group at R^3 and the replacement of the five-membered E-ring by a six-

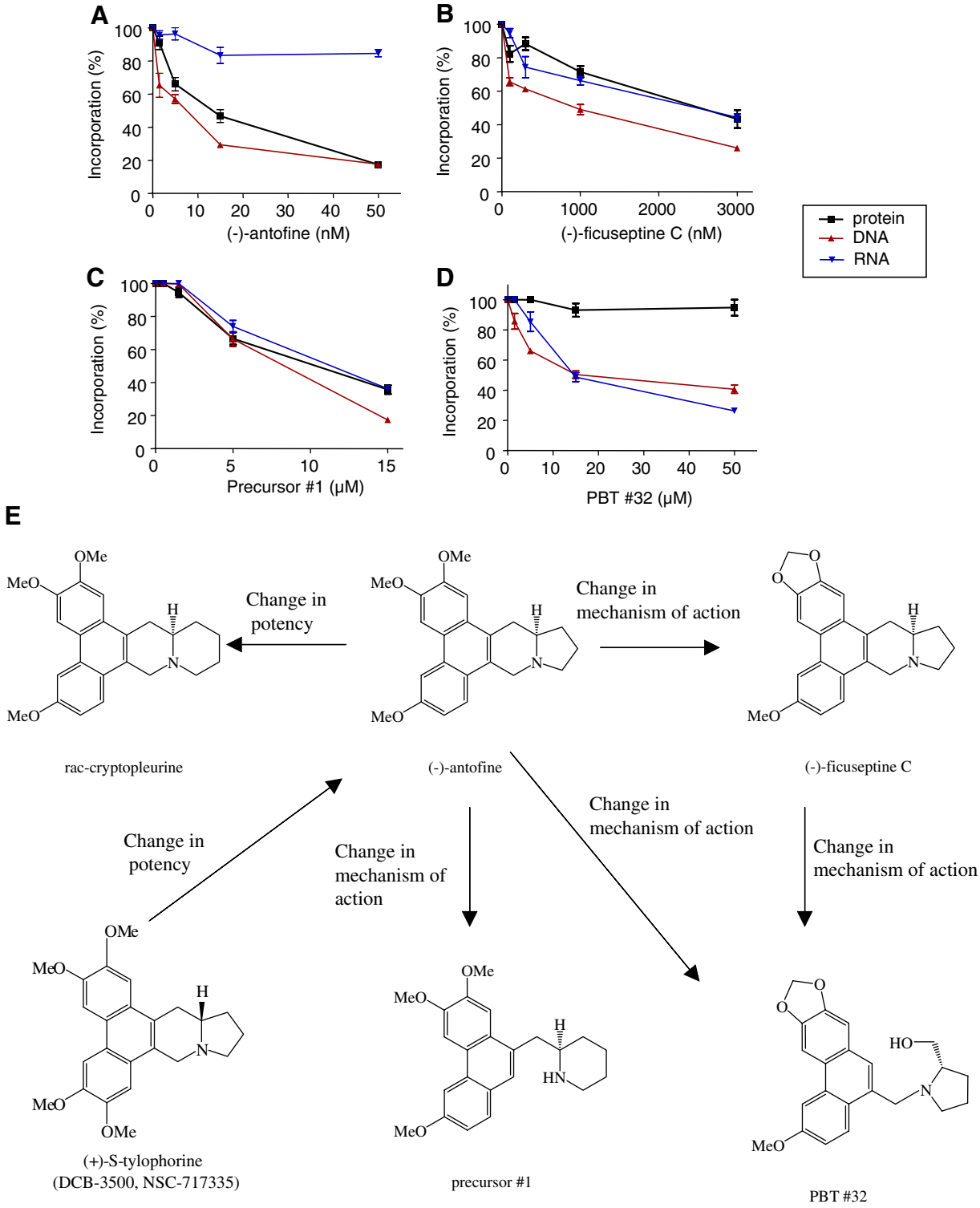

Figure 2. The effects of tylophora alkaloids and phenanthrene-based tylophorine derivatives on L-[^{35}S]-methionine, [^{14}C]-thymidine, and [^{14}C]-uridine incorporation. HepG2 cells were pretreated with serial dilutions of drugs for 5 min and labeled with L-[^{35}S]-methionine, [^{14}C]-thymidine or [^{14}C]-uridine, respectively. Their incorporation percentage (compared with control) is shown. (A) HepG2 cells were pretreated with (−)-antofine 1.5 nM, 5 nM, 15 nM, and 50 nM, respectively. (B) HepG2 cells were pretreated with (−)-ficuseptine C 100 nM, 300 nM, 1 μM and 3 μM, respectively. (C) HepG2 cells were pretreated with precursor #1 0.5 μM 1.5 μM, 5 μM, and 15 μM, respectively. (D) HepG2 cells were pretreated with PBT #32 1.5 μM, 5 μM, 15 μM, and 50 μM, respectively. (E) Schematic description of the SAR of tylophora alkaloids and PBTs.

membered E-ring significantly increased the observed cytotoxicity. Although these compounds are close structural analogs, their cytotoxic potency, selectivity against NF-κB signaling pathway, and their effects against protein, DNA, and RNA synthesis are so different that they should not be considered as the same class of compounds. That is, various tylophora alkaloids may be structural but not functional analogs.

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Supplementary data

Supplementary data associated with this article (Synthesis of phenanthrene-based tylophorine derivatives described in Scheme 1, synthesis of tylophora alkaloids exemplified in Scheme 2, IC_{50}s of the inhibitory effect of tylophora alkaloids and PBTs against endogenous NF-κB, CRE, AP-1 and GRE mediated transcription in HepG2 cells listed in Table S1) can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.054.

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