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Synthesis and Biological Activity of a Photoaffinity Etoposide Probe

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Abstract—The epipodophyllotoxin etoposide is a potent and widely used anticancer drug that targets DNA topoisomerase II. The synthesis, photochemical, and biological testing of a photoactivatable aromatic azido analogue of etoposide also containing an iodo group is described. This azido analogue should prove useful for identifying the etoposide interaction site on topoisomerase II. Irradiation of the azido analogue and an aldehyde-containing azido precursor with UV light produced changes in their UV–visible spectra that were consistent with photoactivation. The azido analogue strongly inhibited topoisomerase II and inhibited the growth of Chinese Hamster Ovary cells. Azido analogue-induced topoisomerase II–DNA covalent complexes were significantly increased subsequent to UV irradiation of drug-treated human leukemia K562 cells as compared to etoposide-treated cells. These results suggest that the photoactivated form of etoposide is a more effective topoisomerase II poison either by interacting directly with the enzyme or with DNA subsequent to topoisomerase II-mediated strand cleavage.

Introduction

The epipodophyllotoxins etoposide and teniposide, like a number of other topoisomerase II targeted antitumor drugs that include the anthracyclines (doxorubicin and daunorubicin), and amsacrine, are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex).[1,2] In this way topoisomerase II is converted into a poison that generates double stranded DNA breaks that are lethal to the cell. Etoposide is one of the most widely used anticancer drugs and is active against small-cell lung cancers, leukemias, and lymphomas.[1,3] Etoposide strongly inhibits religation of the cleavable complex, but has little effect on strand passage and ATP hydrolysis.[3] Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix.[2] Several methods can be used to define drug interaction domains on topoisomerase II.[1] These include: (1) the sequencing of drug resistant mutant topoisomerase II's;[4] (2) competition experiments that take advantage of differences in drug classes;[1,5] and (3) the use of a close analogue of the drug that can be photoactivated to covalently bind to topoisomerase II. The study of mutant enzymes has not, however, clearly defined the etoposide–drug interaction domain because of the lack of direct evidence placing any mutation within the drug binding site, the general lack of critical transfection experiments,[4] and the lack of structural information for the mutant topoisomerase II's.[1] Upon photoactivation aryl azido photoaffinity probes produce an extremely reactive nitrene species that binds to drug or ligand interaction sites.[6,7] For example, a photoactivatable form of amsacrine, 3-azidoamsacrine,[8] has been utilized to demonstrated that the site of drug interaction on DNA is near the site of double strand cleavage.[9] We previously described the synthesis and biological activity of a fluorescent etoposide analogue.[10] In this communication, we describe the synthesis and characterization of the photochemical and biological activity of a photoactivatable azido analogue of etoposide containing an iodo group. In the future, this azido analogue will be used to identify putative etoposide interaction site(s) on topoisomerase II and/or characterize etoposide–DNA interactions. The iodo group on the analogue provides a convenient site for incorporating a radioactive iodine label into the probe. The

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azido analogue may also be useful for further identification and characterization of membrane-associated multi-drug resistance efflux pumps.

Results and Discussion

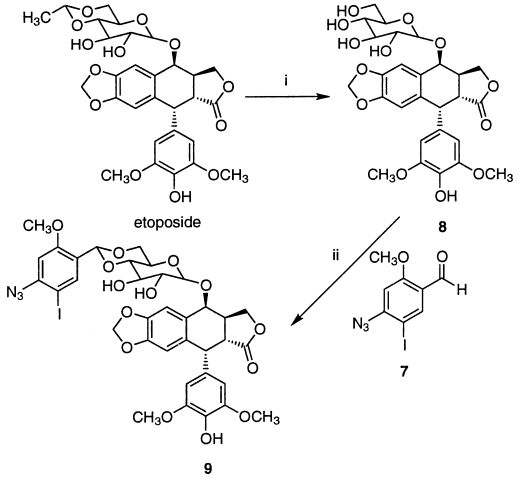
Chemistry

Conversion of 4-amino-2-hydroxybenzoic acid (**1**) to 4-azido-2-hydroxybenzoic acid (**2**) was accomplished by reaction with sodium azide in the presence of sulfuric acid and sodium nitrite using a slight modification of a method that has been described (Scheme 1).[11] Esterification to yield methyl 4-azido-2-hydroxybenzoate (**3**) and iodination to yield methyl 4-azido-2-hydroxy-5-iodobenzoate (**4**) using chloramine-T were carried out as previously described.[12,13] Methyl 4-azido-5-iodo-2-methoxybenzoate (**5**) was prepared by methylation of the phenol with methyl iodide in the presence of K_2CO_3 as briefly described.[14] Reduction of the ester **5** to 4-azido-5-iodo-2-methoxybenzyl alcohol (**6**) was achieved using diisobutylaluminum hydride (DIBALH). Oxidation of the alcohol **6** to 4-azido-5-iodo-2-methoxybenzaldehyde (**7**) was carried out using CrO_3 in dilute sulfuric acid. Hydrolysis of etoposide to form 4′-demethylepipodophyllotoxin β-D-glucopyranoside[15] (**8**) was accomplished in dilute acetic acid/water with close temperature control (Scheme 2). Finally, the condensation of **8** to yield the cyclic acetal target compound 4′-demethyl-1-*O*-[4,6-*O*-(4-azido-5-iodo-2-methoxybenzylidene)-β-D-glucopyranosyl]epipodophyllotoxin (**9**) was carried out in nitromethane using *p*-toluensulfonic acid as a catalyst using a slight modification of a previously described procedure.[16]

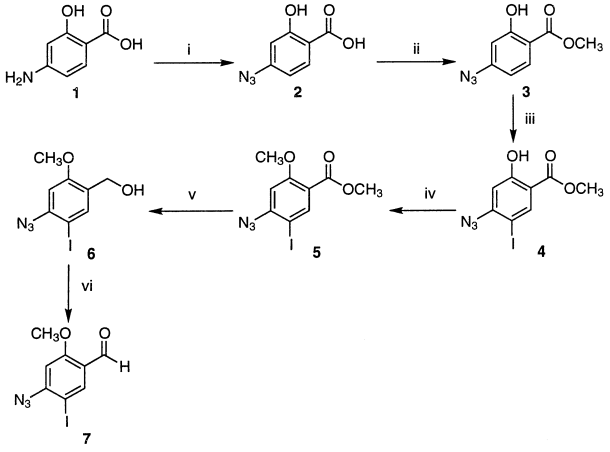
Photochemical kinetics

The effect of UV irradiation on the UV–vis spectrum was examined for both the aldehyde **7** and the target etoposide analogue **9**. This was done both to demonstrate that the target compound **9** could be photolyzed and to define the conditions (wavelength, power, irradiation time) that would be required for biological testing. Aryl azides are well known to undergo UV–vis spectral changes upon photolysis.[8] The spectral changes that occurred upon UV irradiation of a solution of **7** in methylene chloride at 366 nm using a lower power hand lamp for fixed periods of time are shown in Figure 1. Large changes in the spectrum were seen over the whole spectral region. The presence of the same five isosbestic points for each scan suggests that there are only two absorbing species in solution, the reactant **7** and a photolyzed product. Only small spectral changes were seen after 30 s of irradiation, indicating that nearly full photolysis had occurred by this time.

Irradiation of **9** in water under the power and wavelength conditions that were used to obtain the data shown in Figure 1 resulted in only a small spectral changes (data not shown) after several minutes of irradiation. The reduced sensitivity of **9** to photolysis was



Scheme 2. Synthesis of photoaffinity etoposide probe **9**: (i) 20% $AcOH/H_2O$, 70 °C; (ii) *p*-$TsOH/CH_3NO_2$.



Scheme 1. Synthesis of photoaffinity aldehyde intermediate **7**: (i) $H_2SO_4/NaNO_2$, NaN_3 ~0 °C; (ii) CH_3OH/H_2SO_4, reflux; (iii) NaI/chloramine-T/DMF; (iv) K_2CO_3/MeI/acetone, reflux; (v) DIBALH/THF, −40 °C; (vi) CrO_3/H_2SO_4/ether, rt.

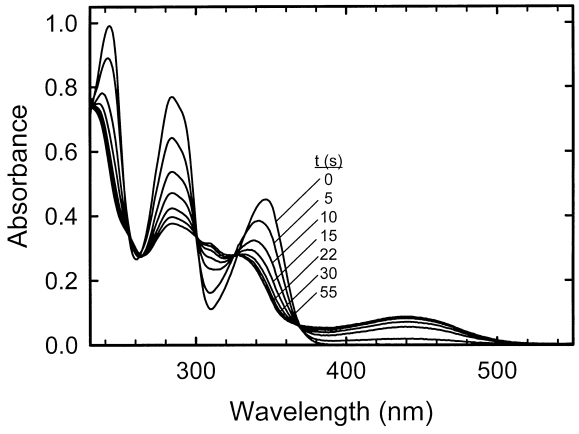


Figure 1. Spectral changes that occurred upon the irradiation of **7** for different times. The aldehyde **7** (40 μM in methylene chloride) was irradiated in a 1-cm silica spectrophotometer cell at 366 nm with a UV lamp (1 mW/cm^2 output) for the times indicated.

most likely due to nonproductive absorption by the two phenyl groups on **9** that do not contain a photolabile azido group, or possibly due to a lower quantum yield for **9**. Results from Figure 2A show the spectral changes that occurred upon 302 nm UV irradiation of **9** dissolved in water using the higher power and lower wavelength transilluminator for fixed periods of time. Because the absorbance changes were relatively small, the difference spectra are also shown in Figure 2B. The changes in the difference spectra indicated that **9** was photolyzed. Two isosbestic points can also be seen in Figure 2A and B, which also suggests that **9** was photolyzed to a single absorbing product. The irradiation of **9** at 302 nm was also carried out in methylene chloride (data not shown) for comparison with the data for **7** in Figure 1. These spectral changes overall were similar to those that were observed for the photolysis of **7** in methylene chloride. The spectral changes in methylene chloride were, however, relatively larger than those observed in water and displayed four isosbestic points. Together, these results indicate that **9** can be photolytically activated by UV light and the data obtained provided the wavelengths, minimum power, and times necessary to produce a photoactive species from **9** for the subsequent biological testing.

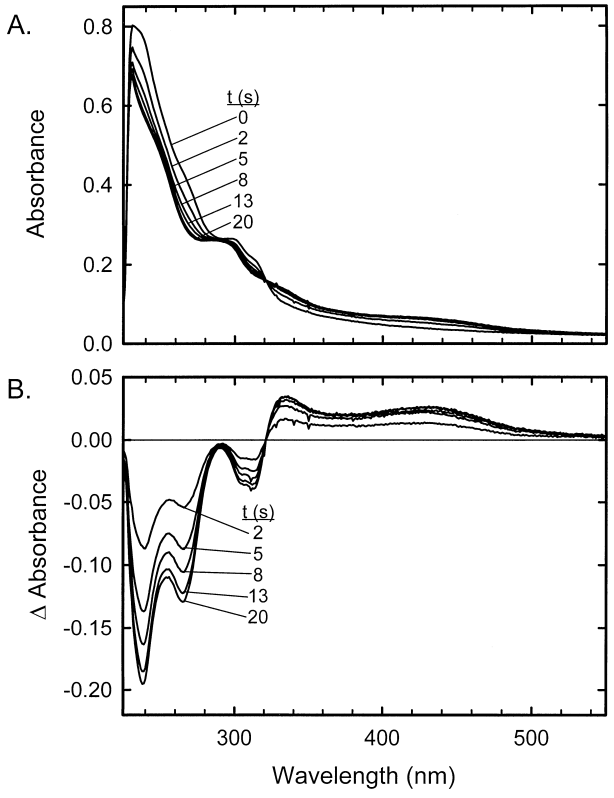


Figure 2. (A) Spectral changes that occurred upon the irradiation of **9** for different times. The target photoaffinity etoposide probe **9** (20 μM) in water was irradiated in a 1-cm silica spectrophotometer cell at 302 nm (8 mW/cm^2 output) for the times indicated (B) difference spectra for the data in (A). The difference spectra were calculated by subtracting the spectrum in (A) at time zero from the spectra recorded at the other indicated times.

Comparison of topoisomerase II inhibition, CHO cell growth inhibitory, and topoisomerase II poisoning effects of 9 and etoposide

For precise comparison of the biological activity of etoposide and its azido analogue, purity of **9** was assured by CHN and I combustion elemental analysis that agreed closely with the theoretical composition (within 0.04, 0.06, 0.19, and 0.24%, respectively). The topoisomerase II inhibitory effects of **9** and etoposide, measured in a 30 min decatenation assay, are compared in Figure 3A. and show that etoposide was 1.6-fold more inhibitory than **9** (IC_{50} value of 6.7 μM) compared to that of etoposide (IC_{50} value of 4.1 μM). The effect of a 72 h continuous exposure of **9** and etoposide on the growth of CHO cells is compared in Figure 3B. In this assay, the target compound **9** was somewhat more cytotoxic than etoposide with an IC_{50} value of 0.30 μM

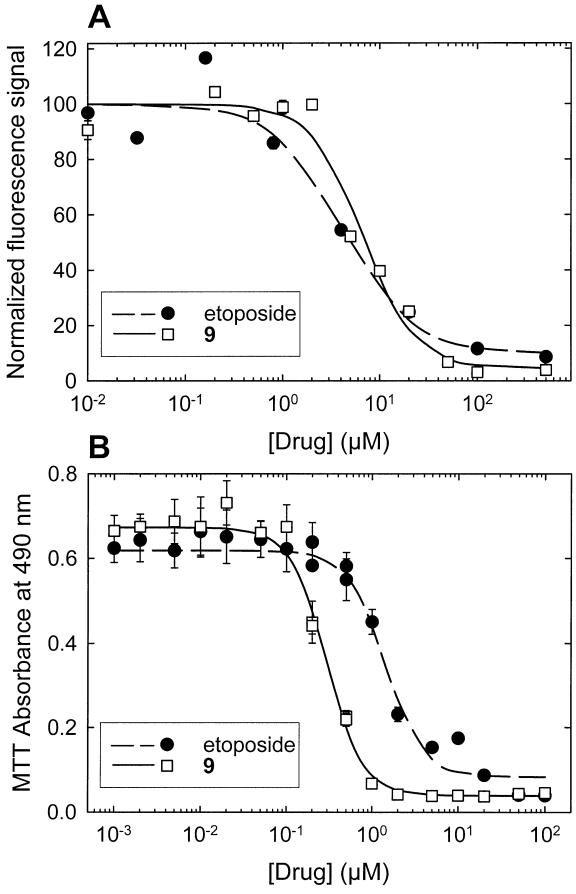


Figure 3. (A) Comparison of the topoisomerase II inhibition by **9** (□, solid line) and etoposide (●, dashed line). The decatenation of kDNA was used to measure the topoisomerase II activity. The lines are non-linear least squares calculated fits of the data to a 4-parameter logistic equation and yield IC_{50}'s of 6.7 and 4.1 μM for **9** and etoposide, respectively. The leftmost points plotted on the axis were determined in the absence of drug. The values shown are from triplicate determinations and where the error bars are not shown they are smaller than the symbol. (B) Comparison of the growth inhibitory effects of **9** (□, solid line) and etoposide (●, dashed line) on CHO cells as measured by MTT assay. The MTT assay was carried out after continuous exposure to the drugs for 72 h. The lines are non-linear least squares calculated (SigmaPlot) fits of the data to a sigmoid four-parameter modified receptor binding logistic equation and yield IC_{50}'s of 0.30 and 1.43 μM for **9** and etoposide, respectively.

compared to that of etoposide of 1.43 μM. The fact that **9** was more growth inhibitory than etoposide, in spite of the fact that it was a slightly weaker inhibitor of topoisomerase II, may be because of the greater lipophilicity of **9** compared to etoposide that could result in enhanced cellular drug uptake. The logarithm of the octanol–water partition coefficient was calculated to be 5.4 for **9** compared to 2.4 for etoposide. The IC_{50} values for **9** and etoposide derived from the 72 h growth inhibitory experiments in CHO cells were 5–10-fold lower than those generated from 30 min in vitro decatenation assays performed using nuclear extracts isolated from CHO cells. We attribute these differences mainly to the longer incubation times in growth inhibitory assays. In addition, in vitro decatenation assays are run under conditions where ATP concentrations are fixed at 1 mM and where high salt nuclear extracts are utilized that contain proteins aside from topoisomerase II that could bind to and modulate drug activity. Hence, the differences in these assays systems can easily account for the differences in IC_{50} values. Taken together, our results are consistent with both **9** and etoposide exerting their activity at the level of topoisomerase II with comparable potency.

The effect of UV irradiation on etoposide- and **9**-induced topoisomerase II-DNA covalent cleavable complex formation was also examined (Fig. 4). Results indicated that UV irradiation of K562 cells at 302 nm for 30 s, after a 60 min incubation in the presence of **9** (10 μM), significantly increased ($p = 0.0083$) drug-induced topoisomerase II–DNA covalent complexes. Etoposide-induced (10 μM) topoisomerase II–DNA covalent complexes were not potentiated subsequent to UV irradiation of drug-treated cells. In the absence of UV irradiation, both etoposide (10 μM) and **9** (10 μM) induced topoisomerase II–DNA complexes to a similar extent compared to DMSO solvent controls (2.75 ± 0.49- and 3.24 ± 0.35-fold for etoposide and **9**, respectively, mean ± SE, $p = 0.49$, Student's *t*-test). These results suggest that **9** targets topoisomerase II and poisons the enzyme more potently after UV irradiation.

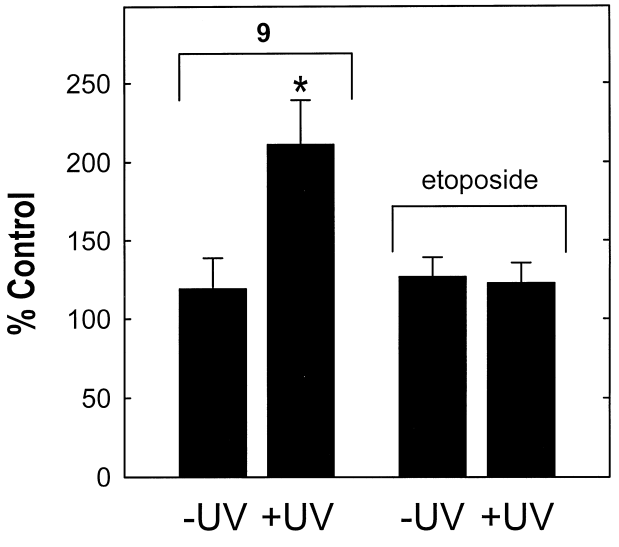


Figure 4. Effect of 30 s of UV irradiation at 302 nm (8 mW/cm^2 output) on etoposide- and **9**-induced topoisomerase II–DNA covalent cleavable complex formation in K562 cells. Cells were treated with drugs or DMSO solvent control for 60 min, exposed 30 s in the absence or presence of UV light (302 nm; 8 mW/cm^2), incubated for a further 40 min, followed by assessment of KCl/SDS-precipitable protein-DNA complexes.[17] The results are expressed as the percentage of control topoisomerase II–DNA complexes formed after the initial 60 min drug incubation. The errors shown are SE's from five separate experiments for etoposide and four experiments for **9**. The asterisk indicates statistical significance by a Student's paired *t*-test ($p = 0.008$) for the UV irradiation for **9** compared to no UV irradiation.

Conclusion

In conclusion, the etoposide analogue **9** that was synthesized was shown to be even more strongly growth inhibitory towards CHO cells than etoposide. The UV–vis spectral changes that occurred upon UV irradiation of the azido compounds **7** and **9** indicated that these compounds underwent facile photolysis in solution. The enhanced activity of **9** after UV irradiation could have resulted from enhanced formation or slower decay of topoisomerase II–DNA covalent complexes. Photactivation of **9** might have resulted in covalent binding of the reactive nitrene species to topoisomerase II directly and/or to cleaved DNA strands as has been demonstrated previously for photoactivated azidoamsacrine.[9] Future work will elucidate the exact mechanism(s) of UV-mediated activation/potentiation of this photoactivatable analogue of etoposide. In particular, synthesis of an ^{125}I-labeled compound **9** is underway and should permit isolation of drug/enzyme complexes subsequent to incubation of CHO cells with ^{125}I-**9** and UV irradiation. In addition, use of etoposide resistant cell lines containing topoisomerase II mutations should be useful to further establish that topoisomerase II is the primary intracellular target for **9**. The etoposide photoaffinity probe **9** should prove useful in elucidating the mechanism of action of this important anticancer drug.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-300 FT instrument. High-resolution mass spectra (HR-MS) were recorded on an Analytical VG 7070E-HF instrument. Melting points were measured on a hot stage instrument and are uncorrected. Merck silica gel (200–400 mesh, 60 Å, Aldrich, Oakville, Canada) was used for all column chromatography. Preparative thin layer chromatography was carried out on Fisher (Nepean, Canada) silica gel glass plates (layer thickness 250 μm, particle size 5–17 μm, pore size 60 Å, plate dimension 20×20 cm). Tetrahydrofuran (THF) was distilled from $LiAlH_4$ under argon. Nitromethane was dried over $CaSO_4$ and then distilled under argon. All reactions involving azido derivatives were carried out in the dark because of their photosensitivity. Combustion elemental analyses were performed by Midwest Microlab LLC (Indianapolis, IN, USA). The octanol-water partition coefficients were calculated based on the frag-

mental constants database (Pallas v. 1.2, CompuDrug International, South San Francisco, CA, USA).

4-Azido-2-hydroxybenzoic acid (2). This compound was prepared from **1** by a slight modification of a method that has been described.[11] The NMR data are reported here for the first time: ^{1}H NMR (DMSO-d_6) δ 6.63 (1H, d, J = 2.1 Hz), 6.66 (1H, dd, J = 2.1, 8.4 Hz), 7.79 (1H, d, J = 8.4 Hz).

Methyl 4-azido-5-iodo-2-methoxybenzoate (5). Methylation of the phenol **4** has been only briefly described.[14] A suspension mixture of **4** (1.45 g, 4.55 mmol), anhydrous K_2CO_3 (3 g), and methyl iodide (2.8 mL, 45.0 mmol) in acetone (50 mL) was heated under argon at reflux for 5 h. The mixture was concentrated under vacuum to remove most of the solvent, diluted with water (30 mL), and then extracted with methylene chloride (3×20 mL). The organic layers were combined, washed with brine (2×30 mL), dried (Na_2SO_4), filtered and concentrated under vacuum to leave a pale yellow solid (1.50 g, 99% yield). ^{1}H NMR of this product indicated >98% purity. It was further purified by recrystallization from methanol: mp 132–134 °C; ^{1}H NMR ($CDCl_3$) δ 3.87 (3H, s), 3.94 (3H, s), 6.68 (1H, s), 8.21 (1H, s); ^{13}C NMR ($CDCl_3$) δ 52.2 (CH_3), 56.4(CH_3), 75.7 (C), 102.3 (CH), 118.3 (C), 142.9 (CH), 146.4 (C), 160.8 (C), 164.3 (C); MS m/z (relative intensity): 333 (M^+, 31) 305 ([M–N_2]$^+$, 100), 290 (65), 276 (30); HR-MS calcd for $C_9H_8O_3N_3I$ 332.9610, found 332.9608.

4-Azido-5-iodo-2-methoxybenzyl alcohol (6). To **5** (1.51 g, 4.53 mmol) in dry THF (30 mL) at −40 °C (CH_3CN/dry ice) under argon, diisobutylaluminum hydride (1.0 M in THF, 13 mL, 13 mmol) was added dropwise, over a period of 20 min. The mixture was stirred at −40 °C for 3.5 h before being quenched with ice-cold 1.0 M HCl (20 mL). After concentration under vacuum to remove most of the THF, the mixture was extracted with ethyl acetate (2×25 mL). The organic solutions were combined, washed with 1.0 M HCl (2×25 mL) and brine (2×25 mL), dried (Na_2SO_4), and concentrated under vacuum to afford yellow crystals (1.21 g, 87% crude yield). ^{1}H NMR spectrum of this product indicated about 92% purity. The product seemed relatively unstable as it turned green after standing at room temperature in the dark for about a week. It was used in the next reaction without further purification. After recrystallization from methanol/water: anal. ($C_8H_8IN_3O_2$) C, H, N, I. ^{1}H NMR ($CDCl_3$) δ 3.89 (3H, s), 4.60 (2H, d, J = 6.2 Hz), 6.63 (1H, s), 7.66 (1H, s); ^{13}C NMR ($CDCl_3$) δ 55.8 (CH_3), 60.6 (CH_2), 76.3 (C), 101.2 (CH), 128.4 (C), 139.1 (CH), 141.6 (C), 158.6 (C); MS m/z (relative intensity): 305 (M^+, 30), 277 ([M–N_2]$^+$, 21), 263 ([M–N_3]$^+$, 21), 249 (27), 217 (100); HR-MS calcd for $C_8H_8IN_3O_2$ 304.9661, found 304.9707.

4-Azido-5-iodo-2-methoxybenzaldehyde (7). The crude alcohol **6** (1.20 g, ca. 3.94 mmol) was suspended in diethyl ether (80 mL) and was stirred vigorously while 5% CrO_3 in 10% aqueous sulfuric acid (39 mL) was added in one portion. After 15 min the red mixture was diluted with cold water (80 mL). The organic layer was separated, washed with water (2×50 mL), 5% aq $NaHCO_3$ (2×50 mL), and brine (2×50 mL), dried (Na_2SO_4), and concentrated under vacuum to leave a brown solid. The product was discolored by boiling in methylene chloride (50 mL) and Darco® charcoal (<100 mesh, 1 g) for 2 min, followed by filtration through Celite. Concentration of the filtrate gave a pale yellow solid, which upon recrystallization from methanol–water afforded pale yellow needles (1.02 g, 74% yield from ester **5**): mp 152–154 °C; ^{1}H NMR ($CDCl_3$) δ 3.97 (3H, s), 6.69 (1H, s), 8.18 (1H, s), 10.24 (1H, s); ^{13}C NMR ($CDCl_3$) δ 56.1 (CH_3), 77.2 (C), 101.7 (CH), 123.5 (C), 139.9 (CH), 148.2 (C), 162.7 (C), 186.7 (C); MS m/z (relative intensity): 303 (M+, 26), 275 ([M−N_2]$^+$, 100), 260 (15), 246 (12); HR-MS calcd for $C_8H_6IN_3O_2$ 302.9505, found 302.9511. Anal. ($C_8H_6IN_3O_2$) C, H, N, I.

4′-Demethylepipodophyllotoxin β-D-glucopyranoside (8). A suspension of etoposide (245 mg, 0.416 mmol) in 20% acetic acid/water (100 mL) was heated at 68–72 °C for 20 h. The resulting solution was concentrated to dryness under vacuum at 30 °C, and further dried under high vacuum. Water (40 mL) was added and the mixture heated at 40 °C until most of the solid had dissolved. It was then cooled to room temperature, stirred vigorously with methylene chloride (50 mL), and then transferred to a separatory funnel. The organic layer was removed, and the aqueous layer further washed with methylene chloride (3×20 mL). The aqueous solution was lyophilized to give a white powder (163 mg, 69% yield): mp 230–232 °C (lit.[15] 225–227 °C); ^{1}H NMR (DMSO-d_6) δ 2.86 (1H, m), 2.94–3.16 (5H, m), 3.42 (1H, m, overlapped with H_2O peak), 3.60 (6H, s), 3.75 (1H, m), 4.24–4.38 (3H, m), 4.48 (1H, d, J = 5.4 Hz), 4.66 (1H, t, J = 5.8 Hz), 4.92 (2H, m), 4.99 (1H, d, J = 4.4 Hz), 5.02 (1H, d, J = 3.2 Hz), 6.00 (2H, AB, Δδ = 2.9, J = 0 Hz), 6.17 (2H, s), 6.52 (1H, s), 7.04 (1H, s), 8.24 (1H, s).

4′-Demethyl-1-*O*-[4,6-*O*-(4-azido-5-iodo-2-methoxybenzylidene)-β-D-glucopyranosyl]epipodophyllotoxin (9). The condensation of **7** with **8** was carried out by a slight modification of a procedure that has been described.[16] A suspension mixture of **8** (76 mg, 0.135 mmol), **7** (84 mg, 0.277 mmol), and *p*-toluenesulfonic acid (4.1 mg, monohydrated, *p*-TsOH) in dried nitromethane (2.0 mL) was bubbled with argon for 15 min, and then stirred under argon at room temperature for 2 days. The suspension was concentrated under vacuum at ~30 °C to give a colorless solid, which was then resuspended in methylene chloride (10 mL), filtered, and rinsed with methylene chloride (5 mL). According to the ^{1}H NMR, the residual solid (32 mg) was found to be predominantly the unreacted glucopyranoside **8** plus traces of unknowns. The filtrate was concentrated to 1 mL, and then chromatographed on TLC plates. The desired band was scraped out, eluted with methanol, and concentrated at ~30 °C to afford **9** as a colorless solid (40 mg, 35% yield): mp 209–214 °C dec. gradually; ^{1}H NMR ($CDCl_3$) δ 2.35 (1H, g-OH, d, J = 1.7 Hz), 2.65 (1H, g-OH, d, J = 1.7 Hz), 2.83–2.97 (1H, m), 3.27 (1H, dd, J = 5.2 Hz, 14.0), 3.42–3.58 (3H, m), 3.71–3.86 (2H, m), 3.77 (6H, s), 3.88 (3H, s), 4.24 (1H, dd, J = 8.3,

9.0 Hz), 4.30 (1H, dd, $J = 5.2$, 10.6 Hz), 4.44 (1H, dd, $J = 9.0$, 10.6 Hz), 4.62 (1H, d, $J = 5.1$ Hz), 4.72 (1H, d, $J = 7.6$ Hz), 4.94 (1H, d, $J = 3.4$ Hz), 5.40 (1H, s), 5.82 (1H, s), 6.00 (2H, AB, $\Delta\delta = 6.0$, $J = 1.2$ Hz), 6.27 (2H, s), 6.56 (1H, s), 6.62 (1H, s), 6.84 (1H, s), 7.92 (1H, s); ^{13}C NMR ($CDCl_3$) δ 37.5 (CH), 41.3 (CH), 43.8 (CH), 56.0 (CH_3), 56.5 (2× CH_3), 66.5 (CH), 68.0 (CH_2), 68.8 (CH_2), 73.1 (CH), 74.0 (CH), 74.5 (CH), 76.5 (CH), 80.6 (CH), 96.1 (CH), 101.4 (CH), 101.7 (CH_2), 102.1 (CH), 107.9 (2× CH), 109.0 (CH), 110.8 (CH), 124.2 (C), 128.3 (C), 130.5 (C), 132.9 (C), 134.1 (C), 138.3 (CH), 143.3 (C), 146.5 (2×C), 147.2 (C), 148.9 (C), 158.0 (C), 175.0 (C); electrospray ionization mass spectrum m/z: 846.3 (M−H, negative mode), 848.1 (M+H, positive mode). Anal. ($C_{35}H_{34}INO_{14}$) C, H, N, I.

Photochemical kinetics and irradiation of drug-treated cells

The photochemical reactions of **7** and **9** were followed spectrophotometrically in 1-cm stoppered silica cells on a Cary 1 spectrophotometer (Varian, Mulgrave, Australia). The UV light sources used, as indicated, were either a UVL-21 hand lamp (1 mW/cm^2 at 366 nm at 75 mm distance) placed at 49 mm from the cell placed on its side or, as indicated, on the surface of a M20E transilluminator (8 mW/cm^2 at 302 nm at the surface), both from UVP (San Gabriel, CA, USA). For the aqueous solution study, **9** was dissolved in DMSO and diluted into water to give a final concentration of DMSO of 0.5% (v/v). At this concentration of DMSO the UV cutoff (absorbance = 1) was 230 nm. The solutions were exposed to the UV light for fixed times and the UV–vis spectra were then recorded.

Experiments on the effect of irradiation on etoposide- or **9**-induced cleavable complex formation in cells were also carried out on K562 cells in open Petri dishes containing 1.5×10^6 cells/mL in a buffer containing 25 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), pH 7.4, containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, and 10 mM glucose. Cells were irradiated under an UVP transilluminator (model TM-15). The liquid height in the Petri dishes was 2 mm in all experiments and the distance from the surface of the UV illuminator to the liquid surface was 13 mm. The UV exposure was at 302 nm at 8 mW/cm^2, measured at the surface of the transilluminator. The cells were exposed to drugs or DMSO solvent for 60 min, irradiated with UV light for 30 s (or not) and then incubated for a further 40 min, followed by processing for assessment of protein–DNA complexes.[17]

Topoisomerase II inhibition assay

The inhibition of topoisomerase II was measured by the ATP-dependent decatenation of kinetoplast DNA (kDNA) obtained from Topogen (Columbus, OH, USA) by a modification of methods previously described.[18,19] Topoisomerase II-containing nuclear extracts were prepared from CHO cells as described.[20] The very high molecular weight network kDNA was separated from the lower molecular weight catenanes by centrifugation. The supernatant was assayed for DNA as the fluorescent DNA–4′,6-diamidino-2-phenylindole complex by flow injection on an HPLC apparatus equipped with fluorometric detection. The IC_{50} values and their SEs for enzyme inhibition were obtained from a non-linear least squares fit of the fluorescence peak area data (obtained from the chromatography software) to a four-parameter logistic equation (SigmaPlot, Jandel, San Rafael, CA, USA).

Cell culture and cytotoxicity assay

CHO cells (type AA8) obtained from the American Type Culture Collection (Rockville, MD, USA) were grown in α-MEM (Gibco BRL, Burlington, Canada) containing 20 mM HEPES (Sigma, St. Louis, MO, USA), 100 units/mL penicillin G, 100 μg/mL streptomycin, 10% calf serum (Gibco BRL) in an atmosphere of 5% CO_2 and 95% air at 37 °C (pH 7.4). Cells in exponential growth were harvested and seeded at 2000 cells/well in 96-well microtiter plates (100 μL/well) and allowed to attach for 24 h. Etoposide and **9** were dissolved in DMSO and added to the wells such that the final concentration of DMSO was 0.5% (v/v) (which was shown to have no significant effect on cell growth) and then made up to a final volume of 200 μL/well. The cells were then allowed to grow for a further 72 h. The cell growth was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-tetrazolium bromide (MTT) assay basically as described.[21] Briefly, 20 μL of MTT (2.5 mg/mL in PBS) was added to each well and the plate was incubated for a further 4 h. After careful aspiration of the media, 100 μL of DMSO was added and the plates were read at 550 nm with reference to the absorbance at 650 nm and appropriate blanks in a Molecular Devices (Menlo Park, CA, USA) plate reader. Six replicates were measured at each drug concentration. The IC_{50} values and their SEs for growth inhibition were obtained from a non-linear least squares fit of the absorbance–drug concentration data to a four-parameter logistic equation. Human K562 cells, obtained from the American Type Culture Collection, were grown in suspension in DMEM (Dulbecco's modified Eagle medium) containing 7% iron-supplemented newborn bovine serum (Hyclone, Logan, UT) and 2 mM L-glutamine (Gibco/BRL, Grand Island, NY, USA). Exponentially growing cells were used for all experiments.

Topoisomerase II–covalent complexes

Topoisomerase II–DNA covalent complex formation in intact K562 cells was measured as previously described.[17] Mid-log growth cells were labelled for 24 h with 0.5 μCi/mL [methyl-^{3}H]thymidine (0.5 Ci/mmol) and 0.1 μCi/mL [^{14}C]leucine (318 mCi/mmol) in DMEM containing 7.5% (v/v) iron-supplemented calf serum. Cells were then pelletted and resuspended in fresh DMEM/7.5% calf serum and incubated for 1 h at 37 °C. Cells were pelletted and resuspended in buffer (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM HEPES and 10 mM glucose at 37 °C at a final cell density of 1.0×10^6 cells/mL for

experimentation. Cells were then incubated with various concentrations of etoposide or **9** alone. Reactions were stopped by adding 1 mL of cell suspension to 10 mL of ice-cold PBS. Cells were then pelletted, lyzed, cellular DNA sheared, and protein–DNA complexes precipitated with SDS and KCl as described.[17]

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