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Apoptotic activities in closely related styryllactone stereoisomers toward human tumor cell lines: Investigation of synergism of styryllactone-induced apoptosis with TRAIL

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ABSTRACT

A related series of styryllactones with small functional and stereochemical variations were compiled for a comparative study of their apoptotic activities toward two tumorigenic and one non-tumorigenic control cell line. While a substantial range of intrinsic activity was observed, the relative order of activity of the different compounds toward the cell types varied somewhat as did the relative ratios of apoptosis and necrosis observed in conjunction with the loss of cell viability. While some of the styryllactones showed substantial activity, a small but significant apoptosis-induced synergism was demonstrated with (−)-altholactone and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).

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1. Introduction

The styryllactones are a family of natural products derived from plants of genus *Goniothalamus* of the family *Annonaceae* indigenous to South East Asia.[1] Many styryllactones have been isolated and well-characterized by spectroscopic and by other chemical analysis[2–4] including unambiguous stereospecific synthesis of the naturally occurring stereoisomers.[5–12]

A number of representative styryllactones have been shown to possess mild to moderate apoptotic activity toward numerous tumor cell lines.[13–15] However, little effort has been made to develop an understanding of either the origin of this activity or whether the apoptotic effect can be linked synergistically with other apoptotic agents. The observation of synergism has been previously made and developed as a useful concept for combination agents in cancer chemotherapy based on two or more different apoptotic activities.[16,17] A significantly reduced synergism toward normal-non-targeted cells compared with malignant cells would also need to be observed to realize the full therapeutic potential in such an approach.

Our recent unambiguous stereochemical synthesis of the styryllactone gonioheptolide A,[18] several closely related diastereomers of gonioheptolide A, and other related styryllactones, including (+)- and (−)-altholactone has allowed us to gain access to an increased diversity of closely related styryllactones. This versatile synthesis has given us an opportunity to expand structure–activity studies significantly and thus to test the importance of subtle stereochemical alterations on apoptotic activity. Further, the concept that any or all of the apoptotic styryllactones can act synergistically with other apoptotic agents acting by different mechanisms can also be examined. Here we report the baseline apoptotic activities of a related group of styryllactone stereoisomers and assess the potential of one of them to act synergistically with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).[19] We have also assessed baseline apoptotic activities of these styryllactones toward different tumor cell lines.

2. Results

Figure 1 shows the structures of styryllactones employed in theses studies. We have previously reported the stereoselective synthesis of (−)-gonioheptolide A **1** starting from L-(+)-tartrate.[18] In the course of that effort we also synthesized and reported the (+)-enantiomer **2** as well as the C-3 epimer **3** and the naturally-occurring (−)-goniofupyrone **4**. The synthetic strategy developed also proved to be useful in the synthesis of other stereoisomers and closely related stereochemically-defined analogues of **1**.

Compound **5** was synthesized from intermediate **8**[18] starting from (−)-diethyl tartrate in the Sharpless asymmetric epoxidation

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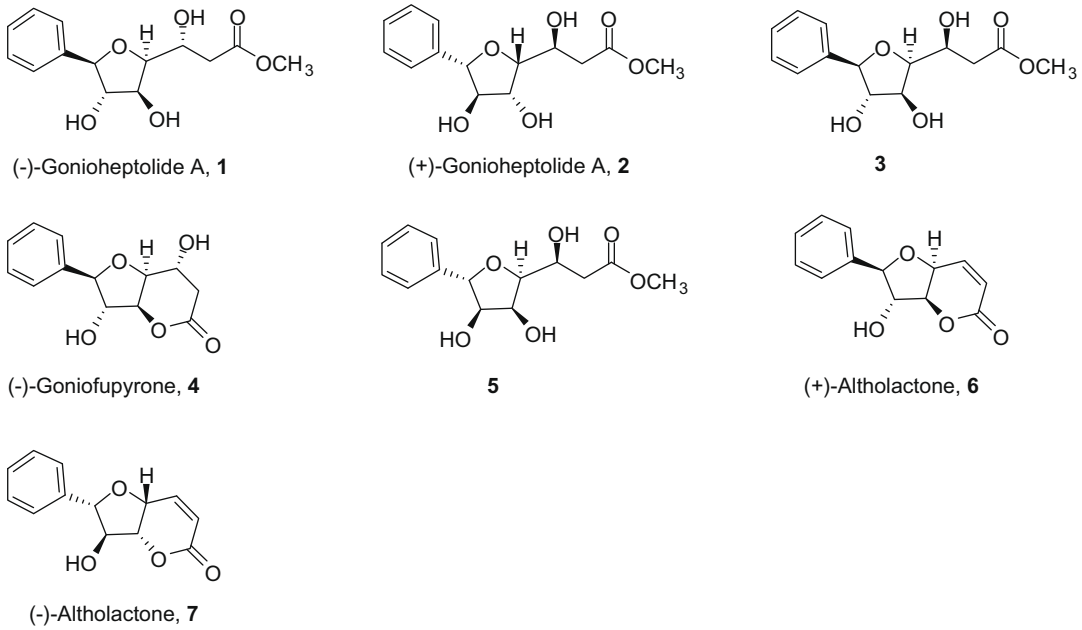
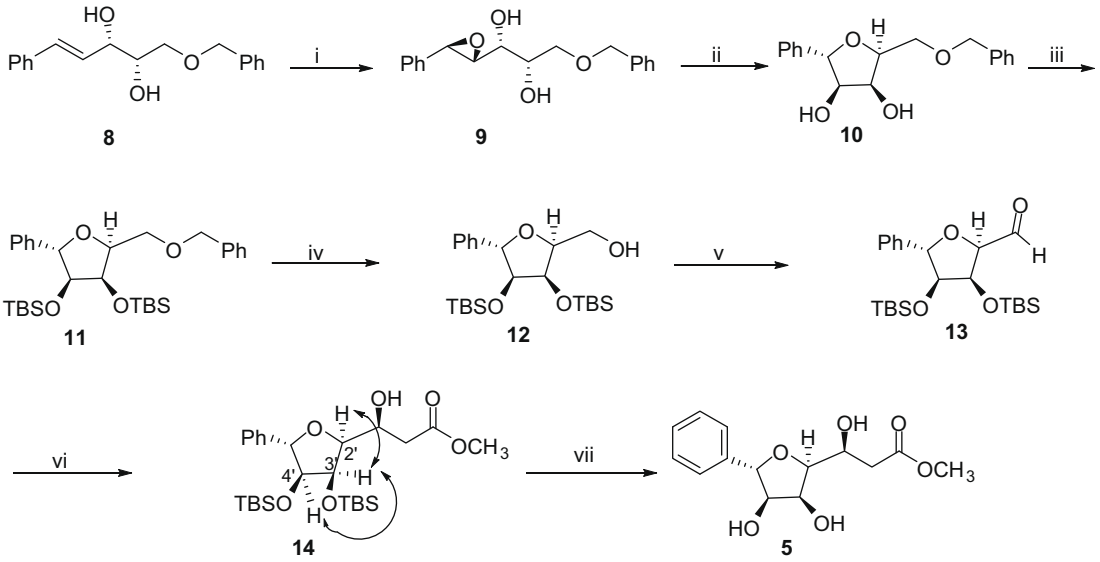


Figure 1. Styryllactones investigated.

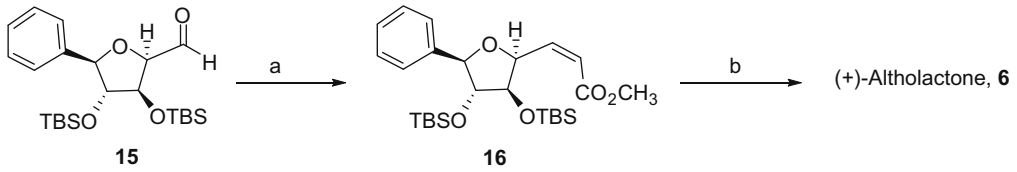
protocol to give intermediate **9** (Scheme 1) by a synthetic strategy similar to that developed earlier.[18] Acid-catalyzed intramolecular cyclization of **9** gave the tetrahydrofuran **10**, the secondary hydroxyl groups of which were protected as silyl ethers. Removal of the benzyl ether function followed by DMP oxidation of the resulting primary alcohol gave the aldehyde **13**, stereoselective aldol reaction of which with the lithium enolate of methyl acetate under kinetically-controlled conditions gave **14**. Finally, mild desilylation using tris(dimethylamino)sulfonium difluorotrimethylsilicate (TAS-F) gave the target compound **5**. The absolute configuration of the newly-generated secondary alcohol carbon of **14** was confirmed as S by Mosher ester analysis, and strong NOE signals observed between C2′ and C3′ and between C3′ and C4′ confirmed the absolute stereochemistry of **14** (also of **5**) as well as the stereochemistry of the epoxide **9**.

We also synthesized the natural (+)-altholactone **6**[5–12] from intermediate **15** which was used in our earlier synthesis of (−)-gonioheptolide A.[18] Compound **15** was transformed into ester **16** using Still-Gennari type HWE olefination. The *Z*-ester spontaneously cyclized to the lactone upon removal of the TBS groups with TAS-F to give (+)-altholactone **6** in 85% yield (Scheme 2). (−)-Altholactone **7** was also synthesized by an identical procedure starting with the enantiomer of **15**.[18]

The proliferation of both tumorigenic and non-tumorigenic human breast cell lines, (MCF-7 and MCF 12A, respectively) was inhibited by (−)-altholactone **7** in a concentration-dependent manner. The other agents tested were comparatively less active inhibitors of cell proliferation, with the exception of (+)-altholactone **6** (Table 1). IC_{50}s for (+)-altholactone were nearly identical for both MCF-7 and MCF-12A. The other agents (**1–5**) were unable



Scheme 1. Reagents and conditions: (a) D-(−)-DET, Ti(O*i*-Pr)$_4$, TBHP, MS 4 Å, CH_2Cl_2, −20 °C, 85%; (b) CSA, CH_2Cl_2, 85%; (c) TBSOTf, 2,6-luitidine, CH_2Cl_2, 88%; (d) H_2, Pd/C, MeOH, 1 atm, 90%; (e) Dess–Martin periodinane, CH_2Cl_2, 75%; (f) CH_3COOCH_3, LDA, THF, −78 °C, 81%; (g) TAS-F, DMF, 70%.



Scheme 2. Reagents and conditions: (a) 18-crown-6-ether, $CH_3O_2CCH_2P(O)(OCH_2CF_3)_2$, $KN(TMS)_2$, THF, −78 °C, 85%; (b) TAS-F, DMF, 72%.

Table 1
Antiproliferative activity of styryllactones on MCF-7 (tumorigenic human breast cells), MCF-12A (non-tumorigenic human breast cells), and CEM (human leukemic T-lymphocytes)

	IC_{50}[a] (μM)		
Compound	MCF-7	MCF-12	CEM
1	Inactive	Inactive	Inactive
2	Inactive	Inactive	Inactive
3	Inactive	Inactive	Inactive
4	Inactive	Inactive	Inactive
5	Inactive	Inactive	Inactive
6	124 ± 40	120 ± 90	19 ± 8
7	100 ± 21	110 ± 60	24 ± 16

[a] Inactive means less than 50% inhibition at 250 μM, the maximum concentration tested.

to achieve 50% inhibition at concentrations up to 250 μM. Nevertheless, the order of decreasing activity was observed to be **5** > **3** > **2** with compounds **1** and **4** demonstrating little or no activity above control even at 60 μg/ml, for both MCF-7 and MCF-12A cells. The activities of the more active agents (**2**, **3**, and **5**) were, in general, 1.5–2-fold greater toward the tumorigenic MCF-7 compared with the non-tumorigenic MCF-12A cells. A leukemic cell line (CEM) was also investigated. CEM cells showed greater susceptibility toward (+)-altholactone **6** (IC_{50} 19 ± 8 μM) and (−)-altholactone **7** (IC_{50} 24 ± 16 μM) than did MCF-7 or MCF-12A cells. However, compounds **1**–**5** were essentially inactive toward CEM cells (Table 1).

Figure 2 illustrates in a flow cytometry plot the ability of (−)-altholactone to induce apoptosis and necrosis in CEM cells. Fluorescence intensity of bound annexin-V-fluorescein isothiocyanate (FITC) (along the *x*-axes) reflects the exposure of phosphatidyl serine. While this negatively charged phospholipid is primarily located within the inner leaflet of the plasma membrane, during early stages of apoptosis, the enzymatic activity that keeps it in place begins to fail, allowing increased binding of annexin-V to phosphatidyl serine that has moved to the external side of the plasma membrane. Once the integrity of the plasma membrane is sufficiently compromised and necrosis ensues, both annexin-V and propidium iodide gain access to the interior of the cell, as reflected by exponentially increased fluorescence along both axes (the upper right quadrant of each plot), indicating a transition between apoptosis and necrosis. While untreated cells displayed minimal binding of propidium iodide and annexin-V to their targets, a 24-h exposure of cells to (−)-altholactone caused a 40% increase in late apoptosis/necrosis, along with a 10% increase in early apoptosis.

It may also be noted that (+)-altholactone primarily induced early apoptosis in the leukemic CEM cells (a 33% increase above than seen in untreated cells), but only a 19% increase in late apoptosis/necrosis occurred (data not shown). Exposure of cells to either (+)- or (−)-altholactone reduced total viability to only 19–20%. Figure 3 illustrates the activities of compounds **1**–**7** toward CEM cells at 120 μM (30 μg/ml) after a 24 h incubation. At these concentrations only the altholactone enantiomers showed substantial activity above background. For example, goniofupyrone produced only a 10% increase in late apoptosis/necrosis.

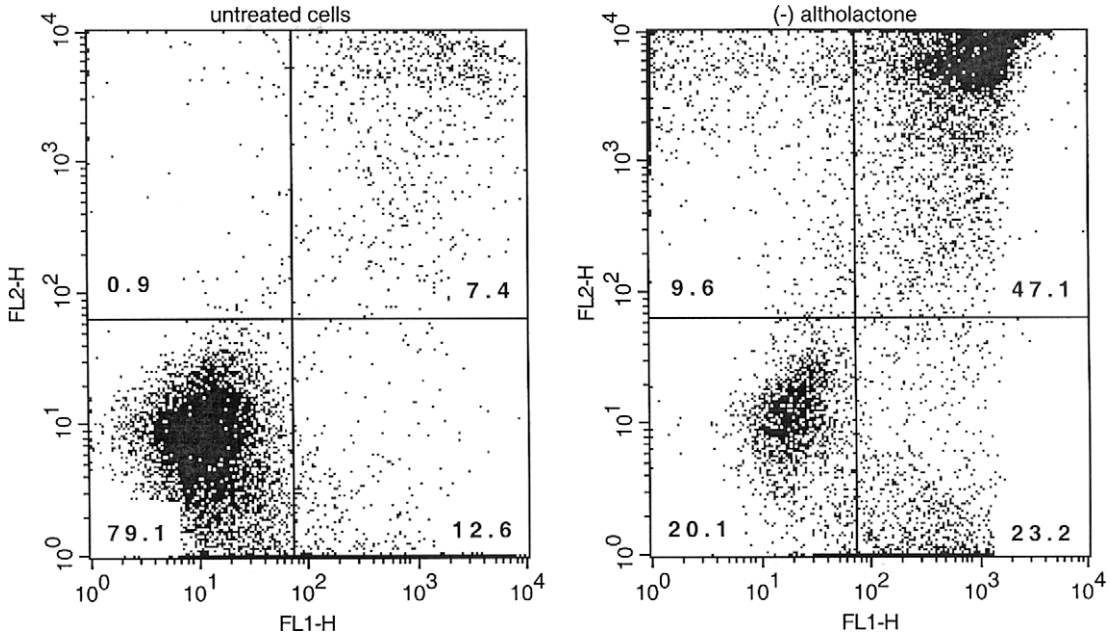


Figure 2. Effects of 120 μM (−)-altholactone (**7**) upon CEM cells after 24 h incubation. FL1-H reflects Annexin-V-FITC fluorescence; FL2-H reflects propidium iodide fluorescence. The percentage of cells in each quadrant is indicated. The lower left quadrant contains viable cells; the lower right quadrant contains early apoptotic cells; the upper right quadrant contains cells that are late apoptotic/necrotic.

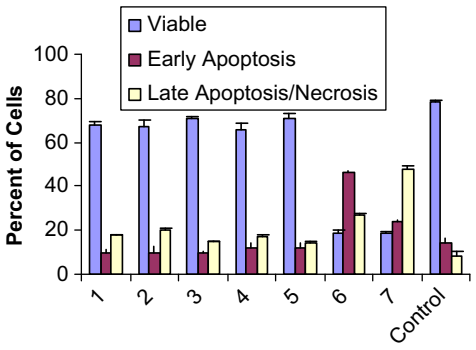


Figure 3. Induction of CEM cell apoptosis/necrosis by styryllactones. Error bars represent standard deviations from three experiments, 10,000 cells each.

We also treated leukemic (CEM) cells with the most potent of the derivatives, and then added TRAIL at concentrations that promote minimal apoptosis. TRAIL is known to activate the extrinsic pathway of apoptosis through the productive cleavage of procaspase-8. Figure 4 illustrates a nominal synergistic activation of apoptosis produced by (−)-altholactone in the presence and absence of TRAIL. At the end of the 24-h incubation and the 2-h experiment, untreated cells were 81% viable; equal numbers of the other cells were either apoptotic or had entered the transition between apoptosis and necrosis. TRAIL alone (added 3.5 h prior to the end of the 24-h incubation) induced a 5% increase in apoptosis, with no increase in necrotic cells. By contrast, the combination of (−)-altholactone with TRAIL produced increased early apoptosis. TRAIL as a potential synergistic agent was also used in conjunction with camptothecin (CPT), which is known to activate the intrinsic pathway of apoptosis following its internalization by target cells.

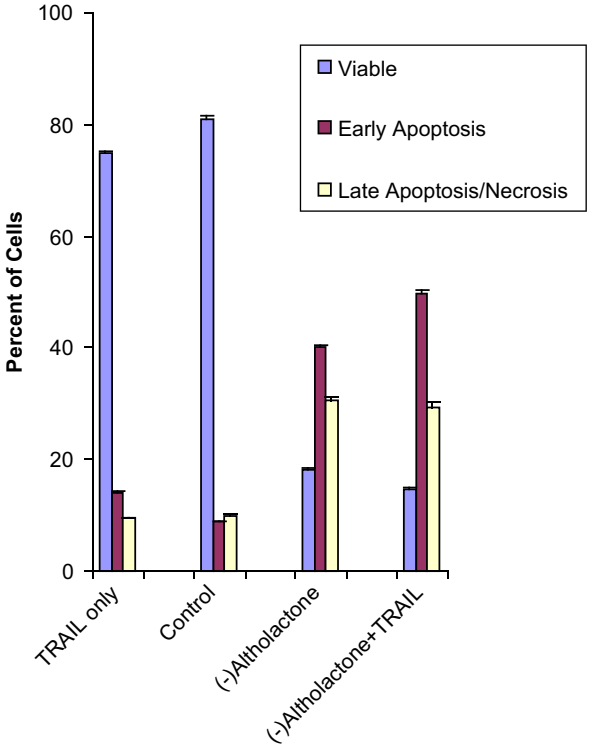


Figure 4. Enhanced early apoptosis produced by the combination of (−)-altholactone (**7**) and TRAIL. Error bars represent standard deviations. The probability that the extent of early apoptosis is the same in the presence or absence of TRAIL is $P < 0.0005$ (Student's *t*-test, 4 degrees of freedom).

Interestingly, a complete absence of synergism was observed between TRAIL and CPT (data not shown). Similarly, (+)-altholactone, which alone had induced early apoptosis, showed no potentiation in the presence of TRAIL.

3. Discussion

Among styryl lactone derivatives, dose-dependent apoptosis was shown by Inayat-Hussain et al.[20] to be induced by (+)-altholactone in HL-60 cells, a human promyelocytic leukemia line with phagocytic activity. With the exception of (+)-altholactone, however, the apoptotic/necrotic activities of our compounds are described here for the first time. Of the compounds studied, (−)-altholactone and (+)-altholactone were shown to be nearly equipotent in their ability to inhibit breast cancer (MCF-7) cell proliferation. Both (−)-altholactone and (+)-altholactone induced extensive apoptosis in human T-leukemic (CEM) cells, but the preponderance of cells in late apoptosis/necrosis due to (−)-altholactone supports the proposition that (−)-altholactone is significantly more cytotoxic than its naturally occurring stereoisomer. With regard to (+)-altholactone cytotoxicity, it may be noted that in mouse T-leukemic cells assayed by changes in the ability of mitochondria to reduce a tetrazolium dye, an IC_{50} of 3 μg/ml was reported by Bermejo et al.[21] This corresponds well with the 10 μg/ml IC_{50} we observed using human T-leukemic cells, particularly in view of the 100-fold difference in susceptibility among tumor cell lines to (+)-altholactone that was noted in the review by Mereyala and Joe.[15] A further difference between the activities of (−)-altholactone and (+)-altholactone was observed when these agents were tested in combination with TRAIL. No potentiation of apoptosis occurred when TRAIL was added to cells incubated with (+)-altholactone or with camptothecin. While synergy between TRAIL and (−)-altholactone was modest, the increase in the relative contribution to early apoptosis suggests that synthetic modifications of the (−)-altholactone scaffold could be fruitful in the development of more effective compounds that will selectively kill cancer cells by TRAIL-potentiated apoptosis.

4. Conclusion

In summary, we have synthesized here and elsewhere,[18] seven variant but closely related stereochemically-defined styryllactones, some of which are available from natural sources but some of which are not available except through a defined chemical synthesis. Each compound has been purified and well-characterized. The apoptotic activities of each of the synthetic agents were evaluated using two tumorigenic cell lines (CEM, leukemic and MCF-7, breast) and one non-tumorigenic cell line (MCF-12A, breast). A small but significant synergism in apoptotic activity with one of the styryllactones ((−)-altholactone) and TRAIL was demonstrated. Further improvements in the synergy noted for TRAIL with modified styryllactones based initially on (−)-altholactone could provide additional improvements in both selectivity and efficacy toward cancer cells. We intend to explore this possibility.

5. Experimental section

5.1. Chemical syntheses

All reactions were carried out under nitrogen atmosphere using anhydrous conditions, unless stated otherwise. The solvents used were ACS grade from Fisher. The reagents used were technical grade purchased from Aldrich and Acros and used without purification. TLC was carried out on 0.20 mm POLYGRAM SIL silica gel plates (Art.-Nr. 805 023) with fluorescent indicator. Davisil

(100–200 mesh) silica gel from Fisher was used in normal phase flash column chromatography. NMR spectra were recorded on INOVA 600, Varian VXRS-40, or Bruker AC-F 300 MHz spectrometers and using residual undeuterated solvent as an internal reference. Optical rotations were measured on an AUTOPOL III 589/546 polarimeter. High-resolution mass spectra were recorded on a Micromass LCT electrospray ionization mass spectrometer at the Mass Spectrometry and Proteomics Facility of the Ohio State University.

5.1.1. (1*R*,2*S*)-3-(Benzyloxy)-1-((2*R*,3*R*)-3-phenyloxiran-2-yl)propane-1,2-diol (9)

^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.39–7.20 (m, 10H), 4.59 (d, 1H, J = 12.0 Hz), 4.54 (d, 1H, J = 12.0 Hz), 3.98–3.93 (m, 1H), 3.92–3.88 (m, 1H), 3.86–3.82 (m, 1H), 3.70–3.63 (m, 2H), 3.2–3.08 (m, 1H), 2.69–2.65 (m, 2H).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.75, 136.73, 128.78, 128.75, 128.59, 128.22, 128.09, 125.93, 73.96, 71.74, 71.45, 70.73, 63.15, 55.60.; HRMS calcd for $C_{18}H_{20}O_4$+Na^+ 323.1259; found 323.1253 (M+Na^+).

5.1.2. (2*S*,3*S*,4*R*,5*S*)-2-(Benzyloxymethyl)-5-phenyltetrahydrofuran-3,4-diol (10)

$[\alpha]_D^{20} = -20.67$ (c 0.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.37–7.32 (m, 10H), 4.93–4.92 (d, 1H, J = 4.8 Hz), 4.66 (d, 1H, J = 12.0 Hz), 4.60 (d, 1H, J = 12.0 Hz), 4.44–4.42 (m, 1H), 4.39–4.37 (m, 1H), 4.04–4.01 (m, 1H), 3.88–3.83 (m, 2H), 3.56–3.55 (d, 1H, J = 10.2 Hz), 3.31–3.29 (d, 1H, J = 6.6 Hz).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 140.55, 137.11, 128.94, 128.76, 128.51, 128.23, 127.87, 125.52, 125.50, 84.93, 78.80, 78.64, 74.46, 72.62, 69.62.; HRMS calcd for $C_{18}H_{20}O_4$+Na^+ 323.1259; found 323.1252 (M+Na^+).

5.1.3. ((2*S*,3*R*,4*S*,5*S*)-2-(Benzyloxymethyl)-5-phenyltetrahydrofuran-3,4-diyl)bis(oxy)bis(*tert*-butyldimethylsilane) (11)

$[\alpha]_D^{20} = +12.0$ (c 0.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.34–7.29 (m, 10H), 4.84–4.83 (d, 1H, J = 6.6 Hz), 4.60 (d, 1H, J = 12.0 Hz), 4.52 (d, 1H, J = 12.0 Hz), 4.44–4.41 (m, 1H), 4.21–4.19 (m, 1H), 3.93–3.91 (m, 1H), 3.73–3.68 (m, 2H), 0.89 (s, 9H), 0.80 (s, 9H), 0.08 (s, 3H), 0.03 (s, 3H), −0.14 (s, 3H).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 141.06, 138.49, 128.56, 128.45, 128.26, 127.91, 127.81, 126.85, 83.89, 80.89, 80.29, 73.69, 73.66, 70.04, 26.24, 26.17, 18.53, 18.29, −3.97, −4.20, −4.51, −5.13.; HRMS calcd for $C_{30}H_{48}O_4Si_2$+Na^+ 551.2989; found 551.2988 (M+Na^+).

5.1.4. ((2*S*,3*R*,4*S*,5*S*)-3,4-Bis(*tert*-butyldimethylsilyloxy)-5-phenyltetrahydrofuran-2-yl)methanol (12)

$[alpha]_D^{20} = -24.8$ (c 0.4, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.34–7.27 (m, 5H), 4.98–4.97 (d, 1H, J = 3.6 Hz), 4.36–4.31 (m, 2H), 4.00–3.99 (t, 1H, J = 4.2 Hz),), 3.90–3.87 (m, 1H), 3.79–3.75 (m, 1H), 2.79–2.77 (dd, 1H, J = 1.8 Hz, 9 Hz), 0.91 (s, 9H), 0.89 (s, 9H), 0.07–0.06 (m, 6H), 0.03 (s, 3H), −0.14 (s, 3H).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 141.06, 138.49, 128.56, 128.45, 128.26, 127.91, 127.81, 126.85, 83.89, 80.89, 80.29, 73.69, 73.66, 70.04, 26.24, 26.17, 18.53, 18.29, −4.24, −4.28, −4.55, −4.69.; HRMS calcd for $C_{23}H_{42}O_4Si_2$+Na^+ 461.2519; found 461.2522 (M+Na^+).

5.1.5. (2*R*,3*R*,4*S*,5*S*)-3,4-Bis(*tert*-butyldimethylsilyloxy)-5-phenyltetrahydrofuran-2-carbaldehyde (13)

^{1}H NMR ($CDCl_3$, 600 MHz): δ 9.74–9.73 (d, 1H, J = 2.4 Hz), 7.38–7.28 (m, 5H), 5.15–5.14 (d, 1H, J = 3.6 Hz), 4.49–4.48 (dd, 1H, J = 3.6 Hz, 6.6 Hz), 4.39–4.37 (dd, 1H, J = 3 Hz, 6.6 Hz), 3.99–3.97 (m, 1H), 0.87 (s, 18H), 0.07 (s, 3H), 0.06 (s, 3H), −0.02 (s, 3H), −0.15 (s, 3H).

5.1.6. (*S*)-Methyl 3-((2*S*,3*R*,4*S*,5*S*))-3,4-bis(*tert*-butyldimethylsilyloxy)-5-phenyl-tetrahydrofuran-2-yl)-3-hydroxypropanoate (14)

$[\alpha]_D^{20} = +13.9$ (c 0.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.33–7.25 (m, 5H), 4.83–4.82 (d, 1H, J = 6 Hz), 4.55–4.51 (m, 1H), 4.37–3.36 (t, 1H, J = 3.6 Hz), 4.00–3.95 (m, 2H), 3.69 (s, 3H), 3.24–3.23 (d, 1H, J = 3 Hz), 2.88–2.85 (dd, 1H, J = 2.4 Hz, 16.2 Hz), 2.51–2.47 (dd, 1H, J = 9.6 Hz, 16.8 Hz), 0.94 (s, 9H), 0.84 (s, 9H), 0.13 (s, 6H), −0.11 (s, 3H), −0.30 (s, 3H).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.69, 140.96, 128.59, 128.03, 126.57, 84.53, 81.60, 80.79, 74.20, 67.42, 51.79, 38.48, 26.25, 26.20, 18.62, 18.29, −3.298, −4.28, −4.61, −4.80.; HRMS calcd for $C_{23}H_{42}O_4Si_2$+Na^+ 461.2519; found 461.2522 (M+Na^+).

5.1.7. (*S*)-Methyl 3-((2*S*,3*S*,4*R*,5*S*)-3,4-dihydroxy-5-phenyltetrahydrofuran-2-yl)-3-hydroxypropanoate (5)

$[\alpha]_D^{20} = -18.0$ (c 0.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.37–7.27 (m, 5H), 4.81–4.80 (d, 1H, J = 7.8 Hz), 4.48–4.42 (m, 2H), 4.12–4.03 (m, 2H), 3.72 (s, 3H), 3.65–3.64 (d, 1H, J = 3 Hz), 3.58–3.57 (d, 1H, J = 3.6 Hz), 2.93–2.92 (d, 1H, J = 8.4 Hz), 2.86–2.83 (dd, 1H, J = 3 Hz, 16.8 Hz), 2.69–2.65 (dd, 1H, J = 9 Hz, 16.8 Hz).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.54, 140.55, 128.76, 128.11, 125.87, 83.61, 81.44, 79.26, 72.52, 68.38, 52.21, 37.76.; HRMS calcd for $C_{14}H_{18}O_6$+Na^+ 305.1001; found 305.1002 (M+Na^+).

5.1.8. (*Z*)-Methyl 3-((2*S*,3*R*,4*R*,5*R*)-3,4-bis(*tert*-butyldimethylsilyloxy)-5-phenyltetrahydrofuran-2-yl)acrylate (16)

To a solution of 18-crown-6-ether (0.273 g, 1.03 mmol) and bis(2,2,2-trifluoroethyl)(methoxycarbonylmethyl)phosphonate (0.065 ml, 0.31 mmol) in THF (3.4 ml) at −78 °C was added $KN(TMS)_2$ (0.63 ml, 0.32 mmol, 0.5 M in toluene). After stirring at the same temperature for 5 min., aldehyde **10** (90 mg, 0.21 mmol) in THF (1 ml) was added. The resulting reaction mixture was stirred at −78 °C for 3 h before quenching it with saturated aqueous NH_4Cl solution. The aqueous layer was extracted with ether and the combined organic extract was dried over anhydrous Na_2SO_4, concentrated and purified by flash chromatography on silica (6% EtOAc/hexanes) to afford compound **11** (86 mg, 85%) as a colorless oil. $[\alpha]_D^{20} = +79.0$ (c 1.0, $CHCl_3$); TLC R_f = 0.61 (silica gel, 20% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.44 (d, 2H, J = 7.2 Hz), 7.29–7.19 (m, 3H), 6.54–6.51 (m, 1H), 5.94 (d, 1H, J = 13.8 Hz), 5.56–5.54 (m, 1H), 4.79 (s, 1H), 4.26 (s, 1H), 4.05 (s, 1H), 3.73 (s, 3H), 0.93 (s, 9H), 0.74 (s, 9H), 0.07 (d, 6H, J = 6 Hz), −0.07 (s, 3H), −0.13 (s, 3H).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.3, 147.6, 141.1, 128.3, 127.4, 126.9, 120.8, 90.1, 86.1, 81.8, 80.3, 51.6, 25.9, 25.8, 18.1, 18.1, −4.2, −4.3, −4.5, −4.8.; HRMS calcd for $C_{26}H_{44}O_5Si_2$+Na^+ 515.2625; found 515.2601 (M+Na^+).

5.1.9. (2*R*,3*R*,3a*S*,7a*S*)-3-Hydroxy-2-phenyl-3,3a-dihydro-2*H*-furo[3,2-*b*]pyran-5(7a*H*)-one, [(+)-altholactone] (6)

To a solution of the *cis* olefin **11** (5 mg, 0.01 mmol) in DMF (0.1 ml) was added dropwise a solution of TAS-F (13.5 mg, 0.05 mmol) in DMF (0.1 ml). The resulting reaction mixture was stirred overnight and diluted with a phosphate buffer solution of pH 7. The organic phase was separated and the aqueous layer was extracted with EtOAc. The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated and purified by flash chromatography on silica (25% EtOAc/hexanes) to afford (+)-altholactone **6** (1.7 mg, 72%) as a white solid. $[\alpha]_D^{20} = +180.5$ (c 0.3, $CHCl_3$); [lit[4] $[\alpha]_D^{25} = +184.7$ (c 0.2, EtOH)]; ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.35–7.30 (m, 5H), 7.0 (dd, 1H, J = 5.4, 10.2 Hz), 6.23 (d, 1H, J = 9.6 Hz), 4.94 (dd, 1H, J = 2.4, 5.4 Hz), 4.73 (d, 1H, J = 6 Hz), 4.65 (t, 1H, J = 5.4 Hz), 4.45 (dd, 1H, J = 2.4, 5.7 Hz).; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.0, 140.4, 138.1, 128.7, 128.4, 126.2,

123.8, 86.4, 85.9, 83.7, 68.2.; HRMS calcd for $C_{13}H_{12}O_4$+Na^+ 255.0633; found 255.0616 (M+Na^+).

5.1.10. (2S,3S,3aR,7aR)-3-Hydroxy-2-phenyl-3,3a-dihydro-2*H*-furo[3,2-*b*]pyran-5(7a*H*)-one (−)-altholactone (7)

Compound **7** was synthesized as described above for **6**.

$[\alpha]_D^{20} = -181.5$ (*c* 0.2, $CHCl_3$); HRMS calcd for $C_{13}H_{12}O_4$+Na^+ 255.0633; found 255.0634 (M+Na^+).

5.2. Cell culture

Adherent human breast cell lines MCF-7 (non-multidrug resistant) and MCF-12A (non-tumorigenic) and the human T-leukemic cell line CEM were obtained from ATCC and cultured in RPMI medium containing 10% fetal calf serum and 2% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were maintained by passage twice each week and were washed and replated/resuspended 24 h prior to each experiment. Only cell populations exhibiting greater than 90% viability by trypan blue exclusion were used.

5.2.1. Treatments

Stock solutions of agents in DMSO at 1 mg/ml were prepared fresh for each experiment and were diluted in cell culture medium to achieve desired concentrations upon addition to the target cells. All three types of cells were incubated 48 h at 37 °C in 96-well microtiter plates to assess inhibition of cell proliferation by measuring changes in protein content in triplicate wells. For measurement of cell death by apoptosis or necrosis, and for assessment of potential synergy with the apoptosis-inducing compound TRAIL, compounds were incubated 24 h at 37 °C with 10^6 CEM cells/ml in 15-ml conical centrifuge tubes.

5.2.2. Cytotoxicity

Cytotoxicity was assessed using sulforhodamine-B (SRB) by measuring the extent of inhibition of protein synthesis in treated cells compared with that in untreated cells. Following 48 h treatment, cells were fixed by the addition of cold (4 °C) 50% trichloroacetic acid (50 μl/well). After a 1-h incubation at 4 °C, plates were gently washed 5x with deionized water and allowed to dry overnight. A 0.4% solution of SRB in 1% acetic acid (50 μl) was added to each well, and plates were incubated 10 min at room temperature. Wells were then rinsed 5× with 1% acetic acid and allowed to dry. To solubilize the SRB dye, 150 μl/well of 10 mM Tris base (pH 10.5) was incubated for 20 min at room temperature and plates were gently vortex-mixed before absorbance at 570 nm was measured using a spectrophotometer (Spectramax-Pro; Molecular Devices, Sunnyvale, CA). Negative control wells (blanks) contained medium with compound, but no cells. Wells containing untreated cells served as positive controls (maximal protein synthesis). To determine the extent of inhibition of protein synthesis, the mean absorbance of the corresponding set of blanks was subtracted from the mean absorbance of wells incubated with each test agent. This value was then divided by the difference between the mean absorbance of the untreated cells and that of the blanks in order to calculate a percent inhibition for each concentration of agent.

5.2.3. Flow cytometry

Following their treatments, human T-leukemic cells (CEM) were washed twice with ice-cold PBS and resuspended in 200 μl Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$). Annexin V-FITC (5 μl; BD Biosciences Pharmingen, San Jose, CA) and propidium iodide (PI, Sigma, St. Louis, MO) (10 μl of a 1 μg/ml solution) were added and cells were incubated shielded from light for 15 min at ambient temperature. After incubation in the dark, additional binding buffer (800 μl) was added to each tube prior to analysis by flow cytometry.[22] A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) with emission wavelengths of 520 nm for FITC and 620 nm for PI was gated on forward light scatter to eliminate subcellular debris. Statistics were calculated online by CellQuest software. The simultaneous use of both fluorescent dyes facilitates distinguishing healthy, apoptotic, and necrotic cells, since PI is normally impermeant and since its fluorescence is enhanced 20–30-fold upon binding intracellular nucleic acids.

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