Dual Effects of Cycloheximide on U937 Apoptosis Induced by Its Combination with VP-16

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In this study, cycloheximide (CHX) and VP-16 alone and in combination (C&V) have been used to strongly trigger apoptosis in U937 cells. The presence of CHX markedly prevented VP-16-induced apoptosis, suggesting that in this process *de novo* protein synthesis is required. But interestingly, C&V had shown more similarities with CHX but not VP-16 alone, including the effects on cell cycle distribution and induction of apoptosis, which occurred more quickly and was steadily enhanced by increasing concentrations of CHX or by *N*-α-tosyl-L-lysyl-chloromethyl ketone (TLCK), a serine protease inhibitor. These results indicate that CHX, not VP-16, is indeed the dominant inducer of U937 apoptosis, when they are coadministered. In particular, VP-16 even promoted CHX-induced apoptosis, but did not alter its selection of cell types. In T-cells resistant to CHX (Molt-4), we have detected no apoptotic response to their combination. These findings may well explain why the inhibitory effects of CHX on apoptosis induced by the same stimuli are usually different according to the cell type used, and also suggest that CHX may have the potential to lower side effects and drug resistance of cancer therapy.

Key words apoptosis; cycloheximide; VP-16; human leukemic U937 cell; cell cycle distribution; *N*-α-tosyl-L-lysyl-chloromethyl ketone (TLCK)

Apoptosis is a highly regulated cell death process with characteristic morphological and biochemical features, including cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation.[1,2] It occurs both during normal development and under certain pathological conditions in metazoans and plays a crucial role.[3] Evasion of apoptosis is an essential hallmark of cancer.[4] Particularly in the last decade, the emerging knowledge of the molecular links between tumorigenesis, apoptosis, and drug resistance has provided the main foundation for chemotherapeutic tumor eradication.[5]

As a typical inhibitor of protein synthesis cycloheximide (CHX, >1 μg/ml) has been widely utilized to test whether *de novo* protein synthesis is required in apoptosis induced by a variety of stimuli, and various results were obtained. CHX can promote TNF-α- and Fas-induced apoptosis,[6,7] but prevents several anticancer agents- and ionizing radiation-induced apoptosis,[8—10] since they have initiated distinct apoptotic pathways, some of which depend on *de novo* gene expression while others do not. However, in different cell lines there were discrepancies in CHX's inhibitory effects on apoptosis induced by the same stimuli, too. For example, CHX has been shown to totally block dexamethasone- and dibutyryl-cAMP-induced apoptosis in thymocytes but have no impact in B lymphocytes.[9] Thus, such discrepancy can not be well explained up to now, as it can not be simply ascribed to the individual diversity of the cell types used.

In fact, Tang *et al.*[11] and Martin *et al.*[12] have indicated that CHX, independently of other stimuli, is also capable of triggering apoptosis with the selection of cell types. This led us to investigate whether this property of CHX would interfere with the display of its own inhibitory effects. As expected, we found that CHX not only was a forceful inhibitor, but also was indeed the dominant inducer of apoptosis, when it was coadministered with VP-16, a clinical chemotherapeutic drug reported to sharply induce apoptosis,[13] to human leukemic U937 cells. Particularly, VP-16 even promoted CHX-induced apoptosis, but did not alter its selection of cell types.

MATERIALS AND METHODS

Cell Culture U937 cells and Molt-4 cells were purchased from ATCC and grown in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone), 2 g/l sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Drug Treatments CHX, VP-16 and *N*-α-tosyl-L-lysyl-chloromethyl ketone (TLCK) were all purchased from Sigma. CHX and VP-16 were prepared in dimethyl sulfoxide (DMSO). TLCK was dissolved at stock solutions of 50 mM in ethanol and stored at −20 °C. For all experiments, exponentially growing cells were seeded at an initial concentration of 1.5×10^5/ml for the following 12 h of culture and exposed to treatments for the times indicated.

Flow Cytometric Analysis of Cell Cycle Distribution, Sub-G_1 Peak and Externalization of Phosphatidylserine (PS) For propidium iodide (PI) staining, cell pellets were fixed by 70% ethanol at −20 °C for at least 12 h. After being washed twice with ice-cold PBS, they were incubated in RNase A/PBS (100 μg/ml) at 37 °C for 30 min. Then intracellular DNA was labeled with PI (50 μg/ml) at room temperature for 15 min. Finally, the analysis of DNA content was performed on a FACSCalibur fluorescence-activated cell sorter (FACS, Becton Dickinson, NJ, U.S.A.). Each histogram was generated using the CELLQuest software with at least 15000 cells. And the percentage of apoptosis was determined from the sub-G_1 events. Moreover, the FITC-labeled antibody to Annexin V (clone FL, Santa Cruz) was also used to identify PS externalization. Cells were collected and washed with ice-cold PBS containing 2 mM Ca^{2+}. Then they were resuspended in 100 μl 1×Binding Buffer (10 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) with 0.5 μg/ml antibodies and incubated in the dark for 15 min at 18—24 °C. After 400 μl 1×Binding

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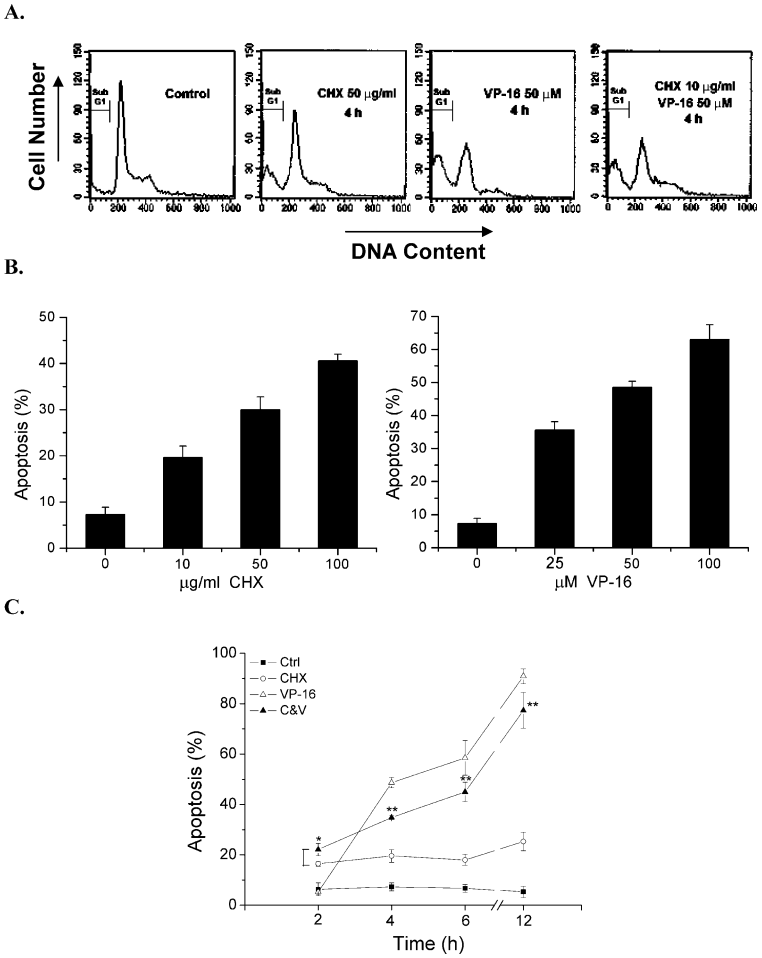

Fig. 1. Effects of CHX and VP-16 Each Alone or in Combination on the Induction of Apoptosis in U937 Cells

U937 cells were incubated with CHX or VP-16 at different concentrations for 4 h and then assayed for sub-G_1 peak by flow cytometry (A and B). Then CHX at 10 μg/ml and VP-16 at 50 μM alone and in combination (C&V) were chosen to display their time courses (C). Cells treated with a drug vehicle (DMSO, Ctrl) served as control. *, $p<0.05$ *versus* the corresponding value in CHX group; **, $p<0.01$ *versus* the corresponding value in VP-16 group.

Buffer was added, the samples were analyzed with FACS within one hour for maximal signal.

Western Blot Cells were harvested and washed twice with ice-cold PBS. The lysates were achieved with TEN-T buffer (150 mM NaCl, 10 mM Tris/HCl pH 7.4, 5 mM EDTA pH 8.0, 1% Triton X-100, 1 mM PMSF, 2 μg/ml aprotinin), and then subjected to 10000×***g*** centrifugation at 4 °C for 20 min. Total protein concentrations in the supernatant were determined by the Bicinchoninic Acid assay (Beyotime biotechnology, China). Proteins were normalized to 50 μg/lane, resolved on 15% SDS-polyacrylamide gel, and subsequently transferred to PVDF sheets and immunoblotted for caspase-3 (clone H-277, Santa Cruz). Blots were then detected with an ECF Western blotting kit. The densities of sample bands were determined with a fluorescence scanner, Storm 860, and analyzed with the ImageQuant software (Amersham Biosciences UK Limited, England).

Statistical Analysis Data are represented as the mean± S.D. of at least three separate experiments. One-way analysis of variance (ANOVA) was performed to determine the significance between groups. The minimum level of significance was set at $p<0.05$.

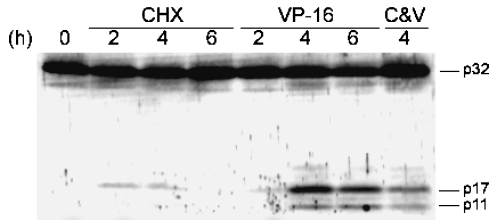

Fig. 2. The Processing of Caspase-3 in U937 Apoptosis

U937 cells were incubated with CHX (10 μg/ml) and VP-16 (50 μM) alone and in combination (C&V) for the indicated times, and then the processing of caspase-3 was measured by Western blot.

RESULTS

Both CHX and VP-16 Induce Apoptosis in U937 Cells CHX[14] and VP-16[13] respectively have been demonstrated to trigger apoptosis in U937 cells. Here, we validated these results through the analysis of sub-G_1 peak (Fig. 1A), an important measure of apoptosis.[15] As shown in Fig. 1B, both of them were dose-dependent, but only VP-16-induced apoptosis was sharply time-dependent, too (Fig. 1C). CHX-induced apoptosis was quicker, and started to occur within 2 h (Fig. 1C). Similar time responses were also observed in the processing of caspase-3 (Fig. 2) characterized by the appearance

of major cleaved products (p17 and p11), suggesting that they were mediated through the activation of caspases.

The Dual Role of CHX To further address their potential interrelationship, CHX (10 μg/ml) and VP-16 (50 μM) in combination (C&V) was then utilized to strongly trigger apoptosis in U937 cells (Fig. 1A). During this process, two time windows were manifested: within 2 h, and after 4 h. After 4 h, C&V-induced apoptosis was significantly lower than VP-16-induced apoptosis (Figs. 1C, 2), suggesting that CHX could prevent VP-16-induced apoptosis. But within 2 h, the result seemed to be the reverse. At this time C&V instead caused a higher induction of apoptosis than CHX or even VP-16 alone did (Figs. 1C, 2). Of course, it would be impossible for CHX to be self-contradictory to inhibit and promote VP-16-induced apoptosis simultaneously, so the cause of this result would be due to the only possibility that VP-16 had also promoted CHX-induced apoptosis. In other words, CHX must have played a dual role in this whole process: first as the inhibitor of VP-16-induced apoptosis, and second as the inducer of the apoptosis that was promoted by VP-16.

C&V Shows More Similarities with CHX Alone, But Not with VP-16 Alone Obviously, it is impractical to believe that the promotion of CHX-induced apoptosis by VP-16 can take place only within 2 h. This raised another question: was apoptosis induced by C&V after 4 h, which was in between that done by CHX and VP-16 alone (Fig. 1C), due to the partial inhibition of VP-16-induced apoptosis by CHX or to the forceful promotion of CHX-induced apoptosis by VP-16, even though the latter was true within 2 h because no response to VP-16 had been detected at this time (Fig. 1C)?

To answer this question, we next compared the similarities between CHX, VP-16 and C&V. To avoid excessive apoptosis causing unwanted difficulties for analysis, CHX at 10 μg/ml, VP-16 at 50 μM and the time point of 4 h were employed in the following experiments. Firstly, cell cycle distribution was analyzed. As shown in Fig. 3A and Table 1, incu-

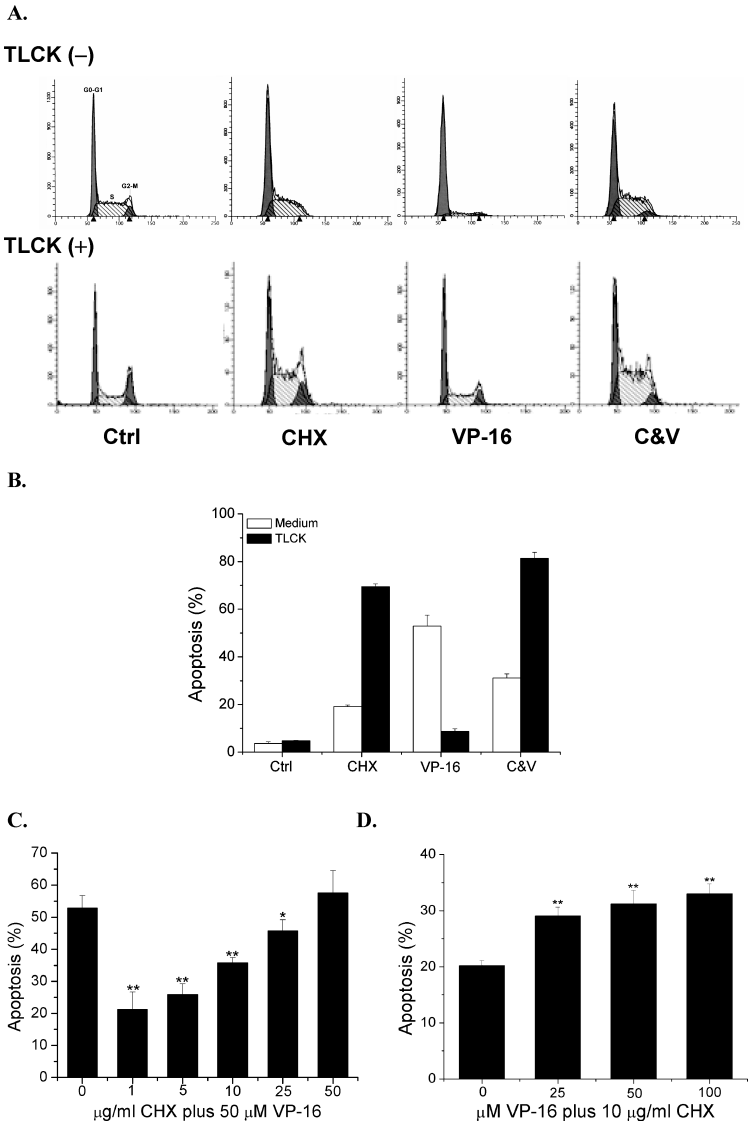

Fig. 3. C&V Shows More Similarities with CHX Rather than VP-16 Alone

U937 cells were incubated with a drug vehicle (Ctrl), 10 μg/ml CHX, 50 μM VP-16 or C&V in the absence (−) or presence (+) of 100 μM TLCK for 4 h. Then cell cycle distribution was analyzed by flow cytometry with the exclusion of cell debris, apoptosis and aggregates (A and Table 1), and apoptosis was evaluated (B and Table 1) by the analysis of sub-G_1 peak. Moreover, U937 cells were also treated with VP-16 (50 μM) in conjunction with a range of CHX concentrations (*e.g.*, 1—50 μg/ml) (C), or with CHX (10 μg/ml) in conjunction with increasing concentrations of VP-16 (*e.g.*, 0—100 μM) (D) for 4 h, and then assayed for sub-G_1 peak. *, $p<0.05$ & **, $p<0.01$ compared with 0 μg/ml CHX (C) or 0 μM VP-16 (D).

Table 1. Cell Cycle Distribution of U937 Cells Treated by Various Stimuli for 4 h

Stimuli	Cell cycle distribution[b]			Apoptosis %
	G_0–G_1	S	G_2–M	
TLCK (−)				
Control	51.05±7.01	42.08±6.30	6.87±0.72	7.29±1.59
CHX (10 μg/ml)	53.43±2.39	45.16±1.85	1.41±0.91**	19.58±2.49
CHX (50 μg/ml)	51.73±2.76	47.12±2.46	1.14±0.64**	29.98±2.79
CHX (100 μg/ml)	52.28±2.50	47.02±2.69	0.70±0.24**	40.55±1.46
VP-16 (50 μM)	82.98±4.82**	13.13±3.48**	3.89±1.35**	48.61±2.03
C&V[a]	48.95±0.27	46.38±0.65	4.67±1.05*	34.82±0.76
TLCK (+)				
Control	41.97±1.77	34.00±0.84	24.02±2.07‡	4.78±0.25
CHX (10 μg/ml)	31.79±2.55	54.35±1.46§	13.86±1.28‡	69.49±1.18
VP-16 (50 μM)	48.42±3.11	38.96±2.68	12.61±1.92‡	8.70±1.11
C&V[a]	29.03±1.44	60.87±4.76§	10.10±3.99‡	81.37±2.59

a) CHX (10 μg/ml) plus VP-16 (50 μM). *b*) Independent of the analysis of apoptosis. $*p<0.05$, and $**p<0.01$ *versus* the corresponding value in control of TLCK (−) group. ‡$p<0.01$ *versus* the corresponding value in TLCK (−) group. §$p<0.01$ *versus* the corresponding value in control of TLCK (+) group.

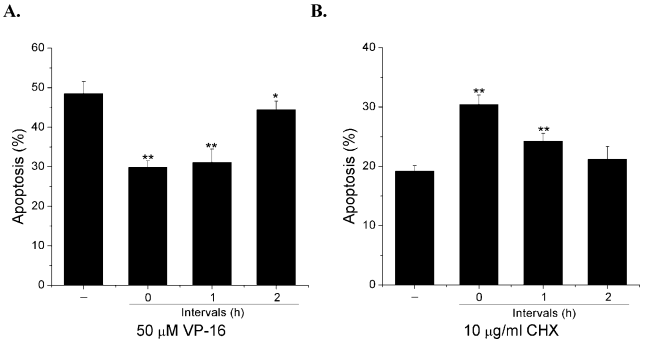

Fig. 4. Effects of Asynchronous Addition of CHX and VP-16 on U937 Apoptosis

U937 cells were incubated with 50 μM VP-16 (A) or 10 μg/ml CHX (B) for 4 h. In the meantime, 10 μg/ml CHX (A) and 50 μM VP-16 (B) respectively were added at indicated intervals. Then apoptosis was evaluated by the analysis of sub-G_1 peak. *, $p<0.05$, and **, $p<0.01$ compared with "—"representative of no CHX (A) or VP-16 (B) addition.

bation of U937 cells with CHX, VP-16 or C&V all resulted in a statistically evident decrease of cells at G_2–M phase. But a significant impact on G_0–G_1 phase or S phase appeared only in the treatment with VP-16, not with CHX or C&V. VP-16 caused a high proportion of G_0–G_1 phase cells (82.98±4.82% *vs.* 51.05±7.01%, $p<0.01$) and a low proportion of S phase cells (13.13±3.48% *vs.* 42.08±6.30%, $p<0.01$) compared with the control. In order to rule out the possibility that different apoptotic extents would affect cell cycle distribution, CHX at 50 and at 100 μg/ml, which had a level of apoptosis close to that of VP-16 and C&V (Table 1), were also tested and showed no difference from CHX at 10 μg/ml (Table 1). Secondly, we examined the responses of CHX-, VP-16- and C&V-induced apoptosis to TLCK, an inhibitor of serine proteases. As shown in Fig. 3B, both CHX- and C&V-induced apoptosis were sharply promoted by TLCK at 100 μM, whilst VP-16-induced apoptosis was strongly inhibited. Interestingly, the treatment of TLCK also uniformly resulted in a marked accumulation of cells at G_2–M phase, but only cells treated with CHX plus TLCK and C&V plus TLCK had a similar cell cycle distribution, with an increased percentage of S phase cells (Fig. 3A, Table 1).

All these results indicated that C&V retained more similarities with CHX alone rather than with VP-16 alone. And CHX, not VP-16, was indeed the dominant inducer of apoptosis when they were coadministered to U937 cells. As the embodiment of the promotion of CHX-induced apoptosis by VP-16, C&V-induced apoptosis was also time- (Fig. 1C) and CHX-concentration-dependent (Fig. 3C). In particular, when CHX concentration reached 50 μg/ml, we even detected a close level of VP-16- and C&V-apoptosis (Fig. 3C), which previously had often been deemed not to have the impact of CHX.[9] Moreover, increasing concentrations of VP-16 only caused a very low-pitched progression of CHX-induced apoptosis (Fig. 3D), suggesting that a lower concentration of VP-16 might be enough to drastically promote CHX-induced apoptosis, and VP-16-induced apoptosis *per se* might be fully inhibited by CHX at the same time.

Effects of Asynchronous Addition of CHX and VP-16 on U937 Apoptosis Next, we investigated whether the synergic effects of CHX and VP-16 on U937 apoptosis would be changed if they were added in tandem. As expected, addition of CHX and VP-16 at 2 h intervals would markedly reduce their synergic effects (Figs. 4A, B). But addition of CHX as late as 1 h after VP-16 significantly inhibited VP-16-induced apoptosis to the same extent as simultaneous addition (Fig. 4A), suggesting that CHX might function in the later stage of VP-16-induced apoptosis.

CHX Completely Prevents PS Externalization Induced by VP-16 in Molt-4 Cells The high selection of cell types is an important property of CHX on the induction of apoptosis.[11,12] So we then investigated whether VP-16 would alter this selectivity of CHX. Molt-4, a T-cell line resistant to CHX, was used in this project and apoptosis was determined by the Annexin V-affinity assay, which relies on PS externalization.[16] As shown in Fig. 5, both CHX and C&V could not cause any PS externalization in Molt-4 cells, but more than 30% PS externalization was induced by VP-16 (50 μM) compared to the control. These results indicated that VP-16 *per se* could not alter CHX's selection of cell types in the induction of apoptosis. And in one way, the full inhibition of VP-16-induced Molt-4 apoptosis by CHX also indirectly demonstrated that CHX, not VP-16, was indeed the dominant inducer of apoptosis.

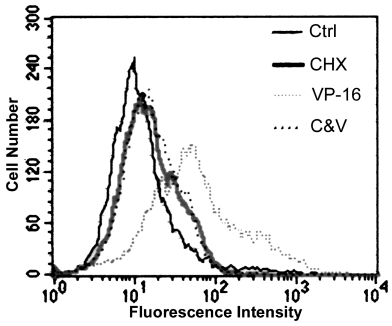

Fig. 5. CHX Completely Prevents PS Externalization Induced by VP-16 in Molt-4 Cells

Molt-4 cells were incubated with a drug vehicle (Ctrl), CHX (10 μg/ml), VP-16 (50 μM) or C&V for 4 h, and then assayed for PS externalization by flow cytometry.

DISCUSSION

The topoisomerase II inhibitor VP-16 (etoposide) is a clinical anticancer agent that has been widely employed to couple DNA damage to apoptosis in various cell lines.[13,17,18] Here, we have demonstrated that it can also induce apoptosis in U937 cells in a dose- and time-dependent manner. In contrast, apoptosis induced by CHX, an inhibitor of protein synthesis, is dose-dependent only. The aberrant time response to CHX has been suggested to be caused by cell adhesion in our recent study (unpublished data). This difference between VP-16- and CHX-induced apoptosis may be associated with their distinct apoptotic pathways. According to previous reports, VP-16 is an activator of the mitochondrial apoptotic pathway,[13] whereas CHX activates a Fas-associated death domain (FADD)-dependent pathway.[11] However, both of them require caspase activation.

Like the inhibition of apoptosis triggered by ionizing radiation or other chemicals,[8—10] CHX can also equally prevent VP-16-induced apoptosis of U937 cells after 4 h (within 2 h, no responses to VP-16 have been detected), when it is added with VP-16 simultaneously or 1 h later, suggesting that the synthesis of new proteins is required in this process and occurs after 1 h. But interestingly, within 2 h C&V instead causes a higher induction of apoptosis than CHX or VP-16 alone does, further indicating that VP-16 can also promote CHX-induced apoptosis. This promotion by VP-16 also extends to beyond 4 h based on the fact that C&V still shows more similarities with CHX alone but not with VP-16 alone. First, unlike the G_2–M arrest caused by VP-16 at 0.5 μM,[18] incubation of U937 cells in the presence of VP-16 at 50 μM will result in a marked accumulation of cells at G_0–G_1 phase. Similar results were also found in camptothecin (CPT)-treated HL-60 cells,[19] probably because apoptosis induced by VP-16 and CPT is limited to the S phase and G_2–M phase cells. In contrast, apoptosis triggered by CHX or C&V is indiscriminate, affecting all phases of the cell cycle, though the inhibition of protein synthesis potentially preventing cells from entering the G_2–M phase may have resulted in the decrease of cells at G_2–M phase. Second, TLCK, a serine protease inhibitor, protects VP-16-treated cells from apoptosis, further suggesting that some serine proteases or/and specific interleukin-1β-converting enzyme-like proteases[20] are also required in VP-16-induced apoptosis, but not in CHX- and C&V-induced apoptosis. As for why TLCK can sharply promote CHX- and C&V-induced apoptosis, no explanation has been proposed yet. But the inhibition of CHX-induced apoptosis in HL-60 cells by two other serine protease inhibitors, 3,4-dichloroisocoumarin (DCI) and *N*-α-tosyl-L-Phe-chloromethyl ketone (TPCK),[21] indicates that serine proteases may be unimportant in CHX-induced apoptosis. Moreover, TLCK can affect the cell cycle distribution by increasing cells at G_2–M phase, also suggesting that it is multifunctional. Third, the increase of C&V-induced apoptosis is CHX-concentration-dependent. In particular, when CHX concentration reached 50 μg/ml, we have even detected that CHX had no impact. Together, these results suggest that CHX, not VP-16, is indeed the dominant inducer of apoptosis when they are coadministered to U937 cells. In other words, apoptosis triggered by C&V may contribute to the forceful promotion of CHX-induced apoptosis by VP-16, but not to the partial inhibition of VP-16-induced apoptosis by CHX.

As for how VP-16 promotes CHX-induced apoptosis in U937 cells, one explanation can come from the fact that VP-16 has been shown to enhance the expression of both Fas receptors involving FADD, and Fas ligands.[22] Since CHX-induced apoptosis is FADD-dependent, thus increased expression of FADD would promote CHX-induced apoptosis. However, two vital conundrums are also raised. First, as an inhibitor of protein synthesis, CHX will inhibit the expression of FADD (data not shown). Second, C&V-induced apoptosis is time-dependent while CHX-induced apoptosis is not. This transformation can not be achieved through simply altering the expression of FADD. Alternatively, we are inclined to propose that VP-16 may promote CHX-induced apoptosis by interfering with the downstream signal pathways of cell adhesion, which has been suggested to prevent CHX-induced apoptosis and develop its aberrant time course in our recent study (unpublished data). Although we still can not point out the details of this process, some kinases such as PI-3K, PKCδ, which have been reported to be activated by VP-16,[17,23] may be included. Nevertheless, additional experiments are needed to clarify these mechanisms.

Although in the above we have ascribed C&V-induced apoptosis of U937 cells to the forceful promotion of CHX-induced apoptosis by VP-16, CHX was also found to totally block VP-16-induced apoptosis in CHX-resistant T-cells (Molt-4). This result not only suggests that VP-16 *per se* can not alter CHX's selection of cell types in the induction of apoptosis, but also advances a latent possibility that the partial inhibition or even lack of impact of CHX that often occurs in CHX-sensitive cell lines may be due to CHX-induced apoptosis, which is enhanced by its concurrent stimuli, and apoptosis induced by these stimuli may be completely blocked by CHX as in cell lines resistant to CHX. That is why the inhibitory effects of CHX on apoptosis induced by the same stimuli are usually different according to the cell type used. Moreover, if we use CHX and other chemotherapeutic drugs such as VP-16 in combination, we would achieve a high percentage of quick and selective induction of apoptosis. This could be an effective way to ameliorate the side effects and drug resistance of cancer therapy.

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