

II selectively affects *de novo* protein synthesis at the cellular level, A549 cells were pretreated with mycotrienin II and then pulse-labeled with [³H]L-leucine, [³H]thymidine, and [³H]uridine in the presence of the compound. Then, the amount of radioactivity incorporated into the acid-insoluble (macromolecular) fractions was measured. Mycotrienin II inhibited the incorporation of [³H]L-leucine in a dose-dependent manner and at the IC₅₀ value of 0.058 μM (Figure 2a), whereas mycotrienin II did not obviously affect the incorporation of [³H]thymidine and [³H]uridine (Figure 2b). When [³H]L-leucine-labeled cell lysates were separated into acid-soluble supernatants and acid-insoluble precipitates, mycotrienin II inhibited the incorporation of radioactivity into the precipitates, but conversely increased the radioactivity incorporation into the supernatants (Figure 2c). Thus, these results suggest that mycotrienin II does not affect the uptake of amino acids across the plasma membrane, but directly prevents translation.

To further investigate whether mycotrienin II directly affects translation, luciferase cDNA or luciferase mRNA was used as a template for transcription and translation in cell-free systems, based on rabbit reticulocyte lysates. The resultant luciferase activity associated with the

protein products was measured. Mycotrienin II suppressed the luciferase activity in the cell-free transcription-coupled translation system (IC₅₀ value: 5.5 μM) (Figure 2d) and the cell-free translation system (IC₅₀ value: 4.1 μM) (Table 1) at equivalent concentrations. These results indicate that mycotrienin II directly prevents translation.

The triene-ansamycin group of compounds inhibits TNF-α/IL-1α-induced ICAM-1 expression and translation

To obtain additional evidence that the inhibition of ICAM-1 expression by mycotrienin II is primarily caused by the prevention of translation, the structure-activity relationship of the triene-ansamycin group of compounds (Figure 3) was investigated. In the inhibitory profiles similar to that of mycotrienin II (Figure 1b), mycotrienin I, trienomycin A, trierixin, quinotrierixin and quinotrierixin HQ inhibited the TNF-α-induced ICAM-1 expression more preferentially than the IL-1α-induced ICAM-1 expression, whereas 13-ketomycotrienin I, 13-ketomycotrienin II and demethyltrienomycinol were totally inactive up to 10 μM (Table 1). Consistent with these data, mycotrienin I, trienomycin A, trierixin, quinotrierixin and quinotrierixin HQ

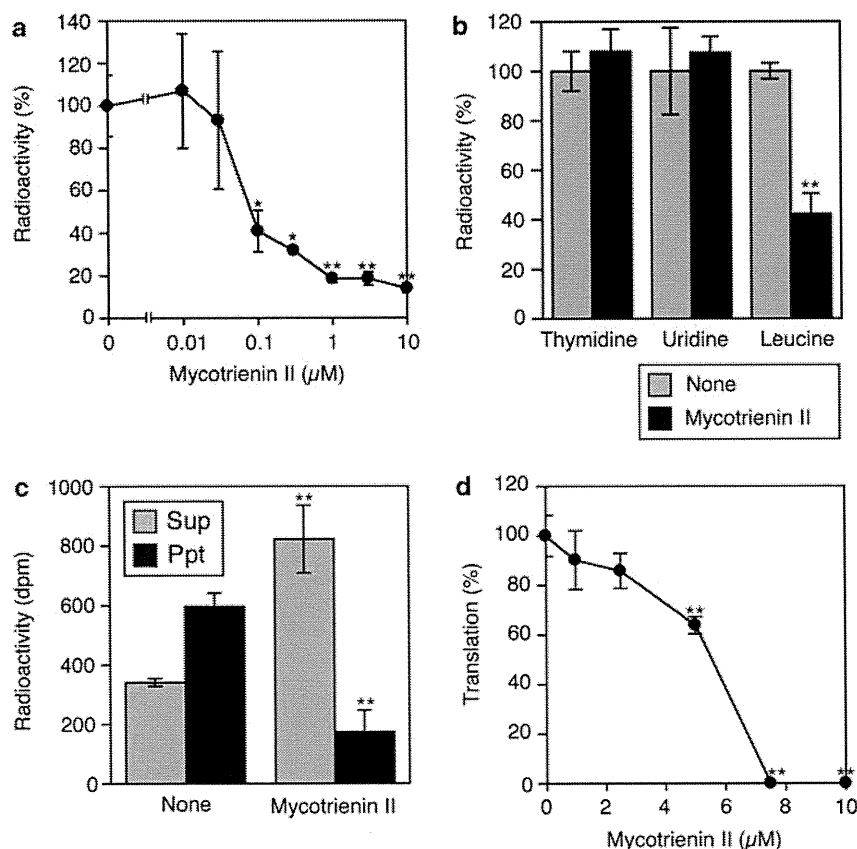


Figure 2 Mycotrienin II directly inhibits *de novo* protein synthesis. (a) A549 cells were pretreated with various concentrations of mycotrienin II for 1 h and pulse-labeled with [³H]L-leucine for 2 h in the presence or absence of mycotrienin II. Radioactivity incorporated into the acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± s.d. (n=3). *P<0.05 and **P<0.01, compared with control. Data are representative of two independent experiments. (b) A549 cells were pretreated with (black bars) or without (gray bars) mycotrienin II (1 μM) for 30 min and then pulse-labeled with [³H]thymidine, [³H]uridine or [³H]L-leucine for 1 h in the presence or absence of mycotrienin II. Radioactivity incorporated into the acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± s.d. (n=3). *P<0.05 and **P<0.01, compared with control. Data are representative of two independent experiments. (c) A549 cells were pretreated with or without mycotrienin II (1 μM) for 1 h and then pulse-labeled with [³H]L-leucine for 2 h in the presence or absence of mycotrienin II. Acid-soluble fractions as supernatants (Sup; gray bars) and acid-insoluble fractions as precipitates (Ppt; filled bars) were collected and measured for radioactivity. Radioactivity (dpm) is shown as means ± s.d. (n=3). *P<0.05 and **P<0.01, compared with control. Data are representative of two independent experiments. (d) Luciferase cDNA was transcribed and then translated by rabbit reticulocyte lysates in the presence of various concentrations of mycotrienin II. Luciferase activity (%) is shown as means ± s.d. (n=3). *P<0.05 and **P<0.01, compared with control. Data are representative of two independent experiments.

inhibited the cell-free translation at comparable concentrations, whereas 13-ketomycotrienin I, 13-ketomycotrienin II and demethyltrienomycinol were inert up to 10 μM (Table 1). These results indicate that the inhibition of ICAM-1 expression by the triene-ansamycin group of compounds is closely related to their ability to prevent translation.

Effect of translation inhibitors on ICAM-1 expression induced by TNF- α and IL-1 α

The above observation that the triene-ansamycin group compounds inhibited the TNF- α -induced ICAM-1 expression more preferentially than the IL-1 α -induced ICAM-1 expression prompted us to investigate the effect of other translation inhibitors on ICAM-1

expression induced by TNF- α and IL-1 α . As reported in our previous paper,¹⁴ acetoxycycloheximide inhibited the TNF- α -induced ICAM-1 expression at much lower concentrations than the IL-1 α -induced ICAM-1 expression (Figure 4a), whereas cycloheximide prevented the TNF- α - and IL-1 α -induced ICAM-1 expression at relatively similar concentrations (Figure 4b). By contrast, anisomycin inhibited the TNF- α -induced ICAM-1 expression more strongly than the IL-1 α -induced ICAM-1 expression (Figure 4c), whereas puromycin and emetine prevented the TNF- α - and IL-1 α -induced ICAM-1 expression at equivalent concentrations (Figures 4d and e). These data indicate that the triene-ansamycin group compounds exert inhibitory profiles similar to acetoxycycloheximide and anisomycin.

DISCUSSION

Cytotrienin A, a structural derivative of mycotrienin II, was initially identified as an inducer of apoptosis.^{17,18} Recently, it has been shown that cytotrienin A inhibits eukaryotic protein synthesis by targeting translation elongation and interfering with eukaryotic elongation factor 1A function.¹⁵ Consistent with this and our findings,^{15,16} mycotrienin II and its structural derivatives inhibited cellular protein synthesis and cell-free translation. Nevertheless, the *de novo* protein synthesis in intact living cells (Figure 2a) was inhibited much more strongly by mycotrienin II than the cell-free translation (Table 1). The difference in sensitivity is in agreement with the observation that cytotrienin A inhibits cellular protein synthesis more strongly than the cell-free translation.¹⁵ This may be because the cell-free translation systems contain larger amounts of proteins that bind to mycotrienin II or cytotrienin A (most likely eukaryotic elongation factor 1A) than living cells, and therefore higher concentrations of the compounds are required for the inhibition of translation.

Nevertheless, the concentrations of mycotrienin II required to inhibit the IL-1 α -induced ICAM-1 expression (Figures 1b) were still higher than those required to inhibit constitutive *de novo* protein synthesis monitored by [³H]L-leucine incorporation (Figure 2a). By contrast, we have shown that the protein synthesis inhibitor cycloheximide prevents cellular protein synthesis and IL-1 α -induced ICAM-1 expression at

Table 1 Biological activities of triene-ansamycin group of compounds

Compound	ICAM-1 expression ^a		Translation ^b
	TNF- α	IL-1 α	
Mycotrienin I	0.57	4.9	6.2
Mycotrienin II	0.3	3.1	4.1
Trienomycin A	0.61	3.0	4.4
13-Ketomycotrienin I	> 10	> 10	> 10
13-Ketomycotrienin II	> 10	> 10	> 10
Trierixin	0.16	0.62	2.2
Quinotrierixin	0.84	4.7	2.8
Quinotrierixin HQ	1.3	6.2	3.7
Demethyltrienomycinol	> 10	> 10	> 10

Abbreviations: ICAM-1, intercellular adhesion molecule-1; IL-1 α , interleukin-1 α ; TNF- α , tumor necrosis factor- α .

^aA549 cells were preincubated with serial dilutions of compounds for 1 h and then incubated with TNF- α (2.5 ng ml⁻¹) or IL-1 α (0.25 ng ml⁻¹) for 6 h in the presence or absence of compounds. ICAM-1 expression (%) was determined by triplicate cultures. The means of IC₅₀ values (μM) were calculated from three independent experiments.

^bLuciferase mRNA was translated by rabbit reticulocyte lysates in the presence of serial dilutions of compounds at 30 °C for 90 min. Luciferase activity (%) was determined by triplicate measurements. The means of IC₅₀ values (μM) were calculated from two independent experiments, except for trierixin.

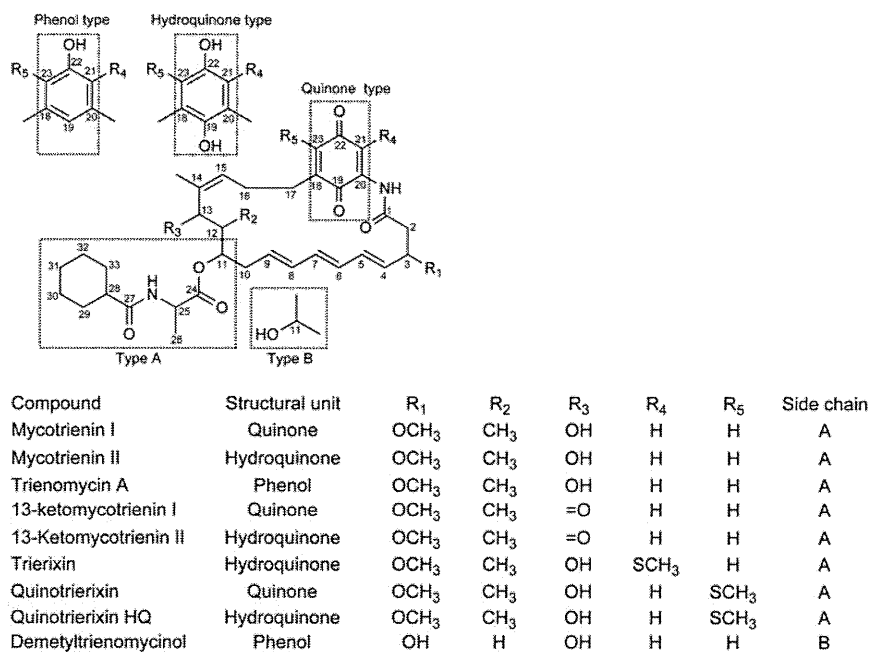


Figure 3 Structures of triene-ansamycin groups of compounds.

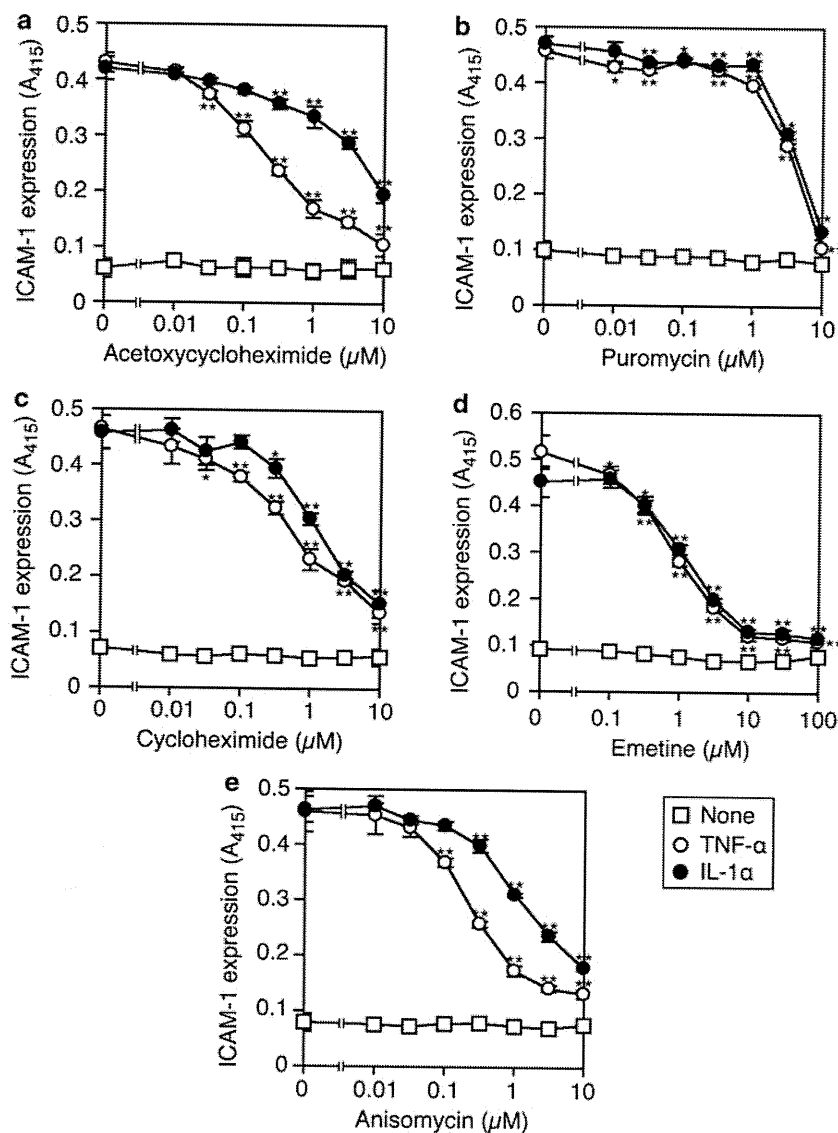


Figure 4 Effect of translation inhibitors on cell-surface intercellular adhesion molecule-1 (ICAM-1) expression induced by tumor necrosis factor- α (TNF- α) and interleukin-1 α (IL-1 α). A549 cells were pretreated with various concentrations of acetoxycycloheximide (a), cycloheximide (b), anisomycin (c), puromycin (d) or emetine (e) for 1 h and then incubated with TNF- α (2.5 ng ml⁻¹; open circles) or IL-1 α (0.25 ng ml⁻¹; filled circles) or without cytokines (open squares) for 6 h in the presence of the compound. ICAM-1 expression (A₄₁₅) is shown as means \pm s.d. ($n=3$). * $P<0.05$ and ** $P<0.01$, compared with control. Data are representative of two independent experiments.

relatively similar concentrations in A549 cells.¹⁴ Eukaryotic elongation factor 1A is indispensable for the translation of all polypeptide-encoded mRNAs in that it has an essential role in the delivery of the aminoacyl-tRNA to the ribosome A site and the subsequent GTP hydrolysis. Therefore, the inhibitory effect of mycotrienin II may be influenced by unidentified intracellular factors and/or conditions other than eukaryotic elongation factor 1A in intact living cells.

The present structure-activity relationship study demonstrated that the hydroxyl group at C-13 is essential for the inhibitory activities of the triene-ansamycin compounds on ICAM-1 expression, as well as the cell-free translation, whereas the difference in the benzenoid moiety (phenol, hydroquinone and quinone) did not alter the inhibitory activities (Table 1). These properties of the triene-ansamycin group are in agreement with the structure-activity relationship study of the inhibitors of endoplasmic reticulum stress-induced X-box binding protein 1 activation.¹⁵ In addition, mycotrienin II and its five

structural derivatives inhibited the TNF- α -induced ICAM-1 expression several times more strongly than the IL-1 α -induced ICAM-1 expression (Table 1). As shown in Figure 3, these characteristic inhibitory profiles were observed with translation inhibitors that potentially induce ribotoxic stress response (acetoxycycloheximide and anisomycin), but not obviously observed with those that induce ribotoxic stress response weakly or not at all (cycloheximide, puromycin and emetine).^{19–23} Ribotoxic stress response caused by translation inhibitors triggers the activation of the mitogen-activated protein kinase superfamily, thereby leading to diverse cellular responses, such as gene expression and apoptosis.^{19–26}

Ectodomain shedding is a critical posttranslational mechanism for the regulation of the function of membrane-anchored ligands and receptors. TNF- α -converting enzyme cleaves many transmembrane proteins, including TNF- α and TNF receptor 1 (TNF-R1), into their soluble forms and has a regulatory role in TNF- α -dependent cellular

responses.^{27,28} We have previously shown that acetoxycycloheximide induces the ectodomain shedding of TNF-R1 by TNF- α -converting enzyme in a manner that is dependent on the activation of extra-cellular-signal regulated kinase and p38 mitogen-activated protein kinase in A549 cells.^{22,23} Consistently, we observed that mycotrienin II induced the upregulation of soluble extracellular TNF-R1 in A549 cells and that it was prevented by the TNF- α -converting enzyme inhibitor TAPI-2 (unpublished data). Thus, mycotrienin II seems to induce the ectodomain shedding of TNF-R1 in a manner similar to acetoxycycloheximide. Because the ectodomain shedding of TNF-R1 by TNF- α -converting enzyme weakens responsiveness to TNF- α , it seems possible that mycotrienin II is able to trigger activation of the mitogen-activated protein kinase superfamily and thereby induce the ectodomain shedding of TNF-R1, leading to the reduction of TNF- α responsiveness. Ribotoxic stress response possibly induced by the triene-ansamycin group compounds may account for the preferential inhibition of the TNF- α -induced ICAM-1 expression rather than the IL-1 α -induced ICAM-1 expression.

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