Biochemical and Biophysical Research Communications **257,** 19–23 (1999)
Article ID bbrc.1999.0401, available online at http://www.idealibrary.com on IDEAL®

A Radicicol-Related Macrocyclic Nonaketide Compound, Antibiotic LL-Z1640-2, Inhibits the JNK/p38 Pathways in Signal-Specific Manner

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Received February 2, 1999

Macrocyclic nonaketide compounds, radicicol and its two analogues, 87-250904-F1 and LL-Z1640-2, have various biological activities. Here we show that these compounds inhibit signal-dependent transcriptional activation with different specificity with distinct mechanism. Although all three compounds inhibited PMA-induced AP-1 transcriptional activity in cell-based reporter assay, these compounds exhibited differential effects in separate transcriptional reporter assays for NF-κB and glucocorticoid receptor. Next we found that one of these compounds, LL-Z1640-2, was a signal-specific inhibitor of the JNK/p38 pathways. In contrast to LL-Z1640-2, radicicol and 87-250904-F1 did not inhibit JNK/p38 activation. Recently, radicicol was reported as an inhibitor of activated-Ras-induced ERK activation. These results indicated that radicicol and LL-Z1640-2 showed distinct specificity to various MAP kinase pathways despite their structural similarity. Furthermore, LL-Z-1640-2 inhibited anisomycin-induced but not TNF-induced JNK/p38 activation, indicating that the inhibition mechanism is signal-specific.

Radicicol-related macrocyclic nonaketide compounds, radicicol, 87-250904-F1 and anibiotic LL-Z1640-2 (Fig. 1), have various biological activities. Radicicol was originally isolated as an anti-fungal antibiotic from fungi (1), and was also known to have several other activities (2, 3). 87-250904-F1 (radicicol analogue A) was reported to reduce IL-1 expression in LPS-treated human monocytic leukemia THP-1 cells (4). Antibiotic LL-Z1640-2 was a natural compound isolated as an anti-protozoan (5). Recently, it was reported that radicicol blocked the activation of mitogen-activated protein kinase (MAPK/ERK) pathway by destabilization of Raf kinase (MAPKKK) resulted from inhibiting chaperone function of HSP90 (6, 7). However the molecular mechanisms of the other radicicol-related compounds were not known.

A common mechanism by which eukaryotic cells sense and respond to extracellular stimuli is via activation of a mitogen-activated protein (MAP) kinase cascade, which consists of three sequentially acting kinases (MAPK-MAPKK-MAPKKK) (8, 9). The JNK/p38 pathways are another cascades of the MAP kinase related group (SAPK: stress-activated protein kinase) that are activated in response to environmental stresses and inflammatory cytokines (8, 9). Both the MAPK (ERK) and the SAPK (JNK/p38) pathways are involved in AP-1 activation by distinct mechanisms: up-regulation of c-Fos or c-Jun, the component of AP-1 complex (10). The ERK pathway leads to the phosphorylation and activation of Elk-1, which is a component of the ternary complex for the c-fos promoter and consequently to the increase of c-Fos expression. The JNK/p38 pathways lead to the phosphorylation and activation of c-Jun.

To understand the molecular mechanisms of the effects by radicicol–related compounds in mammalian cells, we focused on the MAPK (ERK) and SAPK (JNK/p38) pathways. Finally we found that radicicol-related macrocyclic nonaketide compounds, radicicol and its two analogues, 87-250904-F1 and LL-Z1640-2, showed distinct specificity to various MAP kinase pathways despite their structural similarity and that LL-Z-1640-2 inhibited the JNK/p38 pathways signal-specific manner.

MATERIALS AND METHODS

Cell culture and reagent. Human embryonal kidney HEK293 (American Type Culture Collection), Human cervical carcinoma Hela S3 cells (American Type Culture Collection) and murine fibroblast

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0006-291X/99 $30.00

NIH3T3 cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin. Hygromycin resistant cells were maintained at 50 μg/ml hygromycin B. All reagents were purchased from Nacalai Tesque (Kyoto, Japan) except for radicicol, 87-250904-F1 and LL-Z1640-2. Radicicol, 87-250904-F1 and LL-Z1640-2 were isolated and purified from cultures of *Verticillium* sp. AJ117461, *Oidiodendron* sp. AJ117360 and *Curvularia* sp. AJ117361, respectively, at Pharmaceutical Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan.

Reporter plasmids. pTRE-APZ was constructed by replacing the luciferase gene of pGL2-promoter (Promega) with β-galactosidase gene of pSV-β-galactosidase (Promega) and inserting 6 times repeated AP-1 binding sequence (GAGGATGTTATAAAGCATGAGTCAC) derived from human MMP-1 promoter at a XhoI site upstream of a SV40 basic promoter of pGL2-promoter plasmid. pTRE-APZ/hyg was constructed by inserting phospho-glycero-kinase promoter derived hygromycin resistant gene into the BamHI site of pTRE-APZ. pNF-κB-APZ/hyg was constructed similarly to pTRE-APZ/hyg, except that NF-κB binding sequence (GATCTCCAGAGGGACTTTCCGAG) derived from human immunogloblin κ light chain promoter was inserted instead of an AP-1 site. pMMTV-AZ/Hyg was constructed by replacing the AP-1 dependent promoter of pTRE-APZ/hyg with the MMTV-LTR promoter from pMAM-luc-neo (Clontech).

Cell-based reporter gene assay. HEK293 were stably transfected with pTRE-APZ/hyg and pNF-κB-APZ/hyg by a modified calcium-phosphate procedure (11) and selected by growing in media containing 50 μg/ml hygromycin B. Stably transfected cells were also selected by fluorocytometric sorting (12) with β-galactosidase activity induced by 50 ng/ml PMA (or 1 ng/ml IL-1) treatment using the FluoReporter lacZ system (Molecular Probes, Inc., Eugene, OR). After triple fluorocytometric sorting, the cells were provided to assays. Selected HEK293 cells were named as KT cells for AP-1 reporter and as KK cells for NF-κB reporter. KT or KK cells (2×10^4) were plated in 96-well tissue culture plates and cultured for 24 hrs before exposure to 50 ng/ml PMA (or 1ng/ml IL-1) for 16 hrs in the presence or absence of inhibitor compounds. Each compound tested was added to the wells 30 minutes before stimulation. The β-galactosidase activity was determined using the Galacto-Light assay system (Tropix Inc., Bedford, MA). KM cells, which were stably transfected with pMMTV-AZ/Hyg to HEK293 cells, were selected in the same manner as above, except for stimulation by 10^{-7}M dexamethasone. MS-1 cells, which were stably transfected with pMMTV-AZ/Hyg to murine NIH3T3 cells, were selected in the same manner as KM cells.

Immunoblot analysis for detection of JNK/p38 activity. Hela S3 cells (2×10^5) were plated in a 24-well tissue culture plate. Cells were cultured for 24 hrs, then stimulated with 1 μg/ml anisomycin (Nacalai Tesque), 100 ng/ml TNFalpha (PeproTech) or 50 ng/ml EGF (PeproTech) for 30 minutes. The compounds were added to wells 30 minutes before stimulation. Cells were lysed by adding 50 μl of 1 x Laemmlis SDS-PAGE sample buffer containing 1 mM phenyl-methyl-sulphonyl fluoride, 2 mM Na_3VO_4 and 10 mM β-glycero-phosphate. A half volume of cell lysate was electrophoresed on a 5-20% gradient SDS-polyacrylamide gel (ATTO, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane, then immunoblotted with appropriate antibodies. For detection, the blots were incubated with appropriate ECL secondary antibody (horseradish peroxidase-conjugated anti-Ig antibody, Amersham Pharmacia Biotech), and developed using the ECL detection system according to the instruction manuals. Antibodies against phospho-c-Jun, phospho-ATF-2, phospho-JNK, phospho-p38 and phospho MAPK were obtained from New England Biolabs Inc.

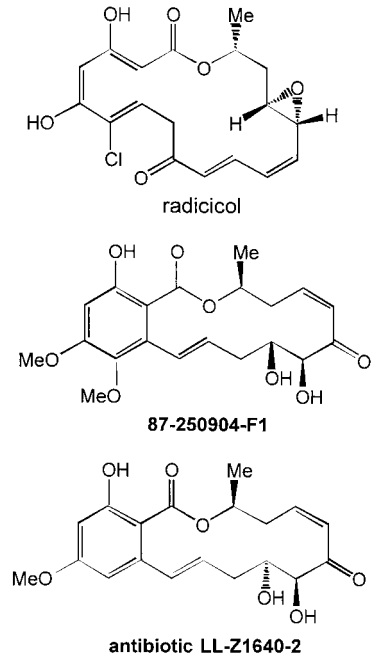

FIG. 1. Chemical structures of macrocyclic nonaketide compounds.

RESULTS AND DISCUSSION

Radicicol-Related Compounds Specifically Repressed PMA-Induced AP-1 Activity in Human Cells

Several experiments were carried out to investigate the effects of three radicicol-related compounds, radicicol, 87-250904-F1 and LL-Z1640-2, on transcriptional activation in mammalian cell-based reporter assays. We found that all three radicicol-related compounds repressed PMA-induced AP-1 activity almost to the basal level in the KT cells, but gave no effects on IL-1-induced NF-κB activity in the KK cells (Fig. 2). These results indicated that radicicol-related compounds were specific inhibitors of the PMA-induced AP-1 activation in mammalian cells. Recently, it was reported that radicicol disrupted the Ras-induced ERK pathway by destabilization of Raf protein (6) that was caused by inhibiting HSP90 chaperone function (7). HSP90 was also known as a glucocorticoid receptor (GR) binding protein and it was indispensable for ligand-induced GR function (13, 14, 15). Next experiments were carried out to investigate the effects of radicicol-related compounds on GR-dependent transcriptional activation. As shown in Fig. 2, among the three compounds, the GR-dependent transcriptional activation was repressed only by radicicol. We also confirmed similar activities in the human cell system; KM cells (data not shown). These results suggest that radicicol is a inhibitor for ligand-dependent GR transactivation by inhibiting chaperone function of HSP90, and that HSP90 would not be a direct molecular target of 87-250904-F1 and LL-Z1640-2. To clarify molecular mechanisms of inhibitory

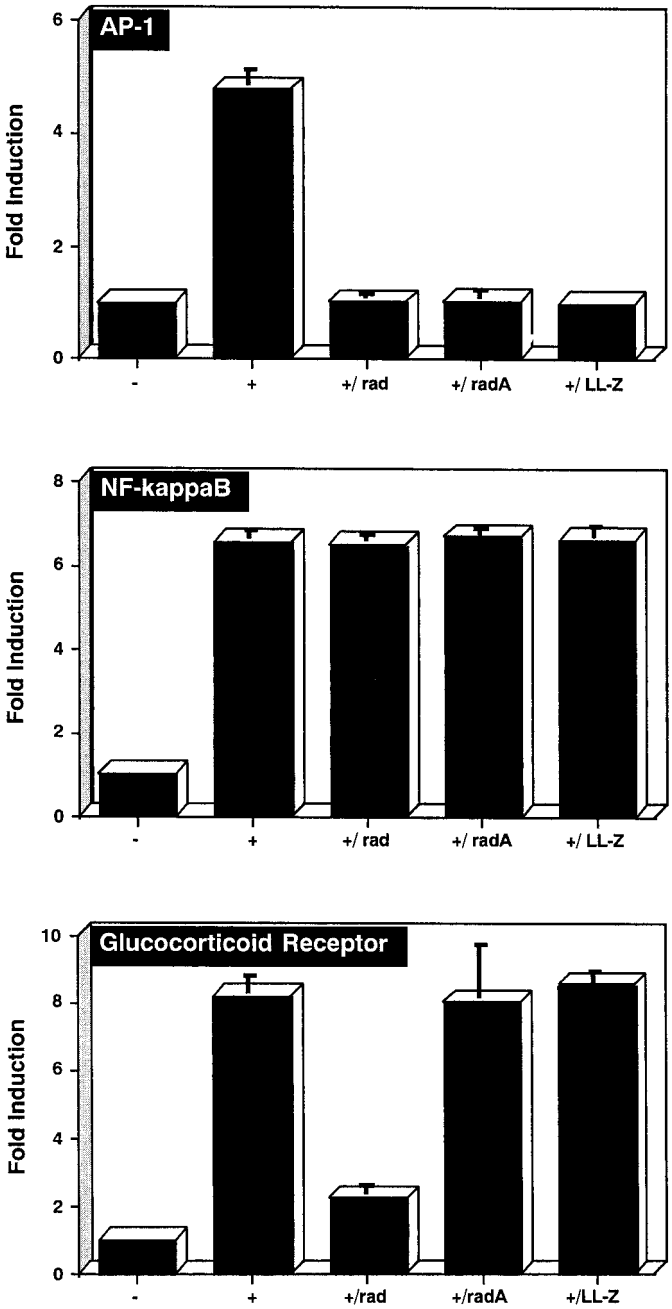

FIG. 2. Macrocyclic nonaketide compounds specifically inhibit AP-1-dependent transcriptional activity. The AP-1-dependent β-galactosidase activity in KT cells (AP-1) induced by 100 ng/ml PMA, the NF-κB-dependent β-galactosidase activity in KK cells (NF-κB) induced by 1 ng/ml IL-1-β and the glucocorticoid receptor-dependent β-galactosidase activity in MS-1 cells (MMTV-LTR) induced by 10^{-7} M dexamethasone was measured 16 hrs after stimulation in the presence of indicated compounds (rad: radicicol, rad A: 87-250904-F1, LL-Z: LL-Z1640-2). Each compound was added 30 minutes before stimulation. Data are the expressed fold induction with the control (no stimulation) value set at 1.00. The (−) is without stimulation and the (+) is with stimulation.

effects of radicicol-related macrocyclic nonaketides on AP-1 activity, we focused on the JNK/p38 pathways (SAPK) signal transduction.

Anisomycin Induced c-Jun Phosphorylation Was Inhibited by LL-Z1640-2, but Not by Radicicol and 87-250904-F

Anisomycin is known as a good agonist for JNK/p38 in many mammalian cells (16, 17). In Hela cells, anisomycin-treatment leads to rapid activation of the JNK/p38 kinase and consequent phosphorylation of c-Jun and ATF2. We used this system for our experiments. Our results indicated that only LL-Z1640-2 (at 100 ng/ml concentration) reduced phosphoryaltion of c-Jun and ATF2 (Fig. 3 (A)) in anisomycin-treated Hela cells. The suppressive activity of LL-Z1640-2 was observed at low concentration (25 ng/ml, Fig. 3 (B)), and at this concentration, this compound showed no obvious effect on cell proliferation (data not shown). Even at higher concentrations, radicicol (Fig. 3 (B)) and 87-250904-F1 (data not shown) had no activity of reducing

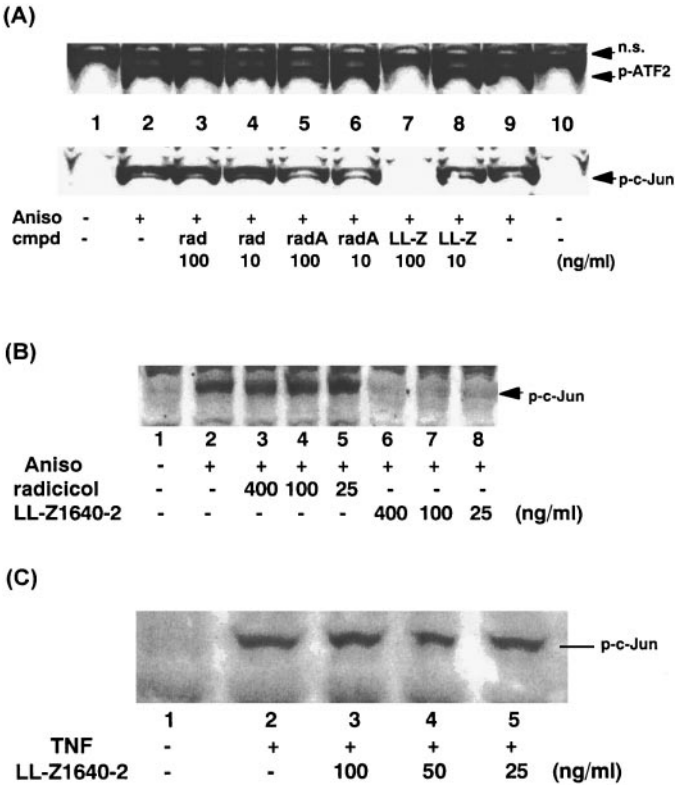

FIG. 3. LL-Z1640-2 inhibits anisomycin-induced JNK/p38 activity in a signal specific manner. (A) Effects of radicicol, 87-250904-F1 and LL-Z1640-2 on 1 μg/ml anisomycin-induced phosphorylation of c-Jun and ATF2 in Hela cells. Immunoblot analysis was performed with anisomycin-treated cell lysates in the presence or absence of the indicated concentration of each compound. The arrows indicate phosphorylated c-Jun and ATF2 bands. (B) Effects of higher dose radicicol and LL-Z1640-2 on 1 μg/ml anisomycin-induced phosphorylation of c-Jun in Hela cells. The arrow indicates the phosphorylated c-Jun band. (C) Effects of LL-Z1640-2 on 10 ng/ml TNF-induced phosphorylation of c-Jun in Hela cells. Immunoblot analysis was carried out as described in Materials and Methods. The arrow indicates the phosphorylated c-Jun band.

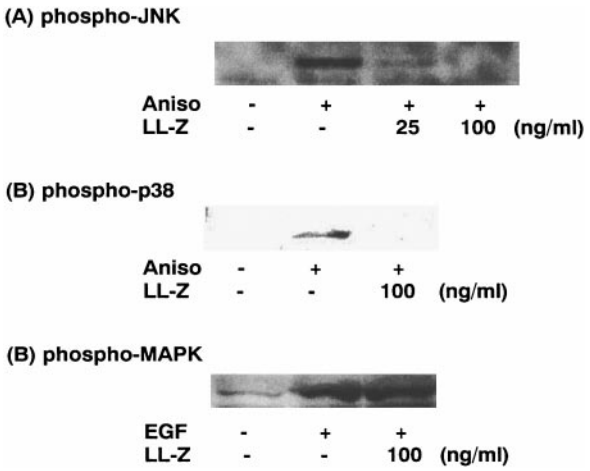

FIG. 4. LL-Z1640-2 inhibits anisomycin-induced JNK/p38 activation at MAPKK level, but not inhibit growth Factor-Induced MAPK Activation. Effects of LL-Z1640-2 on 1 μg/ml Anisomycin-induced phosphorylation of JNK (A), p38 (B) and 50ng/ml EGF-induced phosphorylation of MAPK. Immunoblot analysis was carried out as described in Materials and Methods. The concentration of LL-Z1640-2 is 50 ng/ml in (B) and 100 ng/ml in (C).

the JNK/p38 activities. Recently, it was reported that radicicol disrupted the Ras-induced ERK pathway by destabilization of Raf protein (6) that was caused by inhibiting HSP90 chaperone function (7). In those reports, depletion of Raf protein by radicicol required a relatively long time (more than 24 hrs). In our experiments, just 30 minutes pre-treatment was sufficient for LL-Z1640-2 to inhibit JNK/p38 activity.

The JNK/p38 Pathways INHIBITION by LL-Z1640-2 Are Signal Specific

To determine if LL-Z1640-2 is a direct inhibitor of JNK/p38 themselves or not, we carried out additional experiments. At first, we investigated the inhibitory effects of LL-Z1640-2 on the kinase pathways using another JNK/p38 stimulator, inflammatory cytokine TNF (tumor necrosis factor). As shown in Fig. 3 (C), LL-Z1640-2 failed to inhibit TNF-induced JNK activity in Hela cells. Therefore the inhibitory activity of LL-Z1640-2 is signal specific, requiring specific upstream signals that are involved in anisomycin-induced signaling. These results indicated that 1) LL-Z1640-2 is not a direct inhibitor of the JNK/p38 kinase, 2) LL-Z1640-2 differentiates the two signaling pathways leading to JNK/p38 activation, anisomycin-induced and TNF-induced, and 3) the molecular target of LL-Z1640-2 in mammalian cells located upstream of the JNK/p38 (MAPK) level. To confirm the last point, we examined the inhibitory effects of LL-Z1640-2 at JNK/p38 activator kinase (MAPKK) levels. Anisomycin-induced phosphorylation of JNK and p38 was reduced by LL-Z1640-2 treatment (Fig. 4 (A), (B)). Although LL-Z1640-2 was a strong inhibitor of the JNK/p38 pathway, it had no effect on ERK kinase activation induced by epidermal growth factor (EGF) in Hela cells (Fig. 4 (C)). The results indicates that LL-Z1640-2 interferes with the JNK/p38 signaling pathway at the level or upstream of the MAPKK level, and the molecular target of this compound is involved in anisomycin-induced but not in TNF-induced signaling.

From these results, one can conclude that macrocyclic natural product LL-Z1640-2, which has very similar structural properties with radicicol, has distinct effects on mammalian signal transduction pathway from radicicol. The data presented here indicate that LL-Z1640-2 provides a very useful tool for dissecting the stress-induced signal transduction pathways. The JNK/p38 pathways are involved in many biological responses (e.g., inflammation, immunological responses, environmental stress responses, apoptosis and cell differentiation, etc.) (8, 9, 18) and their deregulation is pathogenic for some specific diseases (inflammatory and immunological disease, neuronal (19) and cardiovascular disorders (20), etc.). LL-Z1640-2 and their relatives have the potential to be therapeutically useful for treating such diseases and useful tools for signal transduction research.

ACKNOWLEDGMENTS

We thank to members of Discovery Laboratories, Pharmaceutical Research Laboratories, Ajinomoto Co., Inc. for helpful discussions and critical reading of this manuscript, and G. I. Warden for technical assistance and comments to improve the manuscript.

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