

Antieosinophilic Activity of Orazipone^[S]

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ABSTRACT

Orazipone is a novel sulfhydryl-reactive compound that has been previously shown to reduce lung eosinophilia in guinea pigs and rats and to inhibit degranulation in mast cells and cytokine production in monocytes and T-cells. However, the effects of orazipone on granulocyte longevity are unknown. Orazipone and its derivative 3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione (OR-2370) reversed interleukin-5-afforded survival of human eosinophils by inducing apoptosis. In contrast, orazipone did not affect granulocyte macrophage-colony-stimulating factor-induced survival of human neutrophils. The effect of orazipone on eosinophil apoptosis is different from that of glucocorticoids in that even high concentrations of

interleukin-5 were not able to overcome the effect of orazipone. Orazipone further enhanced spontaneous apoptosis as well as that induced by CD95 ligation without inducing primary necrosis. OR-2370-induced DNA fragmentation was shown to be dependent on caspases 3 and 6 and c-jun-N-terminal kinase, whereas extracellular regulated kinase, p38 mitogen-activated protein kinase, and phosphatidylinositol 3-kinase as well as caspases 4, 8, and 9 seem not to mediate its actions. Our results suggest that orazipone and its derivative OR-2370 possess strong antieosinophilic activity and thus may have anti-inflammatory efficacy in the treatment of asthma and/or allergic conditions.

Eosinophils are thought to play a critical role in allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis (Giembycz and Lindsay, 1999; Gleich, 2000). In asthmatic patients, activation of eosinophils in the airways is believed to cause epithelial tissue injury, contraction of airway smooth muscle and increased bronchial responsiveness. Apoptosis or programmed cell death is regarded as an important feature in the resolution of asthmatic inflammation (Kankaanranta et al., 2005). Apoptosis is characterized by specific biochemical and morphological changes, including

cell shrinkage, surface blebbing, DNA fragmentation and loss of nucleoli (Kankaanranta et al., 2005), so that the apoptotic cell is phagocytosed intact without release of its contents. In vitro, eosinophil apoptosis is inhibited by cytokines, such as interleukin-3, interleukin-5, and granulocyte macrophage-colony-stimulating factor (GM-CSF) (Giembycz and Lindsay, 1999; Kankaanranta et al., 2005). Eosinophil apoptosis is up-regulated by Fas (CD95/APO-1), a 45-kDa transmembrane protein belonging to the tumor necrosis factor receptor family (Kankaanranta et al., 2005). Eosinophil apoptosis is delayed in patients with asthma or inhalant allergy (Wedi et al., 1997; Kankaanranta et al., 2000a). Furthermore, the number of eosinophils in asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils (Vignola et al., 1999). Thus, pharmacological induction of eosinophil apoptosis is considered an interesting possibility to treat eosinophilic inflammatory conditions such as asthma and/or allergic diseases.

Orazipone and its derivatives OR-1958 and OR-2370 (Fig.

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ABBREVIATIONS: GM-CSF, granulocyte macrophage-colony-stimulating factor; JNK, c-jun-N-terminal kinase; DMSO, dimethyl sulfoxide; Z-, N-benzyloxycarbonyl-; FMK, fluoromethyl ketone; Ac-, N-acetyl-; CHO, aldehyde; MAPK, mitogen-activated protein kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PD098059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole; PI3K, phosphatidylinositol 3-kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; DCB, [(2,6-dichlorobenzoyl)oxy]methane; L-JNK11, GRKKRRQRRR-PP-RPKRPTTLNLFQVPRSQD-amide; L-TAT, RKKRRQRRR-amide, negative control for L-JNK11; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; IL, interleukin; OR-2370, 3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione; OR-1958, 3-(3-chloro-4-methanesulfonyl-benzylidene)-pentane-2,4-dione; OR-2149, 3-(4-methanesulfonyl-benzylidene)-pentane-2,4-dione.

1) are novel anti-inflammatory compounds that exert their effects by forming reversible conjugates with the thiol groups of proteins and glutathione (Wroblewski et al., 1998). Their effect on thiol groups is readily reversible and makes them unique among the other thiol-modulating compounds. Orazipone (OR-1384) has been shown previously to suppress the release of interleukin-1 β , interleukin-8, and tumor necrosis factor- α from human blood monocytes and to suppress oxygen free radical production in polymorphonuclear leukocytes (Nissinen et al., 1997, 1998; Serkkola and Nissinen, 1999). Recently, orazipone and its derivative OR-1958 were shown to inhibit histamine release and tumor necrosis factor- α production in rat and human mast cells (Vendelin et al., 2005). Orazipone and OR-1958 have been shown to reverse the platelet-activating factor-induced pulmonary eosinophilia in a dose-dependent manner in guinea pigs (Aho et al., 2001). Furthermore, orazipone was shown to prevent lung eosinophilia in ovalbumin-sensitized rats after repeated administration with efficacy equal to that of budesonide (Ruotsalainen et al., 2000). However, at the cellular level, the effects of orazipone on eosinophils are not known. In addition to its efficacy in experimentally induced pulmonary inflammation, orazipone has been shown to have anti-inflammatory effects in models of experimental colitis (Wroblewski et al., 1998). The exact intracellular mechanism of the anti-inflammatory action of orazipone remains unknown but may be related to the modulation of intracellular signaling system by inhibition of the function of thiol-containing proteins.

Given the critical role of thiol groups and oxygen radicals in eosinophil apoptosis (Wedi et al., 1999; De Souza et al., 2002; Kankaanranta et al., 2002; Gardai et al., 2003), our aim was to test the possible anti-inflammatory effects of orazipone on human eosinophils. The present study describes the ability of orazipone and its derivative OR-2370 to induce apoptosis in human eosinophils and to reverse interleukin-5-afforded eosinophil survival as well as evaluates their possible mechanisms of action.

Materials and Methods

Granulocyte Purification. Blood (50–100 ml) for eosinophil experiments was obtained from persons with eosinophilia. However, patients with hypereosinophilic syndrome were excluded because of the possibly different signaling in the myeloproliferative variant hypereosinophilic syndrome expressing FIP1L1-PDGFR α -fusion kinase (Schwartz 2003). For neutrophil experiments, blood was obtained from healthy volunteers. Eosinophils and neutrophils were isolated to >99% purity under sterile conditions as reported previously (Kankaanranta et al., 1999, 2000a,b; Zhang et al., 2000, 2002). The cells were resuspended at 10^6 cells/ml and cultured in RPMI 1640 medium, 10% fetal calf serum, and antibiotics. Subjects gave informed consent to a study protocol approved by the ethical committee of Tampere University Hospital.

Determination of Granulocyte Apoptosis. Unless otherwise stated, eosinophil and neutrophil apoptosis was determined by relative DNA fragmentation method and flow cytometry as described previously (Kankaanranta et al., 1999, 2000a,b; Zhang et al., 2000, 2002). The cells showing decreased relative DNA content were considered to be apoptotic, as described previously (Kankaanranta et al., 2000b). Annexin-V binding and morphological analysis was performed as previously reported (Kankaanranta et al., 2000b; Zhang et al., 2002). Oligonucleosomal DNA fragmentation in eosinophils was analyzed by agarose gel DNA electrophoresis as described previously (Kankaanranta et al., 1999, 2000b).

Caspase Activity Assay. Caspase 3/7, 8, and 9 activities in human eosinophils were measured with the use of Caspase-Glo 3/7, 8, and 9 assays according to the manufacturer's instructions. In brief, eosinophils (10^6 cells/ml) were cultured in RPMI 1640 medium, 10% fetal calf serum, and antibiotics in the presence or absence of interleukin-5 (10 pM) and OR-2370 (10 μ M) for 16 h. Equal volumes of Caspase-Glo 3/7, 8, or 9 substrates were added, and the sample was incubated for 1 h at room temperature before measurement of luminescence.

Immunoblot Analysis. Eosinophils were suspended at 10^6 cells/ml and cultured at 37°C. At the time points indicated in Fig. 6, cells were centrifuged at 12,000g for 10 min. The cell pellet was lysed by boiling for 5 min in 30 μ l of 6 \times Laemmli buffer, centrifuged at 12,000g and debris was carefully removed. Samples were then stored at -20°C until immunoblot analysis. For immunoblot analysis, each protein sample was loaded on 10% SDS-polyacrylamide gel and electrophoresed for 2 h at 100 V. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) with semidry blotter, blocked using 5% nonfat dry milk in 20 mM Tris-base, pH 7.6, 150 mM NaCl, and 0.1% Tween 20. Proteins were labeled using specific antibody and subsequently detected using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) Western blotting detection agents and detected by using Fluorchem 8800 equipment and software (Alpha Innotech, San Leandro, CA). Quantification of relevant bands was performed by densitometry. The activated c-jun-N-terminal kinase (JNK) was identified and quantified by Western blot analysis using specific antibody recognizing the dual phosphorylated (i.e., activated) form of JNK. Control time curves with the solvent (0.5% DMSO) were prepared to see the change in JNK activation in similar conditions in the absence of OR compounds. The increase in activation of JNK by OR-2370 is expressed as the phospho-JNK activity in OR-2370-treated cells compared with the simultaneously prepared control cells with the solvent.

Materials. Reagents were obtained as follows: Caspase-Glo 3/7, 8, and 9 assays (Promega), L-JNKI1 (JNK peptide inhibitor 1, L-stereoisomer), and L-TAT control peptide (Alexis Corp., Läufelfingen, Switzerland), Z-Asp-CH₂-DCB (Peptide Institute, Inc., Osaka, Japan), orazipone, OR-2370, OR-1958, OR-1364, and OR-2149 (Fig. 1) (OrionPharma Ltd., Espoo, Finland), phosphospecific JNK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare) and Z-D(OMe)QM-

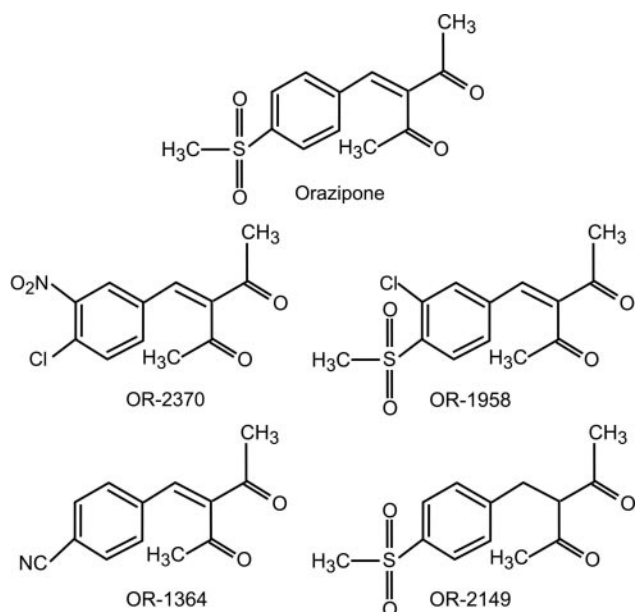


Fig. 1. The chemical structures of orazipone and OR-2370.

D(OMe)-FMK, Z-VEID-FMK, Ac-LEVD-CHO, Ac-IETD-CHO, Ac-LEHD-CHO, Q-VD-OPh, LY294002, PD169316, and wortmannin (Merck, Darmstadt, Germany). Unless otherwise stated, the reagents were obtained as described previously (Kankaanranta et al., 1999, 2000a,b, 2002; Zhang et al., 2000, 2002, 2003). The incubation time is 40 h unless otherwise stated. L-JNK1, L-TAT, PD098059, SB203580, LY294002, PD169316, and wortmannin and caspase inhibitors were added 20 min before OR-2370. Stock solutions of orazipone and OR-compounds, PD098059, SB203580, LY294002, PD169316, wortmannin and caspase inhibitors were prepared in DMSO. The final concentration of DMSO in the culture was 0.5 to 0.75% (1.25% in Caspase 4 and 9 inhibitor experiments). A similar concentration of DMSO was added to the control incubations.

Statistical Analysis. Results are expressed as means \pm S.E.M. Apoptosis is expressed as apoptotic index. Apoptotic index is the number of measured apoptotic cells divided by the total number of measured cells. Statistical significance was calculated by analysis of variance for repeated measures supported by Student-Newman-Keuls test by using InStat software (GraphPad Software, San Diego, CA). Differences are considered significant if $P < 0.05$.

Results

Effects of Orazipone on Interleukin-5-Afforded Eosinophil Survival. Interleukin-5 inhibited human eosinophil apoptosis in a concentration-dependent manner, and maximal inhibition of apoptosis was obtained at 10 pM interleukin-5 (apoptotic indexes, 0.57 ± 0.09 and 0.07 ± 0.02 in the absence and presence of interleukin-5, respectively; $n = 5$, $P < 0.001$). Orazipone increased the number of apoptotic eosinophils in the presence of interleukin-5 (Fig. 2A). This

increase in the number of apoptotic cells was confirmed by increased phosphatidylserine expression on the outer leaflet of cell membrane of interleukin-5-treated cells. The proportion of Annexin-V-positive cells in the absence and presence of orazipone ($40 \mu\text{M}$) was 0.07 ± 0.02 and 0.64 ± 0.10 , respectively; $n = 6$, $P < 0.001$ (Fig. 2, B and C). Furthermore, an increase in the number of eosinophils showing the typical features of apoptosis (such as nuclear coalescence, chromatin condensation, and cell shrinkage) was found with orazipone (apoptotic index, 0.02 ± 0.01 and 0.54 ± 0.12 in the absence and presence of $40 \mu\text{M}$ orazipone, respectively; $n = 6$, $P < 0.001$; Fig. 2, D and E). To further confirm the ability of orazipone to induce eosinophil apoptosis, DNA breakdown, the typical hallmark of apoptosis was analyzed. Orazipone ($40 \mu\text{M}$) reversed the interleukin-5-afforded inhibition of DNA breakdown, and a typical "ladder" pattern was found, indicating the occurrence of apoptotic cell death (Fig. 2F). Two structural sulfhydryl-reactive analogs of orazipone had a similar effect, whereas nonsulfhydryl-reactive analog OR-2149 did not reverse the effect of interleukin-5 on DNA breakdown in eosinophils (Fig. 2F).

Glucocorticoids are known to partially reverse the survival-prolonging action of interleukin-5 on eosinophils. However, this effect of glucocorticoids is abolished when interleukin-5 is used at higher concentrations (Hagan et al., 1998; Zhang et al., 2000, 2002; Druilhe et al., 2003). For example, budesonide ($1 \mu\text{M}$) partly reversed cytokine-afforded survival in the presence of low (1 pM) but not in the presence of higher ($10\text{--}100 \text{ pM}$) concentrations of interleukin-5 (Fig. 3A). To

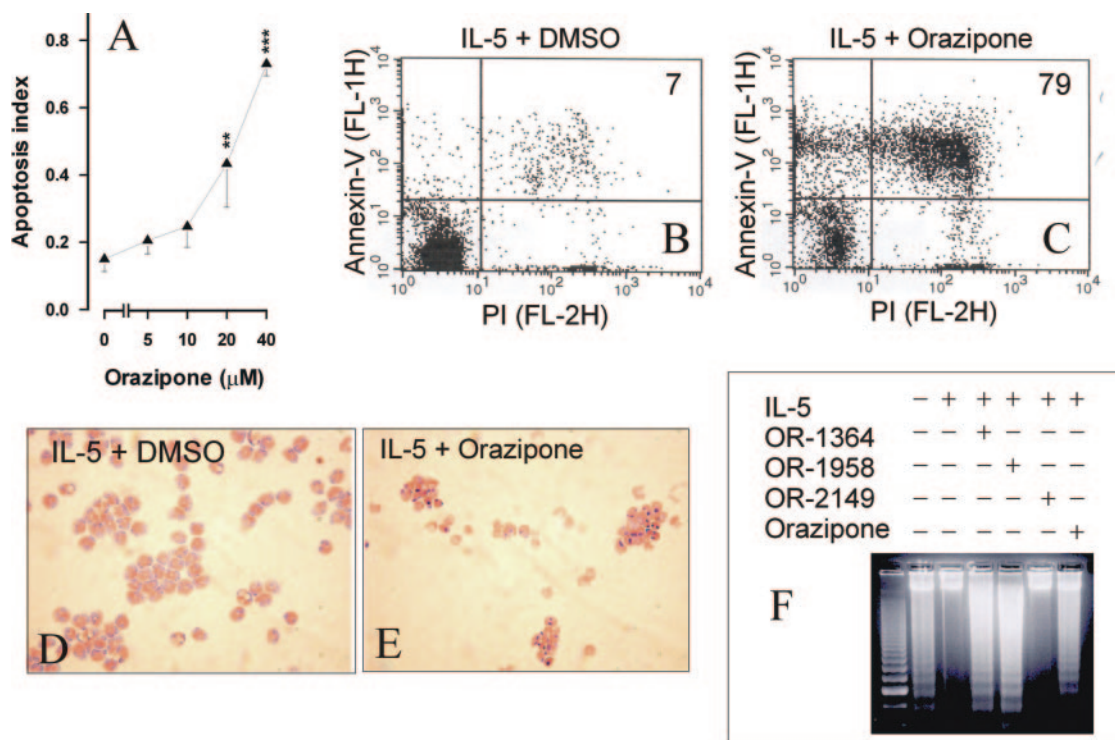


Fig. 2. The effect of orazipone (A) on apoptosis in 10 pM IL-5-treated human eosinophils during culture for 40 h. Each data point represents the mean \pm S.E.M. of six independent determinations using eosinophils from different donors. **, $P < 0.01$; ***, $P < 0.001$ compared with respective solvent control. Representative graphs from Annexin V-FITC (FL-1H) and uptake of propidium iodide (PI: FL-2H) are shown in B and C. B and C, top right, total number of early apoptotic eosinophils (annexin V-FITC⁺ and PI⁻) and late apoptotic eosinophils (annexin V-FITC⁺ and PI⁺). D, morphology of eosinophils cultured for 22 h in the presence of 10 pM IL-5. E, typical morphology of apoptotic and late apoptotic eosinophils cultured in the presence of 10 pM IL-5 and $40 \mu\text{M}$ orazipone. F, the effect of 10 pM IL-5 and $40 \mu\text{M}$ OR-1364, OR-1958, orazipone, and the negative control compound OR-2149 on DNA fragmentation in eosinophils cultured for 22 h. A representative of six (B–E) and three (F) similar experiments using eosinophils from different donors is shown.

evaluate whether the effect of orazipone is similar to glucocorticoids, its effects were studied in the presence of different concentrations of interleukin-5. Interestingly, orazipone (20–40 μM) reversed the effect of interleukin-5 on eosinophil apoptosis even in the presence of high concentrations of interleukin-5 (Fig. 3B). Thus, the effect of orazipone on eosinophil apoptosis seems to be different from that of glucocorticoids so that even high concentrations of the survival-prolonging cytokine interleukin-5 are unable to reduce its effects.

Effect of Orazipone on Fas-Induced Eosinophil Apoptosis. Relatively few compounds exist that are able to reverse the effect of interleukin-5 on eosinophil survival (Kankaanranta et al., 2005). One of those is nitric oxide, which has been shown to reverse the effect of interleukin-5 by inducing apoptosis (Zhang et al., 2003). However, nitric oxide can also reverse the apoptosis inducing effect of Fas in eosinophils (Hebestreit et al., 1998). This prompted us to evaluate whether orazipone has effects on Fas-induced apoptosis. Orazipone (5–40 μM), further enhanced the apoptosis-inducing effect of Fas-ligation in human eosinophils (Supplemental Data file 1).

Effect of Orazipone on Spontaneous Eosinophil Apoptosis. Glucocorticoids are able to enhance apoptosis of cytokine-deprived eosinophils at clinically relevant drug concentrations (Zhang et al., 2000, 2002). Similar to glucocorti-

coids, orazipone (40 μM) was able to enhance apoptosis of cytokine-deprived eosinophils 1.2- to 3-fold as assessed by relative DNA fragmentation assay, morphological analysis, or Annexin-V binding assay ($n = 6$, Supplemental Data file 2).

Effect of Orazipone on Primary Eosinophil Necrosis.

An important feature for a drug possessing anti-eosinophilic activity is that it should not induce primary necrosis that could lead to the release of eosinophil contents to the surrounding tissue. To evaluate this possibility, the effects of orazipone on primary eosinophil necrosis were evaluated by using counterstaining with Annexin-V and propidium iodide, where positive staining with propidium iodide indicates a rupture of the plasma membrane and the absence of staining with Annexin-V indicates that the cell has not undergone apoptosis. Thus, cells showing positive staining with propidium iodide but not with Annexin-V can be considered to have the typical feature of primary necrosis (i.e., the plasma membrane breakdown). In the absence of interleukin-5, the percentages of propidium iodide⁺/Annexin-V⁻ cells were 5 ± 2 and $4 \pm 1\%$ in the absence and presence of 40 μM orazipone, respectively ($n = 6$, $P > 0.05$), and in the presence of interleukin-5 (10 pM), the corresponding percentages were 5 ± 3 and $5 \pm 1\%$ ($n = 6$, $P > 0.05$). Thus, it can be concluded that orazipone does not induce primary necrosis in eosinophils.

Effect of Orazipone on Apoptosis in Human Neutrophils. To exclude a general toxic effect by orazipone on all cell types, the effects of orazipone on apoptosis and cytokine-afforded survival of human neutrophils were studied. Orazipone (5–40 μM) did not affect spontaneous neutrophil apoptosis (Table 1). GM-CSF (70 pM) inhibited human neutrophil apoptosis during culture for 16 h (Table 1). Orazipone (5–40 μM) did not reverse GM-CSF-afforded survival of human neutrophils (Table 1). Ligation of Fas-enhanced neutrophil apoptosis (Table 1). Orazipone did not affect Fas-induced apoptosis in human neutrophils (Table 1). The lack of effect of orazipone (40 μM) on spontaneous apoptosis and GM-CSF-afforded neutrophil survival were confirmed by Annexin-V binding assay. In the absence of GM-CSF, the

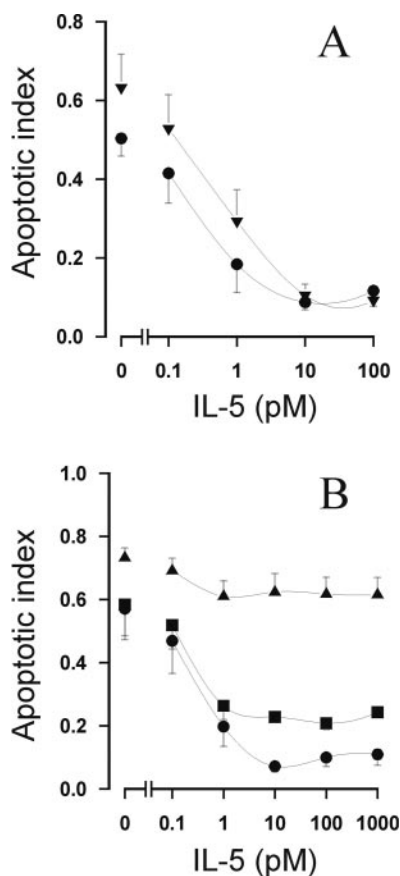


Fig. 3. The effect of budesonide (∇ , 1 μM) (A) and orazipone (\blacksquare , 20 μM ; \blacktriangle , 40 μM) (B) on apoptosis in eosinophils cultured for 40 h in the presence of different concentrations of IL-5. \bullet , the concentration-curve of IL-5 in the presence of solvent control. Each data point represents the mean \pm S.E.M. of four to five independent determinations using eosinophils from different donors. When not visible, error bars are within the symbol size.

TABLE 1

Lack of effect of orazipone on spontaneous or Fas-induced neutrophil apoptosis or GM-CSF-afforded neutrophil survival

Data is expressed as mean \pm S.E.M., $n = 5$. Ligation of Fas was induced by CH-11 monoclonal antibody (100 ng/ml). There were no statistically significant differences between any of the concentrations of orazipone versus solvent control.

	Apoptotic Index
Spontaneous apoptosis	
Solvent control	0.68 ± 0.03
Orazipone 5 μM	0.61 ± 0.05
Orazipone 10 μM	0.63 ± 0.05
Orazipone 20 μM	0.66 ± 0.03
Orazipone 40 μM	0.68 ± 0.04
Fas	
Solvent control	0.84 ± 0.03
Orazipone 5 μM	0.86 ± 0.03
Orazipone 10 μM	0.84 ± 0.05
Orazipone 20 μM	0.80 ± 0.05
Orazipone 40 μM	0.84 ± 0.04
GM-CSF (70 pM)	
Solvent control	0.53 ± 0.05
Orazipone 5 μM	0.55 ± 0.04
Orazipone 10 μM	0.48 ± 0.05
Orazipone 20 μM	0.54 ± 0.04
Orazipone 40 μM	0.57 ± 0.05

apoptotic indexes were 0.78 ± 0.01 and 0.70 ± 0.08 and in its presence 0.73 ± 0.03 and 0.71 ± 0.03 in the absence and presence of orazipone; $n = 4$, $P > 0.05$. Similar to that described in eosinophils, there was no significant induction of primary necrosis in neutrophils (in the absence of GM-CSF, 1 ± 1 and $3 \pm 1\%$ necrotic cells in the absence and presence of $40 \mu\text{M}$ orazipone, respectively, and in the presence of GM-CSF 2 ± 1 and $2 \pm 1\%$ necrotic cells in the absence and presence of $40 \mu\text{M}$ orazipone, respectively).

Effects of Analogs of Orazipone on Eosinophil Apoptosis. Data published previously suggest that structural analogs of orazipone may have improved anti-inflammatory efficacy compared with orazipone (Vendelin et al., 2005). For comparison, structurally related analogs of orazipone were studied. Both OR-1958 and OR-2370 reversed interleukin-5–afforded human eosinophil survival in a concentration-dependent manner by inducing apoptosis (Fig. 4). OR-1958 and another structurally related sulfhydryl-reactive analog OR-1364 reversed interleukin-5 inhibited DNA breakdown similarly to orazipone, whereas a nonsulfhydryl-reactive analog OR-2149 (Nissinen et al., 1997) did not reverse the effect of interleukin-5 on DNA breakdown in eosinophils (Fig. 2F). The ability of OR-2370 to induce apoptosis in interleukin-5–treated human eosinophils was confirmed by showing the increase in the number of Annexin-V positive eosinophils (apoptosis index, 0.09 ± 0.02 and 0.55 ± 0.15 in the absence and presence of $10 \mu\text{M}$ OR-2370, respectively; $n = 6$, $P < 0.001$). Because OR-2370 was found to be even more potent in inducing eosinophil apoptosis than orazipone and OR-1958, OR-2370 was used in further studies to evaluate the mechanisms of orazipone-induced apoptosis in eosinophils.

Role of Caspases in OR-2370-Induced Apoptosis. A pan-caspase inhibitor, Z-Asp-CH₂-DCB (20 – $200 \mu\text{M}$) significantly reversed $10 \mu\text{M}$ OR-2370–induced apoptosis in interleukin-5–treated eosinophils during 40 h of incubation (Table 2). To further evaluate the role of caspases in OR-2370–induced apoptosis, the activities of caspase 3/7, 8, and 9 were measured. During culture for 16 h active caspases 3/7, 8, and 9 were detected. Caspase 3/7 and 9 activities were significantly reduced by interleukin-5 (Fig. 5). OR-2370 significantly increased the activity of caspases 3/7, 8, and 9 in the presence of interleukin-5 (Fig. 5). A broad-range inhibitor of caspases 1, 3, 8, 9, 10, and 12, Q-VD-Oph almost completely

reversed OR-2370–induced apoptosis (Table 2). Q-VD-Oph ($20 \mu\text{M}$) also inhibited caspase 3/7, 8, and 9 activities in human eosinophils in the presence of OR-2370 by 75 to 99% ($n = 2$, data not shown). A more specific inhibitor of caspase 3 [Z-D(OMe)QMD(OMe)-FMK] partly reversed OR-2370–induced apoptosis (Table 2). Inhibitor of caspase 6 (Z-VEID-FMK) also reversed OR-2370–induced apoptosis in eosinophils (Table 2). To evaluate the role of other potential caspases, inhibitors for caspase 4 (Ac-LEVD-CHO), 8 (Ac-ITED-CHO), and 9 (Ac-LEHD-CHO) were investigated. Inhibitors of caspases 4, 8, and 9 did not reverse OR-2370–induced apoptosis (Table 2), although Ac-ITED-CHO and Ac-LEHD-CHO inhibited caspase 8 and 9 activities in human eosinophils by 99 and 65%, respectively ($n = 2$; data not shown). This suggests that even though caspases 8 and 9 are activated by OR-2370 in eosinophils, they do not mediate OR-2370-induced DNA breakdown.

Role of Mitogen-Activated Protein and Phosphatidylinositol 3-Kinases in OR-2370-Induced Apoptosis in Eosinophils. When interleukin-5–treated eosinophils were incubated at 37°C in the presence of OR-2370 ($10 \mu\text{M}$), a time-dependent increase in JNK activity was detected using Western blotting with an anti-pJNK antibody that recognizes the dual phosphorylated (i.e., activated) form of JNK (Fig. 6, A and B). To evaluate the functional role of JNK activation in OR-2370–induced apoptosis in interleukin-5–treated cells, a novel cell-permeable inhibitor peptide specific for JNK, L-JNKI1 (Bonny et al., 2001), was used. L-JNKI1 ($10 \mu\text{M}$), but not the negative control peptide L-TAT, almost completely reversed $10 \mu\text{M}$ OR-2370–induced DNA breakdown in interleukin-5–treated eosinophils (Figs. 6C and 7, A–C). To determine whether JNK activation is central to the OR-2370–induced apoptosis, the effect of L-JNKI1 ($10 \mu\text{M}$) on OR-2370–induced apoptosis was analyzed by using the morphological analysis and measurement of phosphatidylserine appearance on the outer cell membrane using Annexin-V binding assay. Interestingly, L-JNKI1 did not reduce the number of cells showing the typical early signs of apoptosis, such as apoptotic morphology or phosphatidylserine

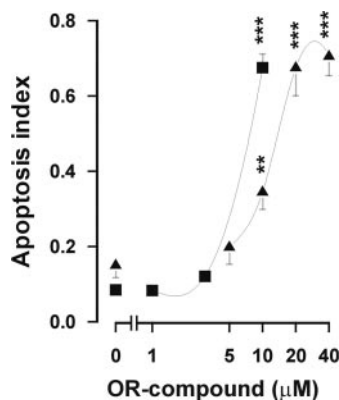


Fig. 4. The effect of OR-1958 (\blacktriangle) and OR-2370 (\blacksquare) on apoptosis in eosinophils cultured for 40 h in the presence of IL-5 (10 pM). Each data point represents the mean \pm S.E.M. of six independent determinations using eosinophils from different donors. **, $P < 0.01$; ***, $P < 0.001$ compared with respective solvent control.

TABLE 2

The effect of caspase inhibition on OR-2370 ($10 \mu\text{M}$)–induced apoptosis in interleukin-5–treated eosinophils

Data is expressed as mean \pm S.E.M., $n = 4$ – 7 . The corresponding apoptotic index in the presence of 10 pM interleukin-5 but in the absence of OR-2370 was 0.11 to 0.25.

	Apoptotic Index
Control	0.46 ± 0.03
Z-Asp-CH ₂ -DCB 20 μM	$0.34 \pm 0.03^{**}$
200 μM	$0.06 \pm 0.01^{***}$
Control	0.58 ± 0.06
Q-VD-Oph 20 μM	$0.16 \pm 0.02^{***}$
Z-D(OMe)QMD(OMe)-FMK 200 μM	$0.39 \pm 0.03^{**}$
Z-VEID-FMK 200 μM	$0.12 \pm 0.02^{***}$
Ac-ITED-CHO 100 μM	0.49 ± 0.07
Control	0.60 ± 0.04
Ac-LEVD-CHO 100 μM	0.55 ± 0.04
Ac-LEHD-CHO 100 μM	0.59 ± 0.04

** $P < 0.01$; *** $P < 0.001$ compared with the respective solvent control in the absence of caspase inhibitors.

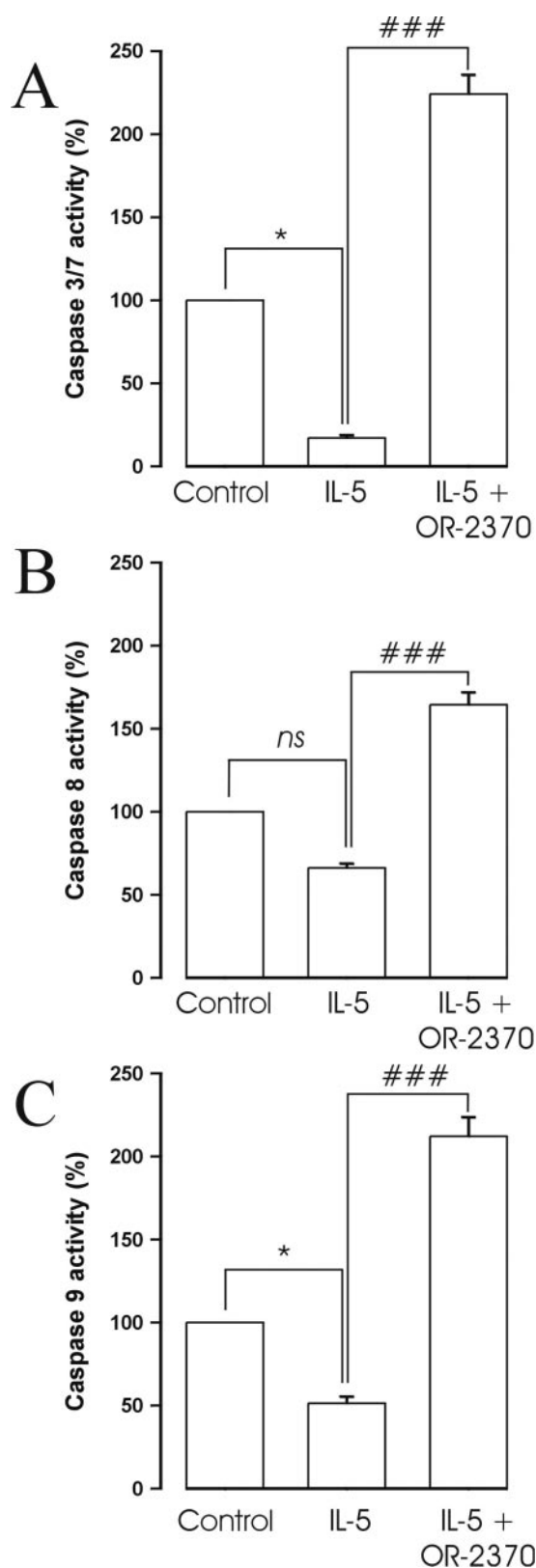


Fig. 5. The effect of 10 μ M OR-2370 on caspase 3/7 (A), caspase 8 (B), and caspase 9 (C) activation in human eosinophils cultured for 16 h in the presence of 10 pM IL-5. Caspase activity was measured with Caspase-Glo assay system. Each data point represents the mean \pm S.E.M. of four independent determinations using eosinophils from different donors. *, $P < 0.05$ compared with respective solvent control in the absence of interleukin-5; ###, $P < 0.001$ compared with the respective solvent control in the presence of interleukin-5.

expression on the outer cell membrane. By using morphological criteria for apoptosis in cells treated with 10 μ M OR-2370 and 10 pM interleukin-5, the apoptotic indexes were 0.64 ± 0.19 and 0.74 ± 0.13 in the presence of 10 μ M L-TAT and 10 μ M L-JNKI1, respectively; $n = 5$, $P > 0.05$) after culture for 20 h (Fig. 7, G–I). Likewise, the proportion of Annexin-V-positive cells was not reduced by L-JNKI1 compared with cells treated with L-TAT (apoptotic indexes, 0.49 ± 0.16 and 0.61 ± 0.14 in the presence of 10 μ M L-TAT and 10 μ M L-JNKI1, respectively; $n = 5$; Fig. 7, D–F). These results suggest that JNK activation is not an early event

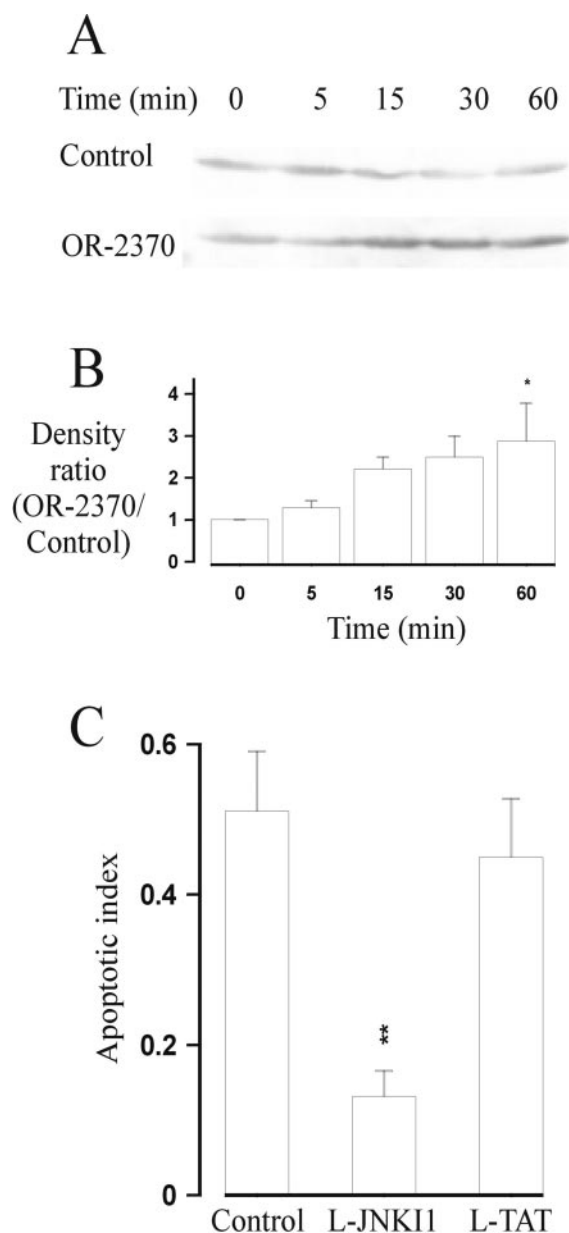


Fig. 6. A, the effect of 10 μ M OR-2370 on phosphorylation of JNK. The upper lane shows JNK phosphorylation in the simultaneously prepared control cells from the same donor treated with solvent control (0.5% DMSO). A typical experiment of six similar is shown. B, density ratio of JNK phosphorylation in OR-2370-treated cells compared with the simultaneously prepared control cells. Mean \pm S.E.M., $n = 6$. *, $P < 0.05$ compared with 0 min timepoint. C, the effect of L-JNKI1 (10 μ M) and the negative control peptide L-TAT (10 μ M) on apoptotic index in 10 μ M OR-2370 and 10 pM IL-5 treated cells. Mean \pm S.E.M., $n = 8$. **, $P < 0.01$ compared with the negative control peptide L-TAT.

mediating orazipone-induced apoptosis; rather, it mediates orazipone-induced DNA fragmentation.

Extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) have been proposed to be involved in the regulation of eosinophil apoptosis (Kankaanranta et al., 1999; Hall et al., 2001). Thus, to evaluate the role of these kinases in OR-2370-induced eosinophil apoptosis, we used a pharmacological approach to inhibit the activity of ERKs with the use of the MAPK kinase inhibitor PD098059 and the activity of p38 MAPK by SB203580 and PD169316. However, PD098059, SB203580, and PD169316 did not affect 10 μ M OR-2370-induced apoptosis in interleukin-5-treated human eosinophils (Table 3).

To evaluate whether activation of phosphatidylinositol 3-kinase (PI3K) could mediate OR-2370-induced apoptosis,

pharmacological inhibitors of PI3K were employed. However, neither LY294002 nor wortmannin reversed OR-2370-induced eosinophil apoptosis (Table 3).

Discussion

In the present study, we showed that the thiol-modulating compounds orazipone and OR-2370 induced apoptosis in interleukin-5-treated human eosinophils and were able to enhance spontaneous eosinophil apoptosis without inducing primary necrotic cell death. In contrast to eosinophils, orazipone does not induce apoptosis in human neutrophils. The mechanism of action of orazipone seems to involve caspases 3 and 6 as well as JNK-mediated DNA breakdown.

Induction of eosinophil apoptosis is currently considered

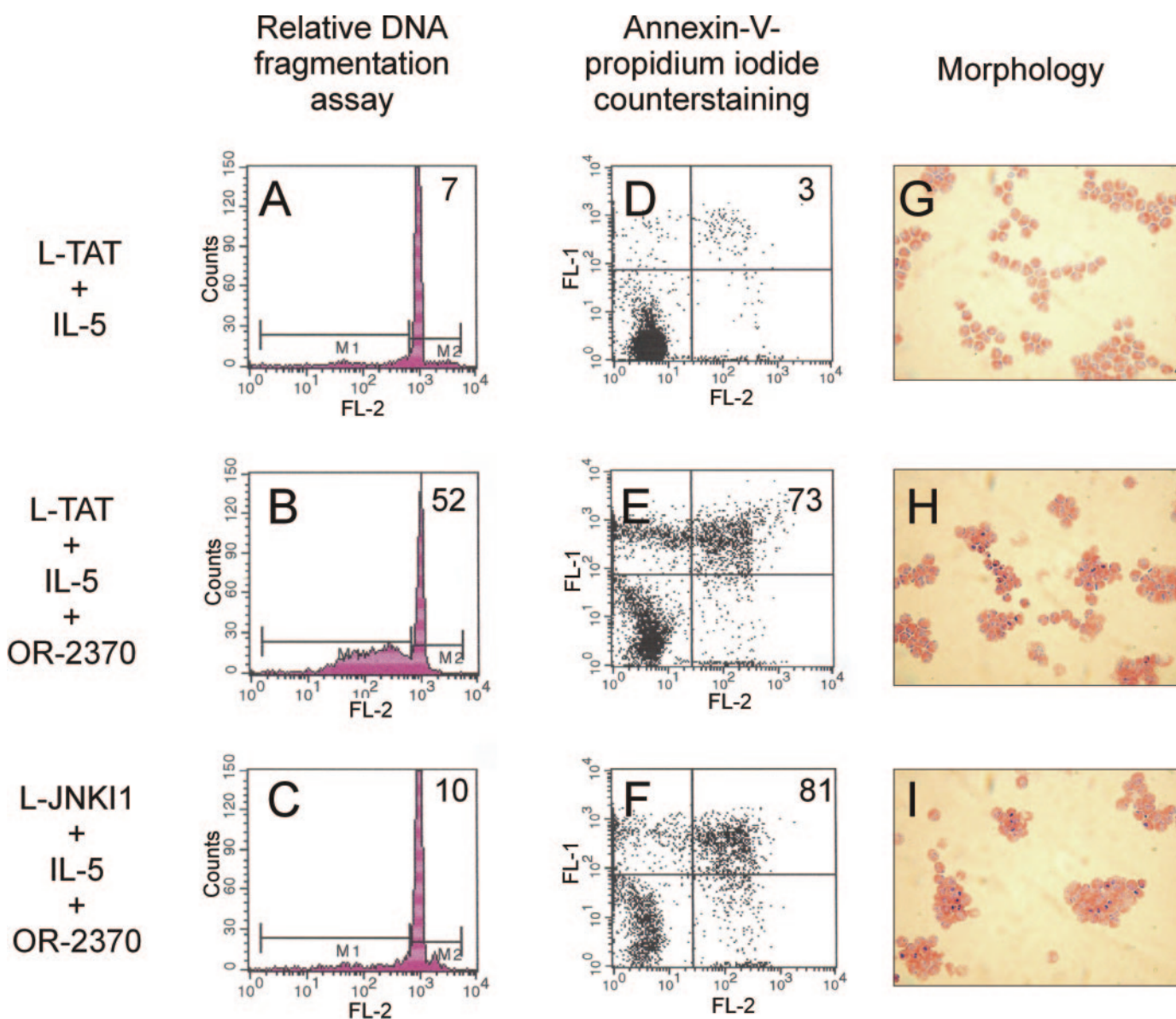


Fig. 7. The effect of 10 μ M L-JNKI1 (C, F, and I) on 10 μ M OR-2370-induced apoptosis in 10 pM interleukin-5 treated eosinophils compared with the negative control peptide 10 μ M L-TAT (B, E, and H). A solvent control incubated in the presence of 10 μ M L-TAT and 10 pM interleukin-5 but in the absence of OR-2370 is shown in (A, D, and G). Representative graphs from relative DNA fragmentation assay of propidium iodide-stained eosinophils (A–C), Annexin V-FITC (FL-1) and uptake of propidium iodide (PI; FL-2) (D–F), and morphological analysis of eosinophils (G–I) are shown. D–F, top right, total number of early apoptotic eosinophils (annexin V-FITC⁺ and PI^{-ve}) and late apoptotic eosinophils (annexin V-FITC⁺ and PI⁺). Representative graphs of experiments with similar results using eosinophils from four to eight different donors are shown.

one of the key mechanisms of the antiasthmatic effectiveness of glucocorticoids (Druilhe et al., 2003; Walker et al., 2003; Walsh et al., 2003; Kankaanranta et al., 2005). Glucocorticoids are able to enhance spontaneous eosinophil apoptosis at clinically relevant drug concentrations (Zhang et al., 2000, 2002). In the present study, we showed that orazipone and OR-2370 were able to enhance constitutive apoptosis of cytokine-deprived eosinophils. More importantly, they were able to fully reverse the interleukin-5–afforded eosinophil survival by inducing apoptosis. Glucocorticoids have been reported to partly reverse interleukin-5–afforded survival, but the effect of steroids falls off as the concentration of interleukin-5 increases (Adachi et al., 1996; Druilhe et al., 2003). In contrast to glucocorticoids, the effect of orazipone was not reversed by higher concentrations of interleukin-5, suggesting that the mechanism of action orazipone is different from that of glucocorticoids and that orazipone may have a better antieosinophilic activity compared with current glucocorticoids.

The exact mechanism of action of orazipone and its derivatives OR-1958 and OR-2370 remains unknown, but they probably exert their effects by forming reversible conjugates with the thiol groups of proteins and glutathione (Vendelin et al., 2005). The ability of orazipone and OR-1958 to inhibit cytokine production is dependent on their ability to react with glutathione in mononuclear cells or activated neutrophils. The effect of orazipone and OR-1958 has been shown to be reversible, and this has been tested by incubating compounds with glutathione and measuring the formation of compound–glutathione adduct and its dissociation back to the parent compound and glutathione (Nissinen et al., 1997). If the double bond in the side chain is reduced, the compounds do not react with glutathione, and they lose their ability to inhibit the activation of these cells (Nissinen et al., 1997). In the present study, we used also OR-2149, which has a reduced double bond in the side chain as a negative control compound. OR-2149 did not induce DNA breakdown or apoptosis in eosinophils. This suggests that the effect of orazipone is related to its ability to modulate thiols.

TABLE 3

The effect of inhibition of ERK (PD098059), p38 MAPK (SB203580, PD169316) and PI3K (LY294002, wortmannin) on 10 μ M OR-2370-induced apoptosis in interleukin-5-treated human eosinophils

Data is expressed as mean \pm S.E.M., $n = 4-7$. The corresponding apoptotic index in the presence of 10 pM interleukin-5 but in the absence of OR-2370 was 0.12 to 0.15.

	Apoptotic Index
PD098059	
0	0.52 \pm 0.06
1 μ M	0.48 \pm 0.05
10 μ M	0.49 \pm 0.05
SB203580	
0	0.38 \pm 0.08
1 μ M	0.41 \pm 0.10
10 μ M	0.43 \pm 0.10
PD169316	
0	0.59 \pm 0.08
0.1 μ M	0.61 \pm 0.09
1 μ M	0.58 \pm 0.11
LY294002	
0	0.59 \pm 0.08
10 μ M	0.66 \pm 0.05
50 μ M	0.72 \pm 0.05
Wortmannin	
0	0.59 \pm 0.08
10 nM	0.59 \pm 0.07
100 nM	0.61 \pm 0.06

Thiol antioxidants such as *N*-acetylcysteine and glutathione have been shown to inhibit spontaneous and sodium arsenite- and Fas-induced apoptosis in human eosinophils (Wedi et al., 1999). Furthermore, we have shown that oxidative stress, especially H₂O₂, enhances spontaneous apoptosis and reverses interleukin-5–afforded eosinophil survival by inducing apoptosis (Kankaanranta et al., 2002). Recently, oxidant-induced mitochondrial injury was reported to be pivotal for eosinophil apoptosis and glucocorticoids were shown to enhance it in a JNK-mediated manner that is in turn inhibited by the survival-prolonging cytokine GM-CSF (Gardai et al., 2003). These data support the idea that eosinophil survival is regulated by thiol-sensitive redox regulation. The present results on the effects of orazipone on eosinophil apoptosis could be explained by formation of reversible conjugates with thiols, thereby preventing their effect on survival. Another possibility for the mechanism of action of orazipone is that it specifically saves some critical thiol-groups from modulation by interleukin-5, which leads to inhibition of interleukin-5–afforded survival.

Regulation of caspase activity is believed to be central during apoptosis. The presence of caspases 3, 6, 7, 8, and 9 and their processing during spontaneous or nitric oxide-induced apoptosis in eosinophils has been described previously (Zangrilli et al., 2000; Dewson et al., 2001; Zhang et al., 2003) and spontaneous eosinophil death can be blocked by broad specificity caspase inhibitors such as Z-Asp-CH₂-DCB or Z-VAD-FMK (Dewson et al., 2001; De Souza et al., 2002). However, the detailed caspase cascades mediating apoptosis in eosinophils remain unknown (Daigle and Simon, 2001). The effect of OR-2370 could be reversed by the broad specificity caspase inhibitors Z-Asp-CH₂-DCB and Q-VD-Oph, suggesting the mediator role of caspases in OR-2370–induced apoptosis. Furthermore, OR-2370–induced apoptosis was reduced by inhibitors of caspase 3 and 6, suggesting their involvement. It is noteworthy that specific inhibitors for caspases 8 or 9 were not able to reverse orazipone-induced apoptosis in IL-5–treated eosinophils even though these inhibitors efficiently suppressed caspase activities in these cells. These results suggest that the effects of orazipone on eosinophils are not mediated via caspase 8 or 9 pathways, but use a caspase pathway involving caspases 3 and 6.

The role of MAPKs and PI3K in the regulation of human eosinophil apoptosis has gained attention (Kankaanranta et al., 1999; Miike et al., 1999; Hall et al., 2001; Gardai et al., 2003; Zhang et al., 2003). There exists some controversy regarding whether ERK pathway is involved in the survival-prolonging action of cytokines (Kankaanranta et al., 1999; Miike et al., 1999; Hall et al., 2001), whereas p38 MAPK seems to be involved in spontaneous eosinophil survival (Kankaanranta et al., 1999). By using pharmacological inhibitors, we were able to exclude ERK, p38 MAPK, and PI3K as targets of OR-2370. Recently, JNK has been proposed to be involved in eosinophil apoptosis induced by dexamethasone (Gardai et al., 2003) and NO (Zhang et al., 2003). OR-2370 enhanced activation of JNK as evidenced by Western blot analysis, showing an increase in the amount of phosphorylated JNK. Inhibition of JNK activity by a specific inhibitor, L-JNKI1, reversed the effect of OR-2370 when apoptosis was measured using the relative DNA fragmentation assay, suggesting that JNK

mediates orazipone-induced apoptosis. However, when the effects of L-JNKI1 on OR-2370-induced apoptosis were analyzed using morphological features of apoptosis and the expression of phosphatidylserine on the outer leaflet of the cell membrane (Annexin-V binding assay), L-JNKI1 was not able to reverse the effect of OR-2370. These results suggest that JNK activity is enhanced in human eosinophils in response to orazipone and mediates orazipone-induced DNA breakdown, but JNK activation is not involved in the early signaling of orazipone-induced apoptosis. The role of JNK in the regulation of apoptosis in other cell types, mainly of malignant nature, has been widely studied, and it has been found to have both pro- and antiapoptotic effects (Lin and Dibling, 2002; Manning and Davis, 2003). The exact relationship between JNK activation and DNA fragmentation/apoptosis in eosinophils remains to be established.

Orazipone and its derivative OR-1958 have been shown to inhibit platelet-activating factor-induced lung eosinophilia in guinea pigs (Aho et al., 2001) and to prevent lung eosinophilia in ovalbumin-sensitized rats after repeated administration with efficacy equal to that of budesonide (Ruotsalainen et al., 2000). Orazipone has been shown to inhibit IL-1 β , IL-2, tumor necrosis factor- α , and IL-8 secretion from monocytes or lymphocytes as well as superoxide release and degranulation in neutrophils and histamine release from mast cells (Wroblewski et al., 1998; Vendelin et al., 2005). In the present study, we found that orazipone induced apoptosis in IL-5-treated human eosinophils in vitro. Whether induction of eosinophil apoptosis explains the ability of orazipone to inhibit lung eosinophilia and to what extent its inhibitory effects on cytokine production and other inflammatory cells contribute to its effects remain currently unknown.

In the present study, we show for the first time that orazipone induces apoptosis in human eosinophils. To exclude the possibility that orazipone induces nonspecific toxicity toward all human cells, we studied the effect of orazipone on spontaneous and Fas-induced apoptosis and GM-CSF-afforded survival of human neutrophils. Orazipone, at concentrations that induced apoptosis in eosinophils, did not affect neutrophil apoptosis at all. Whether orazipone induces apoptosis in any cell type other than eosinophils remains to be evaluated. Orazipone has been successfully administered to healthy volunteers and patients with asthma as an inhalation preparation in two phase I-Ib trials (E. Moilanen, unpublished observations). However, the effects on asthmatic inflammation in vivo in humans remain unresolved.

Taken together, our results suggest that thiol-modulating compounds orazipone and OR-2370 have antieosinophilic activity and are potent candidates for the treatment of eosinophilic inflammatory conditions.

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