(E)-2(R)-[1(S)-(Hydroxycarbamoyl)-4-phenyl-3-butenyl]-2′-isobutyl-2′-(methanesulfonfonyl)-4-methylvalerohydrazide (Ro 32-7315), a Selective and Orally Active Inhibitor of Tumor Necrosis Factor-α Convertase


Received June 6, 2001; accepted March 14, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Tumor necrosis factor-α (TNF-α), a cytokine secreted by inflammatory cells, has been implicated in several inflammatory disease states. (E)-2(R)-[1(S)-(Hydroxycarbamoyl)-4-phenyl-3-butenyl]-2′-isobutyl-2′-(methanesulfonfonyl)-4-methylvalerohydrazide (Ro 32-7315), is a potent, orally active inhibitor of the TNF-α convertase (TACE), an enzyme responsible for proteolytic cleavage of the membrane bound precursor, pro-TNF-α. Ro 32-7315 inhibited a recombinant form of TACE (IC50 = 5.2 nM) with selectivity over related matrix metalloproteinases. In a cellular assay system, THP-1 cell line, and in human and rat whole blood, Ro 32-7315 significantly reduced lipopolysaccharide (LPS)-induced TNF-α release with IC50 values of 350 ± 14 nM (n = 5), 2.4 ± 0.5 µM (n = 5), and 110 ± 18 nM (n = 5), respectively. Oral administration of Ro 32-7315 to Wistar rats caused a dose-dependent inhibition of LPS-induced release of systemic TNF-α with an ED50 of 25 mg/kg. Treatment (days 0–14) of Allen and Hambury’s hooded rats with Ro 32-7315 (2.5, 5, 10, and 20 mg/kg, i.p., twice daily) significantly reduced adjuvant-induced secondary paw swelling (42, 71, 83, and 93%, respectively) as compared with the vehicle group. In the Ro 32-7315-treated group, the reduced paw swelling was associated with improved lesion score and joint mobility. Furthermore, in a placebo-controlled, single-dose study, Ro 32-7315 given orally (450 mg) significantly suppressed ex vivo, LPS-induced TNF-α release in the whole-blood samples taken from healthy male and female volunteers (mean inhibition of 42% over a 4-h duration, n = 6). These data collectively support the potential use of such a compound for the oral treatment of inflammatory disorders.

Tumor necrosis factor-alpha (TNF-α), a cytokine produced primarily by activated monocytes and macrophages, is an important mediator of immuno-inflammatory responses and inducer of other pro-inflammatory cytokines (Vassali, 1992). TNF-α is produced as a 26-kDa membrane-bound pro-TNF-α that undergoes a specific proteolytic cleavage between Ala-76 and Val-77 to release a 17-kDa soluble TNF-α (Kriegler et al., 1988). The enzyme that is responsible for this process is TNF-α convertase (TACE), a metalloproteinase closely related to the matrix metalloproteinases (MMPs; Gearing et al., 1994; McGeehan et al., 1994; Mohler et al., 1994). MMPs are a large family of Zn2+ endopeptidases that include 72- and 92-kDa gelatinases, collagenases, stromelysins 1–3, matrixin, macrophage metalloelastase, and membrane-bound MMP 1–4 (Birkedal-Hansen et al., 1993). They are expressed in immuno-inflammatory conditions such as rheumatoid arthritis (RA), sepsis, and inflammatory bowel disease and are collectively capable of degrading most connective tissue macromolecules under both physiological and pathological conditions (Buchan et al., 1988; Lewis et al., 1997).

RA is a chronic, progressive inflammatory disease that causes substantial morbidity. Although the precise etiology of RA remains unknown, a great deal has been learned about the immunopathophysiology of the disease in recent years. Proinflammatory cytokines such as TNF-α, interleukin (IL)-1, and IL-6 have been suggested to play an important role in the pathogenesis of the RA. In addition to promoting inflammation, these cytokines are capable of directly mediating bone and cartilage destruction, the most prominent feature of RA (Buchan et al., 1988; Feldmann and Maini,

ABBREVIATIONS: DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbant assay; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; Ro 32-7315, (E)-2(R)-[1(S)-(Hydroxycarbamoyl)-4-phenyl-3-butenyl]-2′-isobutyl-2′-(methanesulfonfonyl)-4-methylvalerohydrazide; TACE, tumor necrosis factor alpha convertase; TNF-α, tumor necrosis factor-alpha; RA, rheumatoid arthritis; AHH/R, Allen and Hambury’s hooded rats; Dpa, N-3-(2, 4-dinitrophenyl)-l-2, 3-diaminopropionyl.
Several lines of evidence have identified TNF-α as a key mediator of chronic inflammatory diseases such as RA, inflammatory bowel disease, and sepsis (Esperisen et al., 1991; Tracey et al., 1993). The randomized phase II and III clinical trials using a monoclonal antibody to TNF-α, infliximab, or the soluble TNF-α receptor fusion protein, etanercept, significantly improved the disease activity in RA patients, thus, validating TNF-α as a therapeutic target for RA (Moreland et al., 1997; Maini et al., 1998; Lorenz et al., 2000). The inhibition of TACE offers an alternative point of therapeutic intervention and the possibility of a low molecular weight, orally active agent.

Hydroxamic-acid-based MMP inhibitors have been reported to inhibit endotoxin-induced production of TNF-α by inhibiting the processing enzyme, TACE (Gearing et al., 1994; Mohler et al., 1994). However, there are very limited reports concerning their selectivity for TACE and oral efficacy in preclinical and clinical models. Ro 32-7315 (Fig. 1) is among the first of a new generation of potent, hydroxamic-acid-containing TACE inhibitors with greater selectivity over the MMP family of enzymes. In addition to the potency and selectivity of this compound, a marked improvement in the in vivo biological profile compared with compounds such as BB 1101 (DiMartino et al., 1997; Barlaam et al., 1999) was observed. In this report, we describe the in vitro and in vivo biological profile of Ro 32-7315, a selective inhibitor of TACE. Furthermore, we considered the feasibility of using changes in LPS-induced TNF-α release for the ex vivo evaluation of TACE inhibitor pharmacodynamics. Using this approach, Ro 32-7315 was shown to cause a significant inhibition of TNF-α production ex vivo after oral dosing in healthy volunteers. The findings support the contention that this selective TACE inhibitor may have use as a potential oral treatment for inflammatory diseases.

Materials and Methods

Chemicals

Ro 32-7315 was synthesized in the Medicinal Chemistry Department of Roche Discovery (Welwyn, UK).

Isolated Enzyme Studies

In Vitro TACE Assay. A recombinant form of TACE, which lacked the transmembrane region and cytoplasmic tail, was used in the in vitro assay. The extracellular domain of TACE was subcloned into pVL1393, and the recombinant baculovirus generated was used to infect Sf9 cells. TACE activity was purified by precolumn culture media by Q-Sepharose, concanavalin A Sepharose, and Superdex 75 chromatography. TACE activity was determined by measuring the production of the peptide product VRSSRTPda from the peptide substrate ISPLAQAVRSSRTPda (Dpa is N3-(2, 4-dinitrophenyl)-1-2, 3-diaminopropionyl). The assay was carried out in 10 mM Tris-HCL (pH 8.0), 50 mM NaCl, and 2% octylglycoside and at a substrate concentration of 100 μM. After 60 min at 37°C, the reaction was stopped by adding acetic acid to a final concentration of 1 M. The peptide product was separated from the reaction mixture by reverse-phase high performance liquid chromatography, using a 28 to 70% acetonitrile gradient on a C8 column. The absorbance of the eluate at 360 nm was measured as an index of the amount of product formed.

In Vitro MMPs Assay. The inhibitory activity of Ro 32-7315 against a number of human MMPs was determined using a methodology published by Knight et al. (1992). The IC50 values for Ro 32-7315 were determined for collagenase 1 (using recombinant catalytic domain MMP-1, final assay concentration 800 pM), collagenase 2 (MMP-8, final assay concentration of 250 pM), collagenase 3 (using recombinant catalytic domain MMP-13, final assay concentration of 20 pM), stromelysin (MMP-3, final assay concentration of 1 nM), gelatinase A (using recombinant catalytic domain MMP-2, final assay concentration of 300 pM), gelatinase B (MMP-9, final assay concentration of 250 pM), metalloelastase (MMP-12, final assay concentration of 300 pM), and matrilysin 1 (MMP-7, final assay concentration of 35 pM) using the fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dap-Ala-Arg-NH2 (2 mM; Bachem, Saffron Walden, UK).

The assays were carried out in a total volume of 200 μl of assay buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM CaCl2, and 0.005% Brij 35. The reaction was initiated with the addition of substrate and the emission was monitored continuously for 10 min (excitation at 328 nm and emission at 393 nm; LS50B; PerkinElmer Life Sciences, Boston, MA). The IC50 values were calculated using a curve fit to y = 1/(M0/M1 + 1), where M0 is Ro 32-7315 concentration and M1 is the IC50 value or Km, because the substrate concentration was considerably greater than Km.

Cell-Based Studies

LPS-Induced Cytokine Release from THP-1 Cells. THP-1 cells (5 x 10⁶/ml, American Tissue Culture Collection) in RPMI medium containing 20 mM HEPES buffer, 10% fetal calf serum, and antibiotics were aliquoted (200 μl) into 96-well plates. The cells were incubated for 30 min at 37°C, 95% humidity, and 5% CO₂ with vehicle (0.8% dimethyl sulfoxide (DMSO) final concentration; BDH, Poole, UK) or Ro 32-7315 (10⁻⁹ to 10⁻³ M, final concentration) before the addition of LPS (2 μg/ml final concentration; Salmonella typhimurium; Sigma-Aldrich, St. Louis, MO). After further incubation for 3 h at 37°C (95% humidity and 5% CO₂), the plates were centrifuged (1200 rpm, 4 min, MSE 2900) to sediment the cells. Aliquots of the supernatant were taken for the estimation of TNF-α and IL-8 levels by ELISAs, selective for the human cytokines (R & D Systems, Abingdon, UK). The inter- and intra-assay coefficients of variation were, respectively: TNF-α assay, 6.4%, 4.8%; IL-8 assay, 7.8%, 5.9%.

LPS-Induced TNF-α Release from Rat and Human Whole Blood. Rat blood collected in EDTA (1.6 mg/ml potassium EDTA; Sarstedt, Numbrecht, Germany) was preincubated with either vehicle (0.5% DMSO, final concentration) or Ro 32-7315 (10⁻³ to 10⁻⁹ M, final concentration) at 37°C for 10 min. This was followed by incubation (37°C) of the whole blood with LPS (20 μg/ml, final concentration, Escherichia coli serotype, 0111:B4, Sigma-Aldrich) for 2 h. The blood samples (200-μl aliquots) were centrifuged (4000 rpm, 2 min, Jouan A14), and the plasma was snap frozen and stored at −20°C until rat TNF-α levels were determined by ELISA (R & D Systems). The inter- and intra-assay coefficients of variation for this assay were 8.2 and 9.5%, respectively.

Blood (10 ml) from healthy human volunteers was collected in 1999. The blood samples (200-μl aliquots) were centrifuged (4000 rpm, 2 min, Jouan A14), and the plasma was snap frozen and stored at −20°C until rat TNF-α levels were determined by ELISA (R & D Systems). The inter- and intra-assay coefficients of variation for this assay were 8.2 and 9.5%, respectively.

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 2, 2017
heparinized tubes (10 U/ml) by venipuncture. Aliquots of blood (500 µl) were preincubated with vehicle (0.5% DMSO, final concentration) or Ro 32-7315 (10⁻⁵ to 10⁻¹⁰ M, final concentration) as described above. LPS (2.7 µg/ml, final concentration; S. typhimurium; Sigma-Aldrich) was added, and the samples were further incubated for 2 h at 37°C. At the end of the incubation period, the blood samples were centrifuged (12,500 rpm, 1 min, Jouan A14), and the plasma was removed, snap frozen, and stored at −80°C until TNF-α levels were determined using the human ELISA described above.

The concentration of Ro 32-7315 that caused 50% inhibition of LPS-induced TNF-α release from whole blood and THP-1 cells was computed from the concentration-response curve using a four-parameter logistic function.

In Vivo Studies

LPS-Induced TNF-α Release in Rats. Age-matched, male rats (Wistar, Charles River Breeding Laboratories) were pretreated orally with vehicle (10% succinylated gelatin, 10 ml/kg) or Ro 32-7315 (10–60 mg/kg). Thirty minutes after pretreatment, the rats were given i.p. injections of LPS (100 µg in PBS; E. coli serotype, 0111:B4; Sigma-Aldrich). The rats were bled (via tail vein) at 0 h (pre-LPS challenge), 0.5, 1, 2, 3, and 6 h, and the blood samples were collected into EDTA (potassium EDTA). The blood samples were centrifuged (4000 rpm, 2 min, Jouan A14), and the plasma was collected and frozen at −20°C until TNF-α and Ro 32-7315 levels were determined.

Adjuvant-Induced Arthritis Model. Female AHH/R from rats bred in-house were caged in groups of five and provided with water and a pelleted diet ad libitum. One group of rats was nominated as control and was not dosed or given adjuvant, the other groups were injected with 0.1 ml of adjuvant into the subplantar surface of the right hind paw. Adjuvant was prepared by homogenizing Mycobacterium tuberculosis (heat killed, human strains C, DT, and PN; Central Veterinary Laboratory, Weybridge, UK) in liquid paraffin (1:100; Sigma-Aldrich). The rats were bled (via tail vein) at 0 h (pre-LPS challenge), 0.5, 1, 2, 3, and 6 h, and the blood samples were collected into EDTA (potassium EDTA). The blood samples were centrifuged (4000 rpm, 2 min, Jouan A14), and the plasma was collected and frozen at −20°C until plasma TNF-α and Ro 32-7315 levels were determined.

Ex Vivo Studies in Human Volunteers

LPS-Induced TNF-α Release in Healthy Human Volunteers. In a double blind, randomized, placebo-controlled healthy volunteer study, subjects received single oral doses of either Ro 32-7315 (450 mg, n = 6) or placebo (n = 2). Blood samples (10 ml via venipuncture into heparinized vacutainers, 10 U/ml) were collected at the following times, 0-, 0.5-, 1-, 2-, 4-, 6-, and 8-h postdose administration. Additional blood samples up to 48 h were taken for detailed pharmacokinetic profiling of Ro 32-7315. The heparinized whole blood (500 µl) was incubated with LPS (2.7 µg/ml, final concentration; S. typhimurium) or PBS (In Vitrogen, Carlsbad, CA) control for 2 h at 37°C. After incubation, the whole blood was mixed and centrifuged (12,500 rpm, 1 min, Jouan A14), and the plasma was snap frozen and stored at −20°C until determination of TNF-α Levels by ELISA.

The ex vivo experiments described above were incorporated into the single ascending oral dose study of Ro 32-7315 in healthy volunteers to assess tolerability, safety, pharmacokinetics, and pharmacodynamic response parameters. The Local Ethical Committee approved this investigation and a written, informed consent was obtained from all of the subjects. All of the subjects underwent a screening medical examination within 3 weeks of the study start and during and up to 14 days after dosing. This study was conducted in the Clinical Pharmacology Unit at Roche Products Ltd. (Welwyn Garden City, UK).

Ex Vivo Studies in Human Volunteers

LPS-Induced TNF-α Release in Healthy Human Volunteers. In a double blind, randomized, placebo-controlled healthy volunteer study, subjects received single oral doses of either Ro 32-7315 (450 mg, n = 6) or placebo (n = 2). Blood samples (10 ml via venipuncture into heparinized vacutainers, 10 U/ml) were collected at the following times, 0-, 0.5-, 1-, 2-, 4-, 6-, and 8-h postdose administration. Additional blood samples up to 48 h were taken for detailed pharmacokinetic profiling of Ro 32-7315. The heparinized whole blood (500 µl) was incubated with LPS (2.7 µg/ml, final concentration; S. typhimurium) or PBS (In Vitrogen, Carlsbad, CA) control for 2 h at 37°C. After incubation, the whole blood was mixed and centrifuged (12,500 rpm, 1 min, Jouan A14), and the plasma was snap frozen and stored at −20°C until determination of TNF-α Levels by ELISA.

The ex vivo experiments described above were incorporated into the single ascending oral dose study of Ro 32-7315 in healthy volunteers to assess tolerability, safety, pharmacokinetics, and pharmacodynamic response parameters. The Local Ethical Committee approved this investigation and a written, informed consent was obtained from all of the subjects. All of the subjects underwent a screening medical examination within 3 weeks of the study start and during and up to 14 days after dosing. This study was conducted in the Clinical Pharmacology Unit at Roche Products Ltd. (Welwyn Garden City, UK).

Plasma Drug Level Determination. Concentrations of Ro 32-7315 were measured in plasma samples (rat and human) using a specific high performance liquid chromatographic-mass spectrometric method. The proteins in plasma (20 µl) were precipitated by the addition of 80 µl of methanol containing the tritideuterium form of Ro 32-7315 as an internal standard. After centrifugation and dilution (1:1), the supernatant was injected (40 µl) onto an isocratic chromatography system consisting of a C18 column (5 µm) and precolumn with mobile phase (65% methanol containing 2.5 mM ammonium formate, pH 3.0), which was run at a flow rate of 1 ml/min. The retention times for Ro 32-7315 and the isotopic internal standard were both approximately 2 min. After reduction by postcolumn splitting (4:1), the flow was directed into the mass spectrometer (PE Sciex API3+, PerkinElmer) via a TurboIonSpray interface. The precursor and product ions of Ro 32-7315 (m/z 454.5/421.5 Da and the internal standard, D6-Ro 32-7315 (m/z 457.2/454.2 Da) were monitored, and unknown concentrations were determined by means of calibration standards. The assay sensitivity was at least 5 ng/ml. Mean drug levels are reported as nanograms per milliliter plasma.

The changes in groups of rats receiving different doses of Ro 32-7315 (Figs. 2 and 3) were analyzed using a two-sided Student’s t test. Temporal changes in LPS-induced TNF-α release in human volunteers receiving Ro 32-7315 (Fig. 4) were analyzed with a one way, repeated measures analysis of variance (SigmaStat) using Dun-

![Fig. 2. LPS-induced TNF-α production in male Wistar rats after oral administration of Ro 32-7315 or vehicle. Rats were injected with LPS (1.p.) 30 min after oral dosing with Ro 32-7315 or vehicle. Blood samples were collected over the 6-h duration, plasma was separated, and the TNF-α levels were determined using ELISA, as described under Materials and Methods. Data shown are mean ± S.E.M. (n = 6–9/group). Figures in parentheses are mean plasma Ro 32-7315 levels (nanograms per milliliter, at 1-h time point). *p < 0.05, represents a significant reduction in plasma TNF-α levels compared with vehicle group at the same time points, based on Student’s t test. ■, vehicle; ○, 10 mg/kg; ▲, 20 mg/kg; ●, 30 mg/kg; X, 60 mg/kg.](image-url)
Results

Inhibition of TACE, MMPs, and LPS-Induced TNF-α Release in Vitro. The inhibitory potency of Ro 32-7315 was determined against a recombinant form of TACE using a peptide substrate, ISPLAQAVRSSRTDpa. For selectivity evaluation, Ro 32-7315 was also tested against a number of MMPs. The TNF-α inhibition in cells was determined in LPS-induced THP-1 cells and in rat and human whole blood; the results are summarized in Table 1. Ro 32-7315 demonstrated some selectivity for TACE over other human MMPs (minimum of 2-fold selectivity over MMP 12, metalloelastase, to a maximum of 96-fold selectivity over MMP 1, collagenase 1; Table 1).

In the monocytic cell line, THP-1, Ro 32-7315 inhibited LPS-induced TNF-α release with an IC_{50} of 350 ± 14 nM (n = 5). In the same experiments, the compound had no effect on LPS-induced release of interleukin-8 (IL-8) at concentrations up to 10 μM (not shown), thus, demonstrating that Ro 32-7315 did not cause a general decrease in cytokine production.

The incubation of heparinized, rat whole blood with LPS resulted in a maximal release of TNF-α at 2 h (mean levels of 918 ± 56 pg/ml, n = 5), whereas basal (unstimulated) levels were below the detection limit of the ELISA assay (<5 pg/ml, not shown). Preincubation with Ro 32-7315 (10^{-5}-10^{-8} M), significantly reduced LPS-induced TNF-α release (IC_{50} = 110 ± 18 nM, n = 5). In a similar series of experiments using human whole blood, Ro 32-7315 reduced TNF-α release with an IC_{50} value of 2.4 ± 0.5 μM (n = 5), approximately 7 and 22 times less potent than in the monocytic cell line and rat whole blood, respectively.

LPS-Induced TNF-α Release Studies in Wistar Rats. Figure 2 shows the time course of inhibition of TNF-α release by of Ro 32-7315 dosed orally after an i.p. injection of LPS. Administration of LPS resulted in a transient release of TNF-α in vivo, which peaked between 1 and 2 h, after which time the plasma TNF-α levels declined to baseline (<0.5 pg/ml) by 6-h post-LPS injection. Oral pretreatment with Ro 32-7315 (20–60 mg/kg) resulted in a significant (p < 0.05, compared with vehicle control) dose-dependent reduction in LPS-induced systemic TNF-α release. In the same model, the plasma drug levels were also monitored over the 6-h period (Fig. 2, in parentheses). The inhibition observed was associated with a high plasma exposure of Ro 32-7315 (maximal concentration, C_{max}, of 218 ng/ml and 405 ng/ml, with 30 and 60 mg/kg dose, respectively). These levels are approximately 4- and 8-fold higher, respectively, than the in vitro rat whole-blood IC_{50}.

Adjuvant-Induced Arthritis Model. Adjuvant induced two phases of inflammation in the AHH/R rats, as assessed by increases in hind paw volume. The primary swelling phase (days 0–4) occurred in the injected hind paw, and this represents an acute inflammatory response (not shown). The secondary phase (days 9–14) is a systemic immune response and affects both left (noninjected) and right paws and the nose, ears, forepaws, and tail (Birchall et al., 1994). Ro 32-
The effect of Ro 32-7315 on adjuvant-induced lesion scores and joint mobility in the noninjected paw

Ro 32-7315 was serially diluted from an initial concentration of 10 mM for IC$_{50}$ determination. A constant vehicle concentration (0.5% or 0.8% DMSO) was maintained in the final assay mixture.

The plasma exposure was dose-proportional and was consistent with the inhibitory profile demonstrated by Ro 32-7315. After the i.p. administration of Ro 32-7315 (2.5 to 20 mg/kg, twice daily) causing a significant dose-dependent inhibition of adjuvant-induced secondary paw swelling in the noninjected paws (42, 71, 83, and 93% reduction, respectively, as compared with the vehicle group, n = 10–20/group; Fig. 3). Furthermore, the reduced paw swelling was associated with significant improvements in the lesion score and degree of joint mobility in the paws (Table 2).

Plasma concentrations of Ro 32-7315 were also monitored in the same adjuvant-induced arthritis study. On day 13 of the study, blood samples were collected over a 24-h period in three animals (n = 3/group), and the plasma drug profile was determined. The plasma exposure was dose-proportional and was consistent with the inhibitory profile demonstrated by Ro 32-7315. After the i.p. administration of Ro 32-7315 (2.5 to 20 mg/kg, twice daily), the total exposures (AUC$_{0-24h}$) observed on day 13 were 3,153 ng/h/ml, 5,763 ng/h/ml, 19,040 ng/h/ml, and 26,890 ng/h/ml, respectively. In addition, the maximal exposures observed were well above (≈30-fold) the in vitro rat whole-blood IC$_{50}$ value.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment (i.p., twice daily)</th>
<th>Lesion Score</th>
<th>Joint Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.6</td>
<td>83.7±4.7</td>
</tr>
<tr>
<td>Ro 32-7315</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>5.7*</td>
<td>101±4.6*</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>3.2*</td>
<td>111±3.5*</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>1.0*</td>
<td>111±3.5*</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>1.1*</td>
<td>119±5.9*</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with controls.

**LPS-Induced TNF-α Release in Healthy Volunteers.** Stimulation of TNF-α release from monocytes in human blood has been studied both in vivo and in vitro after LPS challenge (Mohler et al., 1994; DiMartino et al., 1997). The production of human TNF-α has been documented in a number of clinical candidates such as Marimastat, BB 1101, AG 3340, and GW 947 (Gearing et al., 1994; Barlaam et al., 1999). However, these molecules tend to show broad-spectrum inhibition of MMPs (IC$_{50}$ values for inhibition of studies used primary cells in culture or tumor lines (Gearing et al., 1994; DiMartino et al., 1997). Although these systems have provided valuable information, they cannot take account of the complexities that can occur in whole blood such as protein binding, compartmentalization, and possible interactions between different cell types. We have used whole blood as a system to assess the production of human TNF-α. In the present study we have measured inhibition of LPS-induced TNF-α release in whole blood taken from healthy volunteers receiving an oral dose of Ro 32-7315 (450 mg). An initial series of experiments was undertaken to study the baseline and the influence of circadian changes on LPS-induced TNF-α release in whole blood taken from individuals of the same population who were not dosed with Ro 32-7315. For these studies, sequential blood samples (over 24 h) were taken from healthy volunteers (as described under Materials and Methods), and the whole blood was stimulated with LPS in vitro. These studies demonstrated that over a 24-h period, there were no significant changes in the LPS-induced TNF-α release profile (mean levels of 5140 pg/ml and 6930 pg/ml over 24 h for female and male volunteers, respectively; not shown).

The effect of oral administration of Ro 32-7315 to healthy subjects on ex vivo LPS-induced TNF-α release was then studied. In these studies, oral administration of a single dose of the TACE inhibitor, Ro 32-7315 (450 mg), significantly suppressed the LPS-induced TNF-α release in whole blood, in a transient time-dependent manner (Fig. 4). A mean maximal inhibition of 69% (compared with baseline, 0-h value) was demonstrated 1-h post-Ro 32-7315 administration. In the healthy subjects, significant reduction in TNF-α release correlated (r = 0.84) with the plasma concentration of Ro 32-7315 (Fig. 5).

**Discussion**

The results of this study show that Ro 32-7315 is a potent inhibitor of the recombinant TACE (IC$_{50}$ 5.2 nM) and inhibits LPS-induced TNF-α release from a monocytic cell line and in rodent and human whole blood (IC$_{50}$ values of 350, 110, and 2400 nM, respectively). In experimental models, Ro 32-7315 (administered orally and i.p.) significantly reduced systemic TNF-α release and the adjuvant-induced immuno-inflammatory response in a dose-dependent manner. An important finding of the present study is the demonstration of oral bioavailability and the associated efficacy, as assessed by inhibition of ex vivo TNF-α release, in healthy human subjects. This is the first such demonstration for a selective TACE inhibitor.

In the present study, a considerably higher concentration of Ro 32-7315 (11–500 nM range) was required to inhibit selected MMPs compared with recombinant TACE. Of the selected MMPs studied, Ro 32-7315 was most potent against metalloelastase (MMP12, IC$_{50}$ = 11 nM) and least active against collagenase 1 (MMP1, IC$_{50}$ = 500 nM). Numerous potent MMP inhibitors have been described over the past few years, the majority of which incorporate an hydroxamic acid group, as the zinc binding ligand. This approach has resulted in a number of clinical candidates such as Marimastat, BB 1101, AG 3340, and GW 947 (Gearing et al., 1994; Barlaam et al., 1999). However, these molecules tend to show broad-spectrum inhibition of MMPs (IC$_{50}$ values for inhibition of
MMP-1 were 5, 10, and 8.2 nM, respectively; 50- to 100-fold more potent than Ro 32-7315 and TACE (IC_{50} values against TACE are 3.8, 0.2, and 5.5 nM, respectively; Barlaam et al., 1999). Thus, Ro 32-7315 demonstrates a more selective profile for TACE than these compounds.

Bacterial LPS administration is known to induce a rapid TNF-α synthesis from inflammatory cells. This process occurs through rapid up-regulation of TNF-α mRNA expression (Vassali, 1992). LPS-induced TNF-α release was used further to study the in vitro and in vivo biological profile and the mechanism of Ro 32-7315. In the monocytic cell lines, THP-1, and rat and human whole blood, Ro 32-7315 reduced LPS-induced TNF-α release in a dose-dependent manner, with IC_{50} values of 350, 110, and 2400 nM, respectively. In the same experiments Ro 32-7315 had no effect on the release of IL-8. LPS-induced release of TNF-α and other cytokines proceeds through the activation of the transcriptional activator NF-κB (Guha and Mackman, 2001). That Ro 32-7315 does not inhibit IL-8 release indicates that it does not inhibit TNF-α production at the transcriptional level. Furthermore, it was evident that the high intrinsic potency of Ro 32-7315 against recombinant TACE did not translate to the THP-1 cell and whole-blood assays (21- to 461-fold reduction in potencies between the enzyme and the cellular assays). A similar profile has been reported for MMP inhibitors such as BB 1101 and GW 9471 (Gearing et al., 1994, Barlaam et al., 1999). A number of explanations for this reduction in potency in whole-blood cell system versus isolated enzyme have been advanced (Barlaam et al., 1999). It has been reported that all these agents bind extensively to plasma proteins present in the cell-based assays. For example experiments using isolated human mononuclear cells treated with LPS in the presence of human serum (1%) resulted in a 40-fold reduction in activity (Barlaam et al., 1999). Furthermore, the reduced potency in the cellular assays observed in the present study could be due to the fact that the enzyme, TACE, is located in a submembrane compartment (Schlondorff et al., 2000).

In the in vivo experimental model, Ro 32-7315 demonstrated potent inhibitory activity against LPS-induced systemic TNF-α release after an oral administration (ED_{50} of 25 mg/kg). To evaluate the anti-arthritic activity, we used an adjuvant-induced arthritis model that has been shown to be sensitive to anti-TNF-α therapy, Tenefuse (TNF receptor, p55 fusion protein; M. Brewster, personal communication). Ro 32-7315 administered at doses of 2.5, 5, 10, and 20 mg/kg (i.p., twice daily from day 0 to 14) significantly reduced adjuvant-induced secondary paw swelling in a dose-dependent manner. At a dose of 20 mg/kg (twice daily), greater than 90% inhibition was observed. The reduced paw swelling was accompanied with improved joint mobility and reduced adjuvant-induced lesions. Furthermore, the efficacy demonstrated in the immuno-inflammatory model was associated with a high plasma exposure of Ro 32-7315. In comparison, hydroxamic-acid-containing inhibitors of MMP such as BB 1101, have been reported to produce anti-arthritic activity in adjuvant-induced model, however, the degree of inhibition demonstrated was modest (maximal inhibition of 40–56%; DiMartino et al., 1997). The reduced efficacy was attributed to their poor pharmacokinetic profile and the reduced potency for the TNF-α processing enzyme.

The hypothesis that TNF-α plays an important role in the pathogenesis of chronic inflammatory disease such as RA has been confirmed by recent reports on the clinical efficacy of monoclonal antibody, infliximab, and a fusion protein of soluble TNF receptors linked to human immunoglobulin, TNF-Rp75:Fc, Enbrel and TNFRp55:Fc, Lenercept (Moreland et al., 1997, 1998; Maini et al., 1998; McKay et al., 1998). These biologicals significantly reduced signs and symptoms and improved the sense of well being in a large number of RA patients after parenteral administration. The result of anti-TNF treatment on the progression of damage to cartilage, bone, and other connective tissue components in active RA patients remains to be established. Although in collagen-induced arthritis in DBA/1 mice, joint protection has been reported (Joosten et al., 1996). Moreover, the positive clinical results obtained with these parenterally administered anti-TNF-α biologicals provide an impetus to develop orally effective TNF-α inhibitors. The efficacy of Ro 32-7315 was further evaluated in healthy human volunteers by studying the ex vivo, LPS-induced TNF-α release profile and the associated plasma drug exposure. In this study, a high exposure of Ro 32-7315 was observed in human subjects, and this was associated with a significant reduction (up to 69%) in the LPS-induced whole-blood TNF-α release.

These findings suggest that selective inhibitors of TACE, such as Ro 32-7315, may provide an important oral therapy
for chronic inflammatory diseases. An investigation of the effect of Ro 32-7315 on TNF-α release in patients with rheumatoid arthritis would be relevant, because they may respond differently to healthy volunteers. Thus, in rheumatoid subjects, there is evidence of enhanced release of TNF-α after incubation of either whole blood (Zangerle et al., 1992) or isolated monocyte preparations with LPS (Leirisalo-Repo et al., 1995). The magnitude of LPS-induced release of TNF-α in whole blood is under genetic control (Louis et al., 1998), and there is evidence that certain polymorphisms of the TNF gene may underlie rheumatoid arthritis (Verweij, 1999).

References


Address correspondence to: Dr. N. Lad, c/o Teena Bradbury, Roche Product Ltd, 40 Broadwater Rd., Welwyn Garden City, Hertfordshire AL7 3AY UK.
E-mail: teena.bradbury@roche.com