Baseline levels of soluble interleukin-6 receptor predict clinical remission in patients with rheumatoid arthritis treated with tocilizumab: implications for molecular targeted therapy

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Notes

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EXTENDED REPORT

Adalimumab, a human anti-TNF monoclonal antibody, outcome study for the prevention of joint damage in Japanese patients with early rheumatoid arthritis: the HOPEFUL 1 study

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ABSTRACT

Objectives To evaluate the efficacy and safety of adalimumab+methotrexate (MTX) in Japanese patients with early rheumatoid arthritis (RA) who had not previously received MTX or biologics.

Methods This randomised, double-blind, placebo-controlled, multicentre study evaluated adalimumab 40 mg every other week+MTX 6–8 mg every week versus MTX 6–8 mg every week alone for 26 weeks in patients with RA (≤2-year duration). The primary endpoint was inhibition of radiographic progression (change (Δ) from baseline in modified total Sharp score (mTSS)) at week 26.

Results A total of 171 patients received adalimumab+MTX (mean dose, 6.2±0.8 mg/week) and 163 patients received MTX alone (mean dose, 6.6±0.6 mg/week, p<0.001). The mean RA duration was 0.3 years and 315 (94.3%) had high disease activity (DAS28>5.1). Adalimumab+MTX significantly inhibited radiographic progression at week 26 versus MTX alone (ΔmTSS, 1.5±6.1 vs 2.4±3.2, respectively; p<0.001). Significantly more patients in the adalimumab+MTX group (62.0%) did not show radiographic progression (ΔmTSS≤0.5) versus the MTX alone group (35.4%; p<0.001). Patients treated with adalimumab+MTX were significantly more likely to achieve American College of Rheumatology responses and achieve clinical remission, using various definitions, at 26 weeks versus MTX alone. Combination therapy was well tolerated, and no new safety signals were observed.

Conclusions Adalimumab in combination with low-dose MTX was well tolerated and efficacious in suppressing radiographic progression and improving clinical outcomes in Japanese patients with early RA and high disease activity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that is associated with joint damage and progressive disability, an increased risk of morbidity related to comorbid conditions, and substantial socioeconomic costs.[1–3] Given the significant impact biologic therapies have had in the treatment of RA, a paradigm shift has emerged toward earlier inclusion of these therapies in the management of RA.[3 4] Furthermore, international guidelines published in 2010 recommend a treat-to-target goal of remission for patients with early RA in order to mitigate radiographic progression and long-term disability.[5] The efficacy and safety of adalimumab, a tumour necrosis factor (TNF)-α inhibitor, administered as monotherapy or in combination with methotrexate (MTX) for the treatment of RA has been well established in clinical trials conducted in Western countries.[6–12] In early RA, the PREMIER and OPTIMA studies demonstrated that initial combination therapy with adalimumab and MTX was superior to MTX alone in inhibiting radiographic progression and improving clinical symptoms.[6 7 12]

Translating efficacy and safety results of RA Western-based studies to an Eastern populace can be potentially misleading given the genetic, medical and environmental differences (eg, body weight) observed between the two populations.[13] A limited number of studies have evaluated the efficacy or effectiveness and safety of adalimumab in Japanese patients. However, these studies either assessed adalimumab monotherapy in moderate-to-severe RA[14] or were retrospective[15] or postmarketing surveillance studies[16] of adalimumab monotherapy or combination therapy in a population with a wide range of RA duration and prior biologic and MTX experience. Thus, a randomised, placebo-controlled study of adalimumab +MTX combination therapy in MTX-naive Japanese patients with early RA was lacking.

The current study, called adalimumab, a human anti-TNF monoclonal antibody, outcome study for the persistent efficacy under allocation to treatment strategies in early RA, or HOPEFUL 1, was conducted to compare the efficacy and safety of early intervention with adalimumab+MTX versus MTX alone for 26 weeks in inhibiting radiographic progression in MTX-naive Japanese patients with RA.

PATIENTS AND METHODS

Patients aged ≥20 years were evaluated during March 2009 and November 2010 from 94 centres. Eligible patients had RA (1987-revised American College of Rheumatology (ACR) criteria),[17] of ≤2-year duration, a tender joint count ≥10, a swollen joint count ≥8, a C reactive protein (CRP) level ≥1.5 mg/dl or erythrocyte sedimentation rate

(ESR) ≥28 mm/h, and had ≥1 joint erosion or were rheumatoid factor positive. Patients had not previously received MTX, leflunomide or >2 other disease-modifying antirheumatic drugs (DMARDs). Patients who had previously received cyclophosphamide, cyclosporine, azathioprine, tacrolimus or biologic DMARDs (eg, anti-TNF-α therapy) and patients with a chronic infection, interstitial pneumonia, or a history of tuberculosis or malignancy were excluded from the study.

The phase III trial consisted of a randomised, double-blind, placebo-controlled, 26-week phase followed by a 26-week open-label extension phase (clinicaltrials.gov identifier, NCT00870467; only 26-week double-blind data presented). After a 4-week washout period for patients taking eligible DMARDs and a >2-week screening period for all patients, participants were randomised (1 : 1) to receive subcutaneous adalimumab 40 mg or placebo every other week, both administered in combination with oral MTX 6–8 mg/week (adalimumab +MTX vs MTX alone) for 26 weeks. Treatment with MTX was initiated at 6 mg/week and increased to 8 mg/week in patients who did not experience ≥20% decrease from baseline in tender or swollen joint counts on or after week 8, unless investigators indicated a safety concern. In addition, reduction of the MTX dose to 4 mg/week was permitted at the investigator's discretion. All patients received concomitant oral folic acid 5 mg/week. Patients who experienced a >20% increase from baseline in tender and swollen joint counts at weeks 12, 16 or 20 were to discontinue blinded treatment with adalimumab or placebo and were eligible for open-label rescue treatment with adalimumab 40 mg every other week.

The primary endpoint was inhibition of radiographic progression assessed as the change from baseline (Δ) in modified total Sharp score (mTSS) at week 26. All single-emulsion radiographs of the hands (posteroanterior view) and feet (anteroposterior view) obtained from a patient were scored by two independent readers blinded to patient and treatment, as previously described,[6] with the exception that the triquetrum/pisiform joint was not scored for erosions and the first interphalangeal joint was not scored for joint-space narrowing (range, 0–380) (see online supplementary text for more information).

Secondary efficacy endpoints included ACR responses[18 19] by visit; clinical remission (the 28-joint disease activity score with ESR (DAS28-ESR)<2.6) at week 26;[20 21] and change from baseline in the Health Assessment Questionnaire disability index (HAQ-DI)[22] at week 26. Several additional post hoc analyses were conducted, including assessments of the DAS28-CRP, simplified disease activity index (SDAI)[23] and clinical disease activity index (CDAI) scores[24] over time; clinically relevant radiographic progression (ΔmTSS>3); European League Against Rheumatism responses[25] at week 26; and clinical remission, defined as DAS28-CRP<2.6,[26] SDAI≤3.3,[27 28] CDAI≤2.8[28] or meeting Boolean remission criteria,[27] at week 26. Low, medium and high disease activity was also determined using DAS28-ESR, DAS28-CRP, SDAI and CDAI. Adverse events (AEs) and clinical laboratory parameters were routinely monitored during the study. A 28-day follow-up after the completion of or discontinuation from the study and a 70-day follow-up after the last dose of adalimumab administration were conducted to evaluate safety.

Statistics

The primary endpoint was analysed using the Wilcoxon rank sum test for observed data with a separate supportive analysis using linear extrapolation (LE) to impute missing values. Secondary endpoints were analysed using the Fisher's exact test and Wilcoxon rank sum test for discrete variables and continuous variables, respectively. Non-responder imputation was used for binary variables, and the last-observation-carried-forward approach was applied for continuous variables. The safety population included all randomised patients who received ≥1 dose of study medication and had ≥1 efficacy assessment.

To identify baseline predictors of no radiographic progression (mTSS≤0.5) and clinical remission (DAS28-ESR<2.6),

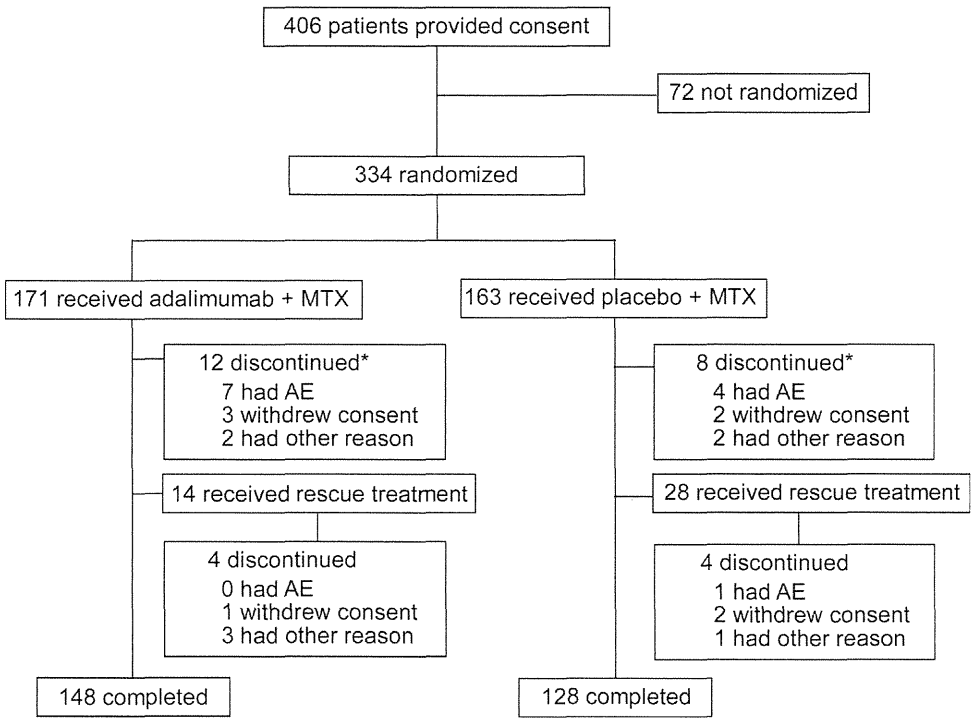

Figure 1 Patient disposition through week 26. *Three adalimumab+MTX patients and one MTX alone patient discontinued from the study by week 26; however, they were included in the efficacy analyses at week 26. AE, adverse event; MTX, methotrexate.

univariate logistic regression analysis was performed, applying 24 baseline demographics and disease characteristics. Significant ($p<0.1$) variables in univariate were included in multivariate models. Last, multivariate models were selected based on model fit statistics (Akaike information criterion and r^2) and clinical significance. Adjusted OR and 95% CIs for selected baseline variables were calculated.

RESULTS

Overall, 334 patients were randomised to treatment and received adalimumab+MTX (n=171) or MTX alone (n=163), and 148 (86.5%) and 128 (78.5%) patients completed the double-blind portion of the study, respectively (figure 1). Demographics and baseline characteristics were well matched between treatment groups (table 1). The mean RA disease duration was 0.3 years, and the majority of patients had ≥1 erosion at baseline and high disease activity. The mean MTX dose during the 26-week study was 6.2±0.8 mg/week in the adalimumab+MTX group and 6.6±0.6 mg/week in the MTX alone group ($p<0.001$). After 26 weeks of treatment, 34.5% (59/171) of adalimumab+MTX patients were receiving MTX 8 mg/week versus 65.0% (106/163) of MTX alone patients ($p<0.001$).

Radiographic progression

Treatment with adalimumab+MTX significantly inhibited radiographic progression (figure 2A) at week 26 versus MTX alone (mean change±SD, 1.5±6.1 vs 2.4±3.2, respectively; $p<0.001$). Results were confirmed by an LE analysis (figure 2A). Changes in radiographic progression during 26 weeks of treatment were also assessed by a cumulative probability plot of ΔmTSS (figure 2B). Fewer adalimumab+MTX patients exhibited radiographic progression (ΔmTSS>0.5), with 62.0% (106/171) of patients showing no radiographic progression versus 35.4% (57/161) of MTX alone patients ($p<0.001$). Furthermore, only 14.0% (24/171) of adalimumab+MTX patients exhibited clinically relevant radiographic progression (ΔmTSS>3) versus 37.3% (60/161) of MTX alone patients ($p<0.001$). In addition, a significantly higher percentage of adalimumab+MTX patients did not experience worsening (≤0.5) in erosion score (73.7% (126/171)) versus MTX alone patients (42.2% (68/161); $p<0.001$). In patients who lacked baseline erosive damage, the continued absence of erosions was reported in more adalimumab+MTX patients versus MTX alone patients (9/9 vs 2/6 patients, respectively; $p=0.01$).

Clinical response

A significantly higher percentage of adalimumab+MTX patients achieved ACR responses versus MTX alone patients at each assessment (figure 3A–C). Significant differences between treatment groups, observed as early as week 2, were maintained through week 26. At week 26, a significantly larger percentage of adalimumab+MTX patients versus MTX alone patients achieved ACR20, ACR50 and ACR70 (figure 3A–C) and ACR90 (12.9% vs 5.5%; $p=0.02$) responses. Significant differences in favour of adalimumab+MTX were also observed from week 2 to 26 for DAS28-ESR, DAS28-CRP, SDAI and CDAI (see online supplementary figure 1A–D). A larger percentage of adalimumab+MTX patients than MTX alone patients demonstrated good or moderate European League Against Rheumatism responses (figure 3D) and were in states of low disease activity or remission after 26 weeks of treatment (figure 3E). Furthermore, a significantly larger percentage of adalimumab+MTX patients versus MTX alone patients satisfied Boolean remission criteria (19.3% vs 8.6%, $p=0.007$). Adalimumab+MTX achieved a 1.8- to 2.2-fold increase in the percentage of patients achieving clinical remission, across all definitions of clinical remission evaluated, versus MTX alone.

Table 1 Demographics and baseline characteristics

Parameter*	Adalimumab+MTX (n=171)	MTX (n=163)
Age±SD (year)	54.0±13.1	54.0±13.2
Females (n (%))	144 (84.2)	128 (78.5)
RA duration±SD (year)	0.3±0.4	0.3±0.4
Weight±SD (kg)	54.4±9.7	56.1±12.3
Previous DMARD use (n (%))	74 (43.3)	87 (53.4)
1 DMARD	57 (33.3)	69 (42.3)
2 DMARDs	17 (9.9)	18 (11.0)
Corticosteroid use at baseline (n (%))	58 (33.9)	49 (30.1)
RF positive (n (%))	146 (85.4)	136 (83.4)
Mean titre±SD (IU/ml)	154.5±202.3	163.7±362.8
Anti-CCP positive (n (%))	145 (84.8)	136 (83.4)
Mean titre±SD (U/ml)	386.2±694.2	241.3±367.2
ESR (mm/h)	59.9±30.1	61.8±29.0
CRP (mg/dl)	2.9±3.0	3.1±3.3
Swollen joint count (n±SD)		
0–28	11.5±4.7	11.8±5.3
0–66	16.5±6.2	17.3±7.7
Tender joint count (n±SD)		
0–28	13.2±5.8	13.2±6.1
0–68	20.7±9.4	21.1±10.2
mTSS	13.6±22.3	13.6±17.4
Erosion score	7.5±11.6	7.3±9.2
Joint space narrowing score	6.2±11.4	6.2±9.4
DAS28-ESR	6.6±0.9	6.6±1.0
DAS28-CRP	5.8±1.0	5.9±1.0
HAQ-DI score	1.1±0.7	1.3±0.8
SDAI score	40.7±12.0	41.4±13.8
CDAI score	37.8±10.9	38.3±12.4
Physician's global assessment of disease activity±SD (mm)	65.8±18.4	66.2±18.8
Patient's global assessment of disease activity±SD (mm)	64.1±24.8	66.4±23.7

*Data are mean±SD unless otherwise indicated.
CCP, cyclic citrullinated peptide; CDAI, clinical disease activity index; CRP, C reactive protein; DAS28-CRP, disease activity score using a 28-joint count and CRP level; DAS28-ESR, disease activity score using a 28-joint count and ESR; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire disability index; mTSS, modified total Sharp score; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, simplified disease activity index.

A significantly larger decrease from baseline in mean HAQ-DI score, indicative of an improvement in physical function, was observed for adalimumab+MTX patients versus MTX alone patients at week 26 (−0.6±0.6 vs −0.4±0.6; $p<0.001$). Although the significant difference between the two groups was small (0.2 units), the percentage of patients achieving normal functionality (HAQ-DI score<0.5) after 26 weeks of treatment was also significantly higher with adalimumab+MTX (figure 3F).

Factors associated with the absence of radiographic progression or with clinical remission

Disease activity or function baseline variables generally were associated with the absence of radiographic progression (ΔmTSS≤0.5) and with clinical remission (DAS28-ESR<2.6) in both treatment groups (see online supplementary text and online supplementary table 1).

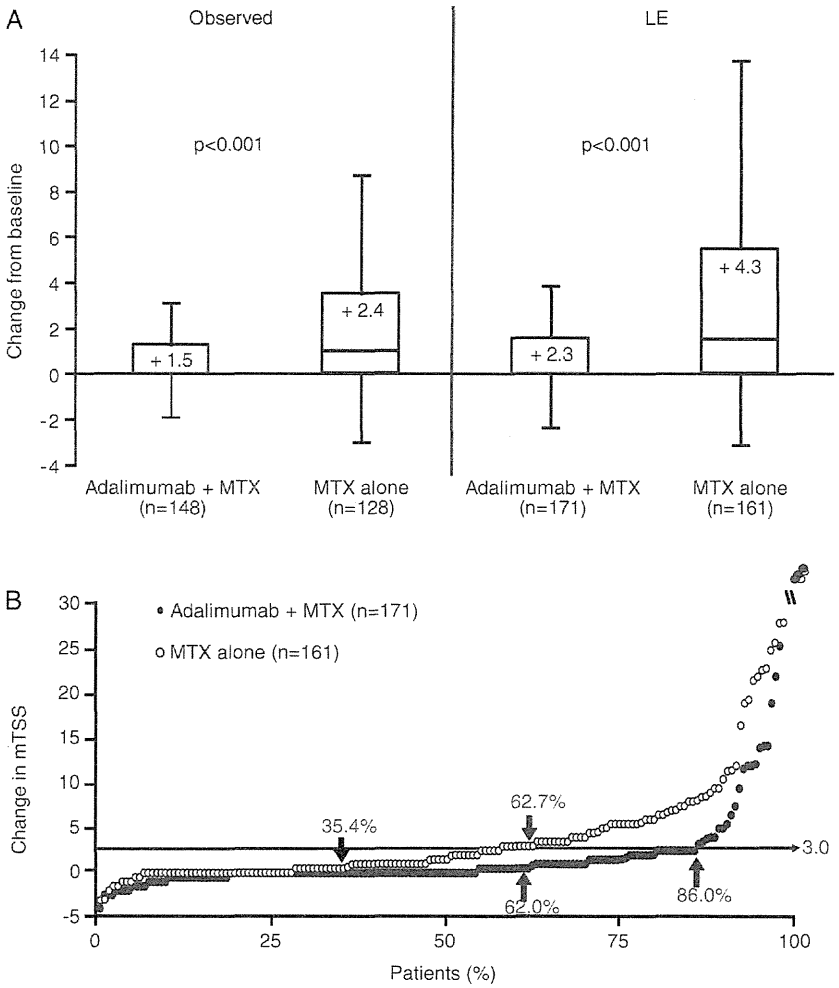

Figure 2 (A) Box plot of change from baseline in mTSS at week 26 with adalimumab+MTX versus MTX alone and (B) cumulative probability plot of mean change from baseline to week 26 in mTSS score (LE). Thickened horizontal lines in (A) indicate median values, the boxes mark the interval between the 25th and 75th percentiles, whiskers indicate the IQR and mean values are reported in the boxes. No radiographic progression (change from baseline in mTSS≤0.5) was reported in 62.0% (106/171) of adalimumab+MTX patients versus 35.4% (57/161) of MTX alone patients (p<0.001). No clinically relevant radiographic progression (change from baseline mTSS≤3) was reported in 86.0% (147/171) of adalimumab+MTX patients versus 62.7% (101/161) of MTX alone patients (p<0.001) (B). LE, linear extrapolation; mTSS, modified total Sharp score; MTX, methotrexate. p Value determined using Wilcoxon rank sum test.

Safety

The mean treatment duration during the double-blind phase was 168.7±36.6 days for adalimumab+MTX patients (mean cumulative adalimumab dose, 477.4±104.5 mg) and 162.8±38.6 days for MTX alone patients. Overall, there were 376 and 302 AEs reported in the adalimumab+MTX group and the MTX alone group, respectively. There were no significant differences in the percentage of patients with AEs in the adalimumab+MTX group (80.7% (138/171)) versus the MTX alone group (71.8% (117/163)), and the incidence of severe AEs was rare (table 2). No significant differences in the incidence of AEs of interest were observed between the two groups, with the exception of injection-site reactions, which were reported in 10.5% of adalimumab+MTX patients and 3.7% of MTX alone patients (p=0.02; table 2). Serious infections were observed in two adalimumab+MTX patients (one case each of pneumonia and infectious enteritis) and one MTX alone patient (*Pneumocystis jiroveci* pneumonia), occurring at rates of 2.5 and 1.4 events per 100 patient-years, respectively. There were no reports of demyelination, tuberculosis or malignancy during the study. One death, due to worsening of interstitial lung disease, occurred in the MTX alone group.

DISCUSSION

The HOPEFUL 1 study was designed to evaluate the efficacy and safety of adalimumab in combination with MTX in Japanese patients with early RA. This is the first description of a clinical trial of anti-TNF therapy+MTX versus MTX alone in MTX-naive Japanese patients with early RA and high disease activity. It is also the first randomised trial evaluating the efficacy of anti-TNF therapy+low-dose MTX versus low-dose MTX alone for the inhibition of radiographic progression in any patient population. This study extends observations from Western studies of adalimumab by demonstrating the superiority of adalimumab+MTX to MTX alone for the inhibition of radiographic progression and improvement in clinical outcomes in Japanese patients with early RA. Moreover, the combination of adalimumab+MTX significantly improved a wide array of clinical and functional disease activity measures and responses versus MTX alone, with improvements observed as early as the first assessment (week 2) and maintained through the 26-week double-blind trial.

Following 26 weeks of treatment, the mean ΔmTSS (primary endpoint) in adalimumab+MTX patients (1.48) in the current study was significantly smaller than observed in MTX alone patients (2.38). In addition, a similar trend in inhibition of radiographic progression in patients with early RA was observed in the OPTIMA study, with a smaller mean ΔmTSS in adalimumab+MTX patients (0.15) versus MTX alone patients (0.96; p<0.001).[12] The difference between the two treatment groups (0.8) at week 26 was similar to the difference observed in the current study (0.9 (observed)).[12] Furthermore, baseline characteristics, including RA duration, in the two studies were generally similar, but the OPTIMA study had a lower percentage of previous DMARD use.

A similar trend in inhibition of radiographic progression in the current study was observed in the PREMIER study, with a

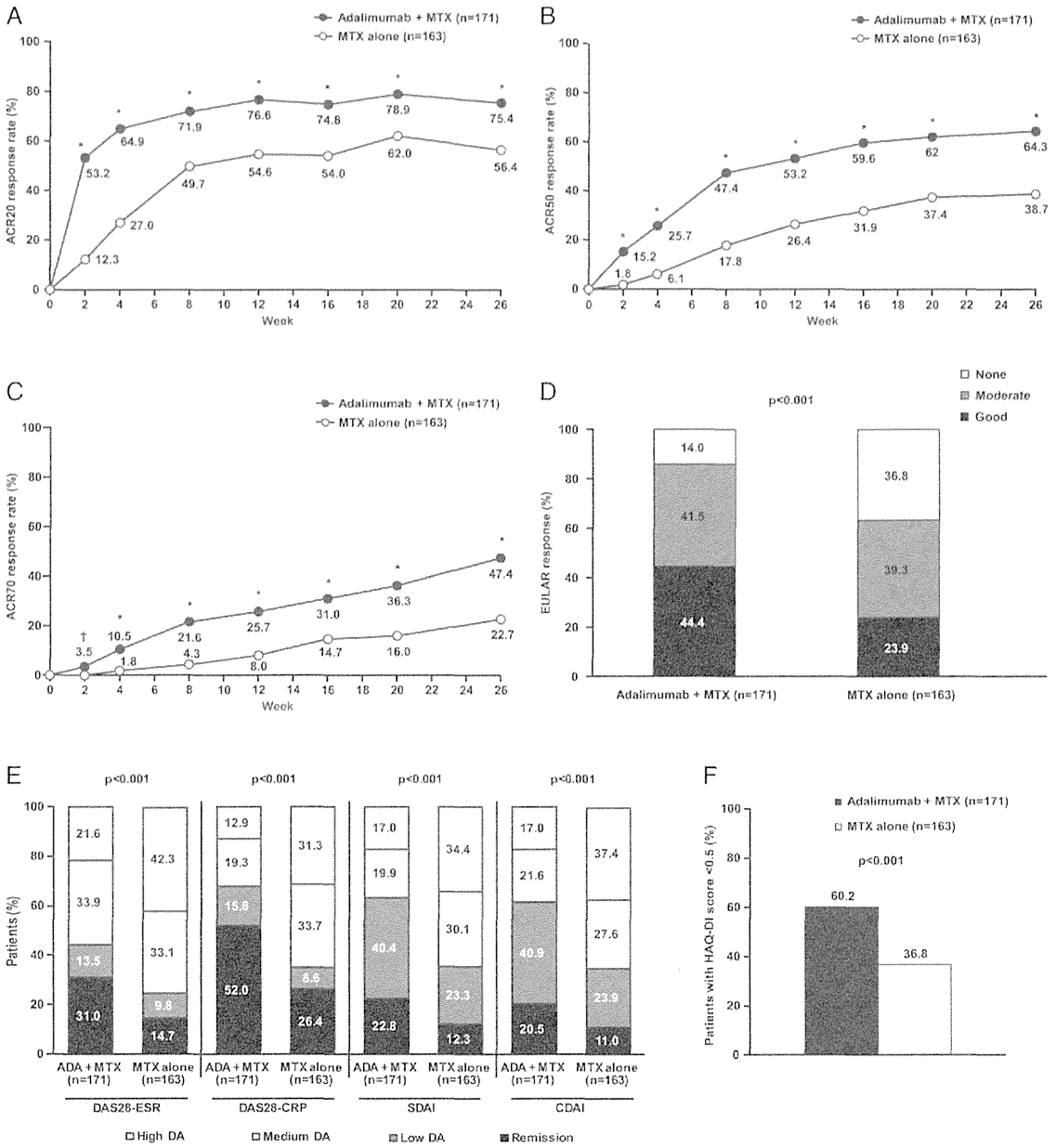

Figure 3 Percentage of patients with an (A) ACR20 response, (B) ACR50 response or (C) ACR70 response over time; (D) the percentage of patients with a EULAR response at week 26; (E) the percentage of patients with low, medium or high disease activity at week 26; and (F) the percentage of patients achieving functional remission (HAQ-DI score<0.5) at week 26. The following values were used to identify remission, low, medium and high disease activity for each clinical assessment in (E): DAS28-ESR or DAS-CRP (<2.6, ≥2.6–<3.2; ≥3.2–≤5.1, >5.1, respectively), SDAI (≤3.3, >3.3–≤11.0, >11.0–≤26.0, >26.0, respectively), and CDAI (≤2.8, >2.8–≤10.0, >10.0–≤22.0, >22.0, respectively). *p<0.001 versus MTX alone. †p=0.03 versus MTX alone. ACR, American College of Rheumatology; ADA, adalimumab; CDAI, clinical disease activity index; DA, disease activity; DAS28-CRP, disease activity score using a 28-joint count and C reactive protein level; DAS28-ESR, disease activity score using a 28-joint count and erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; HAQ-DI, Health Assessment Questionnaire disability index; MTX, methotrexate; SDAI, simplified disease activity index.

smaller mean ΔmTSS in adalimumab+MTX patients (0.8) versus MTX alone patients (3.5; p<0.001). However, the mean difference in radiographic progression between the two treatments groups, although statistically significant, was smaller in the current study (0.9 (observed); 2.0 (LE)) than in the PREMIER study (2.7).

In the current study, the SD for the mean ΔmTSS at week 26 was generally high. When the median ΔmTSS was compared using observed data, results were in good agreement between the PREMIER study (0.0 (adalimumab+MTX) vs 1.3 (MTX alone); data on file) and the current study (0.0 (adalimumab +MTX) vs 1.0 (MTX alone)). Alternatively, the smaller difference in improvement observed in the current study may also be related to the mTSS scoring method used, but this seems unlikely because only two joints assessed in PREMIER were omitted from scoring in the present analysis. The mean duration of RA was also shorter in the current study (0.3 years) versus the PREMIER study (0.7–0.8 years), although the percentage of patients who had previously taken DMARDs was higher (43.3–53.4% vs 31.5–32.5%). There were also slight differences in mean baseline tender and swollen joint counts and CRP levels, which were higher in the PREMIER study and considered

Table 2 Adverse events (AEs)

Parameter	Patients (n (%))	
	Adalimumab+MTX (n=171)	MTX (n=163)
Any AE	138 (80.7)	117 (71.8)
Severe AE	1 (0.6)	1 (0.6)
Serious AE	7 (4.1)	4 (2.4)
Infectious AE	59 (34.5)	48 (29.4)
Serious infection	2 (1.2)	1 (0.6)
AEs leading to study drug discontinuation	7 (4.1)	6 (3.7)
AEs of interest		
Elevated liver function test level	32 (18.7)†	21 (12.9)†
Injection-site reaction	18 (10.5)*	6 (3.7)
Haematological event	7 (4.1)	8 (4.9)
Allergic reaction	1 (0.6)	2 (1.2)
Interstitial lung disease	1 (0.6)	1 (0.6)
Lupus-like syndrome	0	1 (0.6)
Opportunistic infection	0	1 (0.6)

*p=0.02 versus MTX.
†≥94% of events were mild in severity.
MTX, methotrexate.

related to the longer duration of RA at baseline versus the current study. Furthermore, the MTX dose of 6–8 mg/week, although consistent with the dosage commonly administered in Japan at the time the study was conducted, was substantially lower than that commonly administered in Western countries (eg, 15–20 mg/week). In the PREMIER study, MTX was initiated at 7.5 mg/week, increased to 15 mg/week during weeks 4–8, and increased to 20 mg/week starting at week 9. In addition, the mean MTX dose during the 26 weeks of the current study was significantly lower in the adalimumab+MTX group (6.2±0.8 mg/week) versus the MTX alone group (6.6±0.6 mg/week; p<0.001), thereby potentially impacting the ΔmTSS and thus the maximal difference observed between the two treatment groups. Therefore, these multiple differences may have contributed to the small difference in radiographic outcomes between the current study and the PREMIER study. Whether the difference in radiographic outcomes can be explained by differences between Japanese and Western populations remains unclear, although this seems unlikely. Longer-term studies may help elucidate potential differences in outcomes.

Since this study was conducted, the maximum approved MTX dosage in Japan has been increased from 8 to 16 mg/week in patients with RA. Therefore, this study provides important information on the efficacy of low-dose MTX and anti-TNF therapy versus low-dose MTX alone for the inhibition of radiographic progression. Data suggest that patients with early RA who may not tolerate higher doses of MTX will likely benefit from adalimumab+low-dose MTX combination therapy.

Given the lower MTX dose prescribed, one could question whether we might only be seeing natural progression in the MTX only arm. It is ethically difficult to include a true placebo arm in clinical trials of ≥6 months duration for early active RA, particularly when MTX is recommended as first-line therapy to achieve clinical remission/low disease activity. Although an important question to ponder, a placebo arm in long-term clinical trials in early active RA appears to be unrealistic, and further research using highly sensitive and reproducible imaging techniques during a short-term placebo-treatment period in early active RA is warranted.

It is also important to note that the current patient population had severe baseline symptoms, including baseline erosions, despite only several months since RA onset. This scenario is becoming increasingly less common in Western populations due to treat-to-target recommendations and earlier intervention. In Japan, general practitioners are still seeing many early RA patients and referrals to rheumatologists are often delayed. In addition, the diagnosis of RA in this trial was based upon 1987 classification criteria. Thus, these factors may have played a role in the conundrum of more severe baseline clinical symptoms yet shorter mean disease duration.

The clinical results of the current study are supported by the HARMONY study, which retrospectively determined the effectiveness and safety of adalimumab 40 mg every other week with or without MTX (mean dose, 8.5 mg/week) in Japanese patients with RA (mean RA duration, 9.0±9.5 years) with or without prior biologic treatment.[15] Although patients in the HARMONY study had more established disease and the study design was retrospective, adalimumab+MTX patients (n=143) had an improvement from baseline in DAS28-ESR score at week 24 (baseline, 5.3; week 24, 3.3), which was within the range but slightly smaller than the improvement observed in the current study at week 26 (baseline, 6.6; week 26, 3.7; see online supplementary figure 1A). Clinical remission rates for adalimumab+MTX patients were also comparable between the HARMONY study (week 24, 35.0%) and the current study (week 26, 31.0%).

The safety profile of the current study was generally consistent with those in previous clinical studies of adalimumab in patients with RA conducted in Japan.[14–16] There were no reports of demyelination, tuberculosis or malignancy, and there were no statistically significant differences in the incidence of serious AEs, serious infections, opportunistic infections or lupus-like reactions between adalimumab+MTX patients versus MTX alone patients. There was a significantly higher incidence of injection-site reactions for adalimumab+MTX patients versus MTX alone patients, but the incidence (10.5%) was similar to that reported for the 167 adalimumab±MTX patients in the HARMONY study (12.0%). The incidence of injection-site reactions in both of these studies was lower than the 30.8% reported for the 91 adalimumab monotherapy patients (40 mg every other week) in the CHANGE study,[14] possibly related to the immunosuppressive effects of concomitant MTX in the current study and in some of the patients in the HARMONY study.

In the multivariate regression analyses (see online supplementary table 1), lower baseline CRP level was identified as a predictor of radiographic non-progression in adalimumab+MTX patients, whereas normal baseline CRP level (≤0.3 mg/dl) appeared to have an increased likelihood of radiographic non-progression. However, no baseline predictors appeared to predict both the lack of progression and clinical remission. Furthermore, baseline mTSS was not an independent predictor for either treatment group in this study.

Overall, adalimumab+MTX was well tolerated in Japanese patients with early RA with no new safety signals and with a safety and tolerability profile similar to that observed in Western populations. Administration of adalimumab in combination with MTX was efficacious in improving radiographic and clinical responses in MTX-naive patients with early RA, high disease activity and poor prognostic factors (eg, rheumatoid factor positive or with baseline erosive damage) through week 26. Given its radiographic, clinical and functional superiority versus MTX monotherapy, consideration should be given to administration

of anti-TNF-α and MTX combination therapy in patients with early RA and high disease activity.

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ARD

Adalimumab, a human anti-TNF monoclonal antibody, outcome study for the prevention of joint damage in Japanese patients with early rheumatoid arthritis: the HOPEFUL 1 study

Tsutomu Takeuchi, Hisashi Yamanaka, Naoki Ishiguro, Nobuyuki Miyasaka, Masaya Mukai, Tsukasa Matsubara, Shoji Uchida, Hideto Akama, Hartmut Kupper, Vipin Arora and Yoshiya Tanaka

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Structure–activity relationship of celecoxib and rofecoxib for the membrane permeabilizing activity

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) achieve their anti-inflammatory effect by inhibiting cyclooxygenase activity. We previously suggested that in addition to cyclooxygenase-inhibition at the gastric mucosa, NSAID-induced gastric mucosal cell death is required for the formation of NSAID-induced gastric lesions in vivo. We showed that celecoxib exhibited the most potent membrane permeabilizing activity among the NSAIDs tested. In contrast, we have found that the NSAID rofecoxib has very weak membrane permeabilizing activity. To understand the membrane permeabilizing activity of coxibs in terms of their structure–activity relationship, we separated the structures of celecoxib and rofecoxib into three parts, synthesized hybrid compounds by substitution of each of the parts, and examined the membrane permeabilizing activities of these hybrids. The results suggest that the sulfonamidophenyl subgroup of celecoxib or the methanesulfonylphenyl subgroup of rofecoxib is important for their potent or weak membrane permeabilizing activity, respectively. These findings provide important information for design and synthesis of new coxibs with lower membrane permeabilizing activity.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines.[1] NSAIDs are inhibitors of cyclooxygenase (COX), a protein essential for the synthesis of prostaglandins (PGs), which have a strong ability to induce inflammation. However, NSAID use is associated with gastrointestinal complications, such as gastric ulcers and bleeding. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications.[2] Thus, understanding the mechanism of NSAID-induced gastric lesions and its application to design and synthesis of new NSAIDs with reduced adverse effects on the gastric mucosa is important.

The inhibition of COX by NSAIDs was initially thought to be responsible for the adverse gastric side effects manifested by such treatment, because PGs have a strong protective effect on the gastric mucosa. Thus, after the identification of two subtypes of COX (COX-1 and COX-2), which are responsible for the majority of COX activity at the gastric mucosa and in inflammatory tissues, respectively,[3,4] selective COX-2 inhibitors (most of which are coxibs, such as celecoxib and rofecoxib) were developed as NSAIDs with reduced adverse gastric side effects.[5–7] However, due to the observation that rofecoxib was associated with an increased potential risk of cardiovascular thrombotic events,[8,9] this NSAID was withdrawn from the market. At first, this increased risk was believed to be due to the class effect of selective COX-2 inhibitors, because prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2.[10–12] However, some clinical studies showed that the potential risk of cardiovascular thrombotic events was indistinguishable between celecoxib users and classic NSAID users.[13,14] Thus, it is possible that the increased potential risk of cardiovascular thrombotic events is not due to the class effect of selective COX-2 inhibitors, but rather is a specific characteristic of rofecoxib. While mechanisms to explain this rofecoxib-specific increase in the potential risk of cardiovascular thrombotic events have been proposed,[15–17] a definitive explanation for this increase has not yet been forthcoming.

It is now believed that the inhibition of COX by NSAIDs is not the sole explanation for the adverse gastric side effects of NSAIDs, given that the increased incidence of gastric lesions and the

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decrease in PG levels induced by NSAIDs do not always occur in parallel.[18–20] We proposed that, in addition to COX-inhibition at the gastric mucosa, NSAID-induced gastric mucosal cell death is required for the formation of NSAID-induced gastric lesions in vivo.[21,22] Furthermore, we reproduced NSAID-induce cell death in cultured gastric mucosal cells in vitro[22–26] and showed that the primary target of NSAIDs for the induction of cell death is the cytoplasmic membrane. Moreover, a close relationship between membrane permeabilizing activity and cell death-inducing activity among various NSAIDs was shown.[23,25] Thus, decreasing the membrane permeabilizing activity of NSAIDs may be another strategy to synthesize safer NSAIDs for the gastric mucosa. In fact, we recently reported that screening for NSAIDs with lower membrane permeabilizing activity resulted in the identification of an interesting new NSAID, fluoro-loxoprofen, which has much lower membrane permeabilizing and gastric ulcerogenic activities compared with clinically used NSAIDs.[27–31] These results suggest that NSAIDs with lower membrane permeabilizing activity could be therapeutically beneficial. Thus, it is important to understand how the membrane permeabilizing properties of NSAIDs are affected by their structure–activity relationship.

We previously reported that celecoxib showed the most potent membrane permeabilizing and cytotoxic activities among the NSAIDs we tested.[23,25] We also reported that the cytotoxic activity of rofecoxib is much lower than that of celecoxib.[21] As these results suggested that the membrane permeabilizing activity of rofecoxib is lower than that of celecoxib, our objective here was to confirm this hypothesis.

Furthermore, to identify how the structure–activity relationship of coxibs affects their membrane permeabilizing activity, we synthesized hybrid compounds from celecoxib and rofecoxib and examined their membrane permeabilizing activities. The results suggest that the sulfonamidophenyl subgroup of celecoxib and the methanesulfonylphenyl subgroup of rofecoxib are important for determining the membrane permeabilizing activities of these NSAIDs.

2. Chemistry

The synthetic route for target compounds **3–5** is outlined in Scheme 1. Pyrazole compounds **3–5** were synthesized by the condensation of appropriate 1,3-diketones and hydrazine. The reaction of 4,4,4-trifluoro-1-*p*-tolylbutane-1,3-dione **9** with 4-methylphenylhydrazine hydrochloride **11**, 4,4,4-trifluoro-1-phenylbutane-1,3-dione **10** with **11**, or **10** with 4-sulfamoylphenylhydrazine hydrochloride **12** afforded target compounds **3**, **4** or **5**, respectively.

The synthetic route for target compounds **6–8** is outlined in Scheme 2. Furanone compounds **6–8** were synthesized by the condensation of a phenylacetic acid analog and phenacyl bromide. The reaction of **13** with **15**, **14** with **17** or **14** with **16** in the presence of triethylamine afforded the phenacyl phenylacetate products **18**, **19** or **20**, respectively. Treatment of intermediates **18–20** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) provided 3,4-diphenyl-2(5*H*)furanone **21** or target compounds **7** or **8**. chlorosulfonylation of **21** by the reaction with chlorosulfonic acid followed by sulfonamidation using ammonium hydroxide gave target compound **6**.

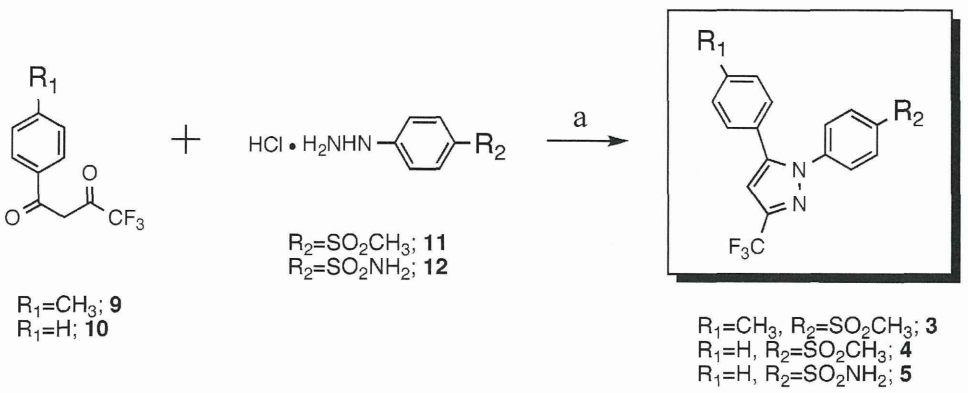
The final compounds were characterized by nuclear magnetic resonance (NMR), infrared spectroscopy (IR), high resolution mass spectra (HR-MS) and elemental analysis.

3. Results and discussion

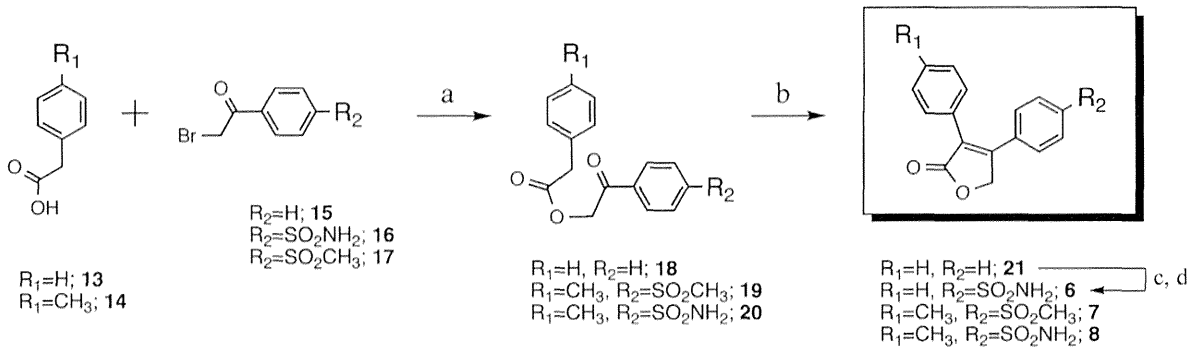
The chemical structures of celecoxib and rofecoxib exhibit some similarities (Fig. 1) and can be divided into three parts (A–C in Table 1); part A, methylphenyl for celecoxib, phenyl for rofecoxib; part B, trifluoromethylpyrazole for celecoxib, furanone for rofecoxib; part C, sulfonamidophenyl for celecoxib, methanesulfonylphenyl for rofecoxib. Thus, in addition to celecoxib and rofecoxib, there are six possible combinations of these three parts that could be used to obtain hybrid compounds of celecoxib and rofecoxib (compounds **3–8** in Table 1). We synthesized these six compounds and tested their membrane permeabilizing and COX-inhibitory activities.

To begin with, we used calcein-loaded liposomes to compare the membrane permeabilizing activities of celecoxib and rofecoxib. As calcein fluorescence is very weak at high concentrations due to self-quenching, the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes causes an increase in fluorescence by diluting the calcein.[25] As shown in Figure 2, celecoxib and rofecoxib increased the calcein fluorescence in a dose-dependent manner. Compared with celecoxib, however, a rofecoxib concentration about 100 times higher was required to increase the fluorescence by the same amount. Figure 2 shows that rofecoxib has a much lower membrane permeabilizing activity than celecoxib.

We next examined the membrane permeabilizing activities of the six hybrid compounds in a similar manner. As shown in Figure 3, all of the hybrid compounds increased the calcein fluorescence in a dose-dependent manner. To compare the membrane permeabilizing activity of these compounds, we used the EC_{50} (half-maximal effective concentration) index, which is defined as the concentration of each compound required for 50% of the calcein in loaded liposomes to be released (Table 2). Comparison of the EC_{50} index of **3**, **5** and **8** (compounds with one part substitution from celecoxib) showed that the membrane permeabilizing activity of **3** was much lower than that of **5** or **8**, suggesting that part



Scheme 1. Synthesis of pyrazole compounds **3–5**.



Scheme 2. Synthesis of furanone compounds **6–8**.

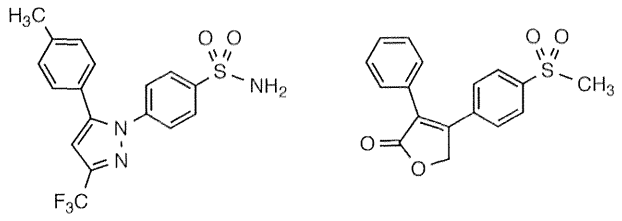
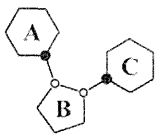
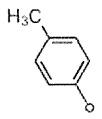
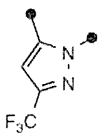
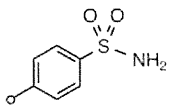
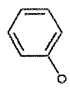
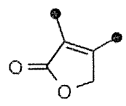
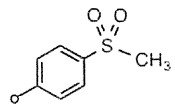
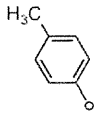
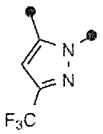
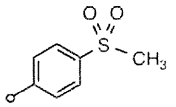
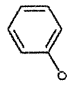
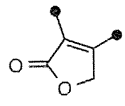
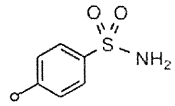
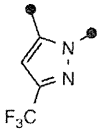
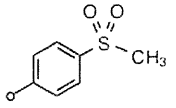
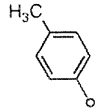
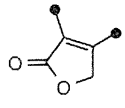
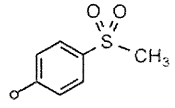
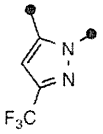
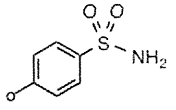
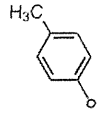
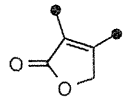
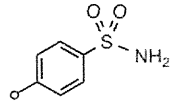


Figure 1. Structures of celecoxib and rofecoxib.

C of celecoxib (the sulfonamidophenyl subgroup) is the most important subgroup determining its high membrane permeabilizing activity. On the other hand, comparison of the EC_{50} index of **4**, **6** and **7** (compounds with one part substitution from rofecoxib) revealed that the membrane permeabilizing activity of **6** was higher than that of **4** or **7**. In this case, part C of rofecoxib (the methanesulfonylphenyl subgroup) was also seemed to be the most important subgroup determining its low membrane permeabilizing activity. Thus, part C seems to be important for determining the permeabilizing activity of these coxibs. The fundamental structural requirement underlying the ability of molecule to permeabilize membrane is a shape in which clusters of hydrophobic and hydrophilic parts are spatially organized with appropriate distance, because this structure enable the hydrophobic part to be located near the surface of membrane, resulting in perturbation of membrane structure. In the present case, because the sulfonamidophenyl subgroup is more hydrophilic than the methanesulfonylphenyl subgroup, the former but not the latter produces hydrophilic part. The methanesulfonylphenyl subgroup is hydrophobic enough to be totally buried within the lipid bilayer structure and therefore, does not affect the membrane structure drastically. On the other hand, the sulfonamidophenyl subgroup is relatively hydrophilic, which allows the compound to face membrane surface, resulting in perturbation of the membrane structure.

Table 1
Structures of celecoxib, rofecoxib and their hybrid compounds (**3–8**)



	A	B	C		A	B	C
Celecoxib				Rofecoxib			
3				**6**			
4				**7**			
5				**8**			

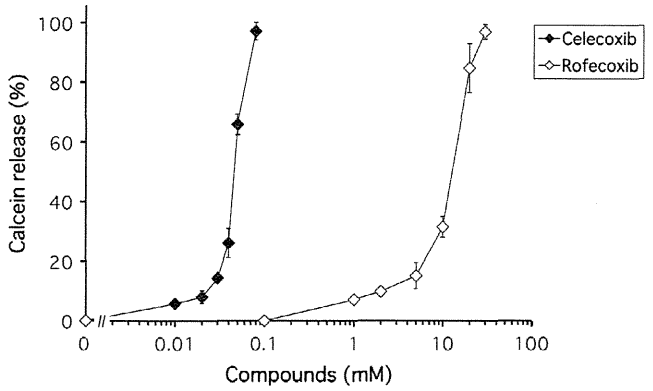


Figure 2. Membrane permeabilization by celecoxib and rofecoxib. Calcein-loaded liposomes were incubated for 10 min at 30 °C with the indicated concentration of each compound. The release of calcein from the liposomes was determined by measuring fluorescence intensity as described in the experimental section. Triton X-100 (10 μM) was used to establish the 100% level of calcein release. Values are mean ± SD (n = 3).

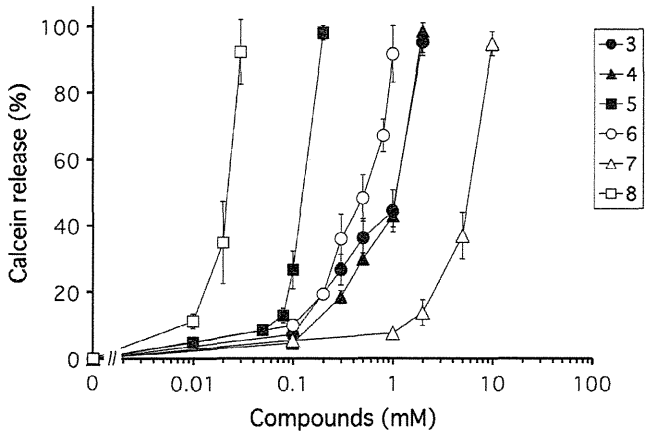


Figure 3. Membrane permeabilization by pyrazole compounds **3**–**5** and furanone compounds **6**–**8**. Experiments and data analysis were performed as described in the legend of Fig. 2. Values shown are mean ± SD (n = 3).

The results presented in Figures 2 and 3 thus provide important information for the design and synthesis of new coxibs with lower membrane permeabilizing activity.

The ionization of these compounds would not be related to their membrane permeabilizing activities. This is because pK_a values of all compounds (celecoxib, rofecoxib, **3**– **8**) are higher than 10 and membrane permeabilizing assay was performed under the conditions of pH = 6.8. Furthermore, such ionization would increase the osmotic pressure outside vesicles and thus, would not stimulate the release of calcein from vesicles.

The inhibitory effects on COX-1 and COX-2 of these compounds were compared by using the IC_{50} (half-maximal inhibitory concentration) index, which is defined as the concentration of each compound required for 50% inhibition of each enzyme. The IC_{50} values for COX-1 and COX-2 of celecoxib and rofecoxib were roughly similar to those reported previously,[32] and the IC_{50} values for COX-1 of all the hybrid compounds were relatively high (Table 2). With the exception of **4** and **6**, the IC_{50} values for COX-2 of the hybrid compounds were within the range seen for celecoxib and rofecoxib, and all the hybrid compounds (except 6) showed COX-2 selectivity (Table 2). Since both **4** and **6** are compounds with one part substitution from rofecoxib, the structure of rofecoxib rather than that of celecoxib appears to be sensitive to modification in relation to COX-2 inhibitory activity.

Table 2
Membrane permeabilizing activities and inhibitory activities on COX-1 and COX-2 of celecoxib, rofecoxib and their hybrid compounds (**3**–**8**)

Compounds	EC_{50} (mM)	IC_{50} (μM)		COX-1/COX-2	EC_{50}/IC_{50}
	Calcein release	COX-1	COX-2		
Celecoxib	0.050	117	0.07	1670	0.71
Rofecoxib	12.6	>500	0.36	>1380	35.0
3	1.44	>500	0.13	>3830	11.1
4	1.30	>500	6.84	>73	0.19
5	0.12	213	0.16	1330	0.75
6	0.48	>500	>500	–	–
7	6.23	>500	0.30	>1680	20.8
8	0.024	385	0.11	>3500	0.22

The EC_{50} value for membrane permeabilization (concentration of each compound required for 50% release of calcein) was calculated based on the data shown in Figures 2 and 3. The inhibitory effect of each compound on COX-1 and COX-2 was examined using purified ovine COX-1 and human recombinant COX-2 as described in the experimental section. The values of IC_{50} (concentration of each compound required for 50% inhibition) were estimated from the sigmoid-like dose-response curve (4-parameter logistic curve model) drawn using logistic-curve fitting software (ImageJ 1.43u; National Institutes of Health, USA), and the COX-1/COX-2 ratios of IC_{50} values were calculated. EC_{50} for membrane permeabilization/IC_{50} for COX-2 inhibition was shown. Values are mean (n = 3).

Among the six hybrid compounds, **4** and **6** can be eliminated as candidates for future clinical use based on their relatively weak inhibitory activity on COX-2 (Table 2). On the other hand, **5** and **8** can be eliminated as candidates based on their relatively potent membrane permeabilizing activity (Table 2). To further compare the potential value of these compounds, we calculated the value of the EC_{50} index for calcein release/IC_{50} index for COX-2 (Table 2). Compounds **3** and **7** appear as the most likely selections as candidates for future clinical use based on this index (Table 2). As described in the introduction section, rofecoxib was withdrawn from the market due to an observed increased potential risk for cardiovascular thrombotic events,[8,9] which may not be a drug class effect but actually something characteristic of rofecoxib alone. According to this hypothesis, it could be postulated that **3** and **7** might have fewer adverse effects associated with their use. Nevertheless, because the mechanism underlying the increased cardiovascular thrombotic events remains to be elucidated, the potential risk of these compounds has not yet been tested without a large-scale clinical study.

4. Conclusion

We here found that rofecoxib has very weak membrane permeabilizing activity compared with celecoxib. Furthermore, analysis of the membrane permeabilizing activities of hybrid compounds derived from celecoxib and rofecoxib suggested that the sulfonamidophenyl subgroup of celecoxib and the methanesulfonylphenyl subgroup of rofecoxib are important for their potent or weak membrane permeabilizing activity, respectively.

5. Experimental section

5.1. Chemistry

All solvents and reagents were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) or Wako Pure Chemical Industries (Tokyo, Japan) and used without further purification. Fourier transform IR spectra were recorded as films with NaCl plates on a JASCO FT/IR-480 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on VARIAN 400- or 500-MR spectrometer (Agilent Thechnologies Japan, Tokyo, Japan) operating at 400 MHz, in a ca. 2% solution of $CDCl_3$ or DMSO-d_6. Coupling constant (J) values are estimated in hertz (Hz) and spin multiples are given as s (singlet), d

(double), m (multiplet), and br (broad). Mass spectra were detected with an electrospray ionization time-of-flight (ESI-TOF) mass spectrometer (Bruker MicroTOF, Bruker, Bremen, Germany) in the negative mode. The progress of all reactions was monitored by thin-layer chromatography (TLC) with silica gel glass plates (60 F_{254}) (Merck Ltd, Tokyo, Japan), and spots were visualized with ultraviolet (UV) light (254 nm) and stained with 5% ethanolic phosphomolybdic acid. Column chromatography was performed using Silica gel 60 N (Kanto Chemical Co., Tokyo, Japan). Elemental analyses were performed for C, H and N (Central Service Research Center, Keio University) and were within ±0.4% of the theoretical values. Melting points (mp) were obtained using a Yanaco melting point apparatus MP-J3 (Yanaco, Kyoto, Japan) without correction. Celecoxib and rofecoxib were from LKT Laboratories Inc. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. (Tokyo, Japan).

5.1.1. General procedure for preparation of compounds 3–5

Phenylhydrazine hydrochloride (**11** or **12**) was added to a stirred solution of the dione (**9** or **10**) in ethanol (30 mL), and the mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The resulting residue was dissolved in AcOEt (50 mL) and washed with brine. The organic fraction was dried over Na_2SO_4 and filtered. The filtrate was concentrated in vacuo and the residue was purified by silica gel chromatography (*n*-hexane/AcOEt, 2:1) to afford pyrazole compounds **3**–**5**.

5.1.2. 1-(4-Methanesulfonylphenyl)-5-*p*-tolyl-3-(trifluoromethyl)-1*H*-pyrazole (3)

Compound **3** was synthesized from **9** (500 mg, 2.2 mmol, 1.0 equiv) and **11** (774 mg, 3.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 35%); mp 125.1–126.1 °C; IR (film) *ν*: 1160, 1325 (SO_2), 2930 (C-H), 3015 (Ar-H) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 2.36 (s, 3H, Ar-CH_3), 3.04 (s, 3H, SO_2CH_3), 6.72 (s, 1H, pyrazole-H4), 7.10 (d, *J* = 8.0, 2H, *p*-tolyl-H3, -H5), 7.16 (d, *J* = 8.0, 2H, *p*-tolyl-H2, -H6), 7.52 (d, *J* = 8.0, 2H, methanesulfonylphenyl-H2, -H6), 7.91 (d, *J* = 8.0, 2H, methanesulfonylphenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m/z*): 379.0706 ($[M-H]^-$, Calcd for $C_{18}H_{14}F_3N_2O_2S$: 379.0728). Anal. Calcd for $C_{18}H_{15}F_3N_2O_2S$: C, 56.84; H, 3.97; N, 7.36. Found: C, 56.64; H, 3.75; N, 7.20. IR and ^1H NMR spectral data for **3** were consistent with reported results.[33,34]

5.1.3. 1-(4-Methanesulfonylphenyl)-5-phenyl-3-(trifluoromethyl)-1*H*-pyrazole (4)

Compound **4** was synthesized from **10** (500 mg, 2.3 mmol, 1.0 equiv) and **11** (567 mg, 2.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 50%); mp 135.2–136.1 °C; IR (film) *ν*: 1162, 1320 (SO_2), 2935 (C-H), 3020 (Ar-H) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) *δ*: 3.04 (s, 3H, SO_2CH_3), 6.77 (s, 1H, pyrazole-H4), 7.22–7.23 (m, 2H, phenyl-H2, -H6), 7.35–7.41 (m, 3H, phenyl-H3, -H4, -H5), 7.51 (d, *J* = 8.5, 2H, methanesulfonylphenyl-H2, -H6), 7.91 (d, *J* = 8.5, 2H, methanesulfonylphenyl-H3, -H5); ^{13}C NMR ($CDCl_3$, 500 MHz) *δ*: 44.28, 106.6, 125.5, 128.4, 128.7, 129.0, 129.5, 139.8, 143.2, 144.0, 144.3, 145.1; HR-ESI-TOF/MS (negative, *m/z*): 365.0550 ($[M-H]^-$, Calcd for $C_{17}H_{12}F_3N_2O_2S$: 365.0572). Anal. Calcd for $C_{17}H_{13}F_3N_2O_2S$: C, 55.73; H, 3.58; N, 7.65. Found: C, 55.58; H, 3.60; N, 7.44.

5.1.3.1. 1-(4-Sulfonamidophenyl)-5-phenyl-3-(trifluoromethyl)-1*H*-pyrazole (5).

Compound **5** was synthesized from **10** (500 mg, 2.3 mmol, 1.0 equiv) and **12** (560 mg, 3.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 66%); mp 164.1–165.2 °C; IR (film) *ν*: 1165, 1325 (SO_2), 3025 (Ar-H), 3680 (N-H) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) *δ*: 4.92 (br s, 2H, NH_2), 6.75 (s, 1H, pyrazole-H4), 7.22–7.24 (m, 2H, phenyl-H2, -H6), 7.32–7.40 (m, 3H, phenyl-H3, -H4, -H5), 7.45 (d, *J* = 8.5, 2H, 4-sulfonamidophenyl-H2, -H6), 7.88 (d, *J* = 8.5, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m/z*): 368.0622 ($[M-H]^-$, Calcd for $C_{16}H_{12}F_3N_2O_2S$: 368.0681). Anal. Calcd for $C_{16}H_{13}F_3N_2O_2S$: C, 52.17; H, 3.56; N, 11.41. Found: C, 52.12; H, 3.45; N, 11.28. ^1H NMR spectral data for **5** were consistent with reported results.[35,36]

5.1.4. General procedure for preparation of compounds 21, 7 and 8

To a stirred solution of phenylacetic acid (**13** or **14**) and triethylamine in dry CH_3CN, phenacyl bromide (**15**–**17**) in dry CH_3CN was added dropwise at room temperature. The reaction mixture was stirred for 1 h and was concentrated in vacuo. The resulting residue was re-dissolved in AcOEt (50 mL) and washed with 1 M HCl (20 mL). The organic fraction was dried over Na_2SO_4 and filtered. The filtrate was evaporated under reduced pressure to give crude product (**18**–**20**) that was used in the next step without further purification.

DBU (1.0 equiv) in dry CH_3CN (2 mL) was added dropwise to a stirred solution of the crude intermediate (**18**–**20**, 1.0 equiv) in dry CH_3CN (8 mL) at 0 °C. After stirring at 0 °C for 15 min, the mixture was poured into dilute HCl solution and the product was extracted with AcOEt. Evaporation of the solvent and purification of the residue by silica gel chromatography (n-hexane/AcOEt, 2:1) yielded the furanone compounds **21**, **7** or **8**.

5.1.4.1. 3-Phenyl-4-(4-sulfonamidophenyl)-2(5*H*)-furanone (6).

Compound **6** was prepared by chlorosulfonylation in chloroform and sulfonamide formation using ammonium hydroxide in ethanol of **21** that was obtained from phenylacetic acid (**13**, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-sulfonamidophenacyl bromide (**15**, 1.03 g, 3.7 mmol, 1.0 equiv) via the intermediate **18** by the method described previously.[36] Colorless needle-like crystals (yield 22%, three steps); mp 248.2–249.5 °C; IR (film) *ν*: 1145, 1320 (SO_2), 1740 (C=O), 3030 (Ar-H), 3230 (N-H) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) *δ*: 5.42 (s, 2H, CH_2), 7.35–7.53 (m, 5H, phenyl-H), 7.42 (br s, 2H, NH_2), 7.52 (d, *J* = 8.6, 2H, 4-sulfonamidophenyl-H2, -H6), 7.85 (d, *J* = 8.6, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m/z*): 314.0495 ($[M-H]^-$, Calcd for $C_{16}H_{12}NO_4S$: 314.0487). Anal. Calcd for $C_{16}H_{13}NO_4S$: C, 60.94; H, 4.16; N, 4.44. Found: C, 61.01; H, 4.02; N, 4.30. IR and ^1H NMR spectral data for **6** were consistent with reported results.[37,38]

5.1.4.2. 3-(4-Methylphenyl)-4-(4-methanesulfonylphenyl)-2(5*H*)-furanone (7).

Compound **7** was synthesized via the intermediate **19** from 4-methylphenylacetic acid (**14**, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-methanesulfonylphenacyl bromide (17, 1.03 g, 3.7 mmol, 1.0 equiv). Yellow needle-like crystals (yield 67%, two steps); mp 174.5–175.4 °C; IR (film) *ν*: 1150, 1320 (SO_2), 1750 (C=O), 3040 (Ar-H), 2930 (C-H) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) *δ*: 2.38 (s, 3H, Ar-CH_3), 3.07 (s, 3H, SO_2CH_3), 5.17 (s, 2H, CH_2), 7.20 (d, *J* = 8.1, 2H, *p*-tolyl-H3, -H5), 7.28 (d, *J* = 8.2, 2H, *p*-tolyl-H2, -H6), 7.52 (dd, *J* = 6.8, 2.0, 2H, methanesulfonylphenyl-H2, -H6), 7.92 (dd, *J* = 6.8, 2.0, 2H, methanesulfonylphenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m/z*): 327.0718 ($[M-H]^-$, Calcd for $C_{18}H_{15}O_4S$: 327.0691). Anal. Calcd for $C_{18}H_{16}O_4S$: C, 65.84; H, 4.91. Found: C, 66.12; H, 5.00. IR and ^1H NMR spectral data for **7** were consistent with reported results.[39]

5.1.4.3. 3-(4-Methylphenyl)-4-(4-sulfonamidophenyl)-2(5*H*)-furanone (8).

Compound **8** was synthesized via the intermediate **20** from 4-methylphenylacetic acid (14, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-sulfonamidophenacyl bromide (16, 1.03 g, 3.7 mmol, 1.0 equiv). Yellow needle-like crystals (yield 67%, two steps); mp 218.5–219.2 °C; IR (film) *ν*: 1140, 1325 (SO_2), 1735 (C=O), 3025 (Ar-H), 3220 (N-H), 2925 (C-H)cm^{-1}; ^1H NMR (DMSO-d_6,

400 MHz) δ: 2.32 (s, 3H, Ar-CH_3), 3.32 (s, 3H, SO_2CH_3), 5.36 (s, 2H, CH_2), 7.22 (d, J = 8.0, 2H, *p*-tolyl-H3, -H5), 7.24 (d, J = 8.0, 2H, *p*-tolyl-H2, -H6), 7.44 (brs, 2H, NH_2), 7.54 (d, J = 8.8, 2H, 4-sulfonamidophenyl-H2, -H6), 7.80 (d, J = 8.8, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m/z*): 328.0694 ([M−H]$^-$, Calcd for $C_{17}H_{14}NO_4S$: 328.0644). Anal. Calcd for $C_{17}H_{15}NO_4S$: C, 61.99; H, 4.59; N, 4.25. Found: C, 62.05; H, 4.68; N, 4.42.

5.2. Membrane permeability assay

Permeabilization of calcein-loaded liposomes was assayed as described previously,[25] with some modifications. Liposomes were prepared using the reversed-phase evaporation method. Egg phosphatidylcholine (PC) (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, dissolved in 1.5 mL of diethyl ether and added to 1 mL of 100 mM calcein-NaOH (pH 7.4). The mixture was then sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A (10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4) (pH 6.8) containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 mL buffer A. A 30 μL aliquot of this suspension was diluted with buffer A to 20 mL and the diluted suspension was then incubated at 30 °C for 10 min in the presence of each compound. The release of calcein from liposomes was determined by measuring the fluorescence intensity at 520 nm (excitation at 490 nm). The EC_{50} value was estimated from non-linear regression plots with the average of triplicate experiments for each compound; Triton X-100 (10 μM) was used to establish the 100% level of calcein release.

5.3. COX-inhibition assay

The inhibitory effect of each compound on COX-1 and COX-2 activity was examined using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA), including purified ovine COX-1 and human recombinant COX-2 according to the manufacturer's procedures.

Acknowledgments

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Supplementary data

Supplementary data (^{1}H NMR spectra of final new compounds for **4** and **8**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.032. These data include MOL files and InChiKeys of the most important compounds described in this article.

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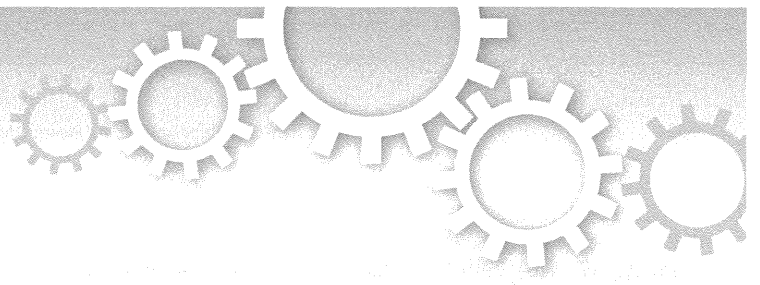

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Superiority of pulmonary administration of mepenzolate bromide over other routes as treatment for chronic obstructive pulmonary disease

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We recently proposed that mepenzolate bromide (mepenzolate) would be therapeutically effective against chronic obstructive pulmonary disease (COPD) due to its both anti-inflammatory and bronchodilatory activities. In this study, we examined the benefits and adverse effects associated with different routes of mepenzolate administration in mice. Oral administration of mepenzolate caused not only bronchodilation but also decreased the severity of elastase-induced pulmonary emphysema; however, compared with the intratracheal route of administration, about 5000 times higher dose was required to achieve this effect. Intravenously or intrarectally administered mepenzolate also showed these pharmacological effects. The intratracheal route of mepenzolate administration, but not other routes, resulted in protective effects against elastase-induced pulmonary damage and bronchodilation at a much lower dose than that which affected defecation and heart rate. These results suggest that the pulmonary route of mepenzolate administration may be superior to other routes (oral, intravenous or intrarectal) to treat COPD patients.

Chronic obstructive pulmonary disease (COPD) is a serious health problem and the most important etiologic factor of which is cigarette smoke (CS). COPD is currently the fourth leading cause of death in the world and its prevalence and mortality rates are steadily increasing[1]. This disease state is defined by a progressive and not fully reversible airflow limitation associated with an abnormal inflammatory response-mediated permanent enlargement of the pulmonary airspace[1–3]. Thus, for the clinical treatment of COPD, it is important not only to improve the airflow limitation by bronchodilation, but also to suppress disease progression by controlling inflammatory processes.

Bronchodilators (β_2-agonists and muscarinic antagonists) are currently used for the treatment of COPD owing to their ameliorating effects on airflow limitation[2,4,5]. Steroids are also used to suppress inflammatory processes in COPD patients; however steroids do not significantly modulate disease progression or mortality[5,6], because the inflammation associated with COPD tends to be resistant to steroid treatment[7]. Thus, the development of new types of anti-inflammatory drugs to treat COPD is paramount.

The number of drugs reaching the marketplace each year is decreasing, mainly due to the unexpected adverse effects of potential drugs being revealed at advanced clinical trial stages. For this reason, we proposed a new strategy for drug discovery and development (drug re-positioning)[8]. In this strategy, compounds with therapeutically beneficial activity are screened from a library of approved medicines to be developed for new indications. The advantage of this approach is that there is a decreased risk for unexpected adverse effects in humans because the safety aspects of these drugs have already been well characterized in humans[8]. From a library of approved medicines, we screened compounds that prevent elastase-induced pulmonary emphysema in mice, and selected mepenzolate bromide (mepenzolate)[9], which is an orally administered muscarinic receptor antagonist used to treat gastrointestinal disorders (such as peptic ulcers and irritable bowel syndrome)[10–12]. We showed that mepenzolate not only exerts an anti-inflammatory effect via a muscarinic receptor-independent mechanism, but also a bronchodilatory effect via a muscarinic receptor-dependent mechanism[9].

Oxidative stress, such as superoxide anion, is believed to play a major role in abnormal inflammation in COPD patients and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays an important role in the production of superoxide anions[13]. The body contains a number of endogenous anti-oxidant proteins such as superoxide dismutase and glutathione S-transferase, with a decrease in these proteins reported to be involved in the pathogenesis of COPD[14,15]. We reported that mepenzolate not only suppressed the elastase-induced production of superoxide anions and NADPH oxidase activation but also stimulated the expression of superoxide dismutase and glutathione S-transferase, suggesting that mepenzolate suppresses elastase-induced pulmonary emphysema via decrease of oxidative stress[9]. Based on these results, we proposed that mepenzolate could serve as a candidate drug for the treatment of COPD.

The route of administration of each particular drug is an important factor to be taken into account when considering its final clinical application. Most muscarinic receptor antagonists currently used for treating COPD patients are administered via the lung[16] because the systemic administration of this type of drug frequently results in adverse effects on cardiac and intestinal functions (such as arrhythmia, heart palpitations and constipation). In this way, we chose the pulmonary route of mepenzolate administration (intratracheal administration or inhalation) in our previous study on mice[9]. On the other hand, since mepenzolate was approved for use as an orally administered drug, the development of this drug to be taken orally for COPD would be more convenient compared to other administration routes. Thus, to determine the appropriate route of mepenzolate administration for possible use by COPD patients, we examined here the effect of different administration routes on this drug's beneficial and adverse effects in mice. When administered intratracheally, mepenzolate showed protective effects on elastase-induced pulmonary damage at a much lower dose than that which affected fecal pellet output and heart rate. With respect to the other administration routes (oral, intravenous and intrarectal), mepenzolate showed protective and adverse effects at similar doses. These results suggest that the pulmonary administration route for mepenzolate may be superior to other routes to treat COPD patients.

Results

Effect of different administration routes of mepenzolate on pulmonary damage and airway resistance. We recently reported that the intratracheal administration or inhalation of mepenzolate suppressed porcine pancreatic elastase (PPE)-induced inflammatory responses, pulmonary emphysema, alteration of lung mechanics, and respiratory dysfunction[9]. As a first step in the present study, we confirmed these effects of intratracheally administered mepenzolate.

As shown in Fig. 1a, the total number of leucocytes and the individual number of neutrophils in bronchoalveolar lavage fluid (BALF), which serve as indicators of pulmonary inflammatory responses, increased after the PPE treatment; this increase was partially suppressed by the simultaneous intratracheal administration of mepenzolate (38 or 190 μg/kg). Histopathological analysis revealed that while PPE administration damaged the alveolar walls and increased mean linear intercept (MLI), this effect could again be partly suppressed by the administration of mepenzolate (38–940 μg/kg; Fig. 1b and c). The alteration of lung mechanics associated with pulmonary emphysema is characterized by a decrease in elastance[17]. PPE treatment decreased both total respiratory system elastance (whole lung elastance, including the bronchi, bronchioles and alveoli) and tissue elastance (elastance of alveoli), both of which were partially restored by simultaneous mepenzolate administration (Fig. 1d). PPE treatment also decreased the $FEV_{0.05}$/FVC ratio (Fig. 1d), which is homologous to the FEV_1/FVC ratio in humans[18,19]. Mepenzolate administration restored the $FEV_{0.05}$/FVC ratio towards control values (Fig. 1d). The bronchodilation activity exerted by mepenzolate was monitored by its inhibitory effect on the increase in airway resistance induced by methacholine[9]. As shown in Fig. 1e, the methacholine-induced increase in airway resistance was completely suppressed by the intratracheal administration of mepenzolate, with the dose required to decrease the airway resistance (0.3 μg/kg) being much lower than that required to protect the pulmonary tissue against PPE-induced damage (38 μg/kg, Fig. 1c). The results in Fig. 1 are thus consistent with those reported previously[9].

We subsequently examined the effects of orally administered mepenzolate on the same parameters as those described above. As shown in Fig. 2a–c, orally administered mepenzolate protected against PPE-induced inflammatory responses and pulmonary emphysema; however, the dose required to achieve this protective effect (190 mg/kg) was much higher than that found when the drug was administered intratracheally (Fig. 1a–c). Orally administered mepenzolate also suppressed PPE-induced alterations of lung mechanics but did not significantly affect respiratory dysfunction (Fig. 2d). The bronchodilatory effect of orally administered mepenzolate was also observed only at higher doses (Fig. 2e) compared with that obtained with intratracheal mepenzolate administration (Fig. 1e). Furthermore, in contrast to the results for intratracheal administration, orally administered mepenzolate showed both bronchodilatory and protective effects against PPE-induced pulmonary disorders at roughly similar doses (Fig. 2).

We also examined the effects of intravenously administered mepenzolate. As shown in Fig. 3a–c, this route of mepenzolate administration (10 μg/kg) protected against PPE-induced inflammatory responses and pulmonary emphysema. Compared to the intratracheal administration, although the effective dose was slightly lower via the intravenous route, the extent of amelioration was not as apparent (Fig. 3a–c). Furthermore, intravenous administration of the highest dose of mepenzolate tested for this route (100 μg/kg) did not protect against PPE-induced pulmonary damage (Fig. 3a and c), nor did it significantly restore the lung mechanics and respiratory function, both of which were affected by the PPE treatment (Fig. 3d). These results demonstrate that intravenously administered mepenzolate is not as effective against PPE-induced pulmonary damage as that achieved via the intratracheally administered route. On the other hand, almost complete inhibition of the methacholine-induced increase in airway resistance was observed with the intravenous administration of mepenzolate (Fig. 3e). These results suggest that the protective effects of mepenzolate against PPE-induced pulmonary damage and its bronchodilatory effect are independent of each other.

Monitoring of the mepenzolate level in blood and tissue after administration of the drug via different routes. High performance liquid chromatography (HPLC) analysis was used to determine the level of mepenzolate in plasma and tissue. We initially examined the plasma level of mepenzolate after its intravenous administration, with the detected levels of the drug increasing in a dose-dependent manner (Fig. 4a). Examination of the time-course profile showed that mepenzolate was clearly detectable at 1 min, significantly reduced after 5 min, and undetectable 30 min following its intravenous administration (Fig. 4b), suggesting that mepenzolate is very unstable in blood. We then performed similar analyses to determine plasma mepenzolate levels after oral administration of the drug. As shown in Fig. 4c, mepenzolate could be detected in the plasma only when a very high dose (940 mg/kg) of the drug was administered via this route. Furthermore, the peak level was achieved 30 min after oral administration (Fig. 4d). In contrast, when mepenzolate was administered via the intratracheal route, it could be detected at a relatively lower dose (10 mg/kg) (Fig. 4e). Furthermore, the detection was very rapidly (at 1 min) (Fig. 4f). These results suggests that the efficiency of absorption into the circulation is higher for the intratracheal route of administration than the oral route. We also tried to detect mepenzolate in the lung tissue of treated mice, with the drug detected following

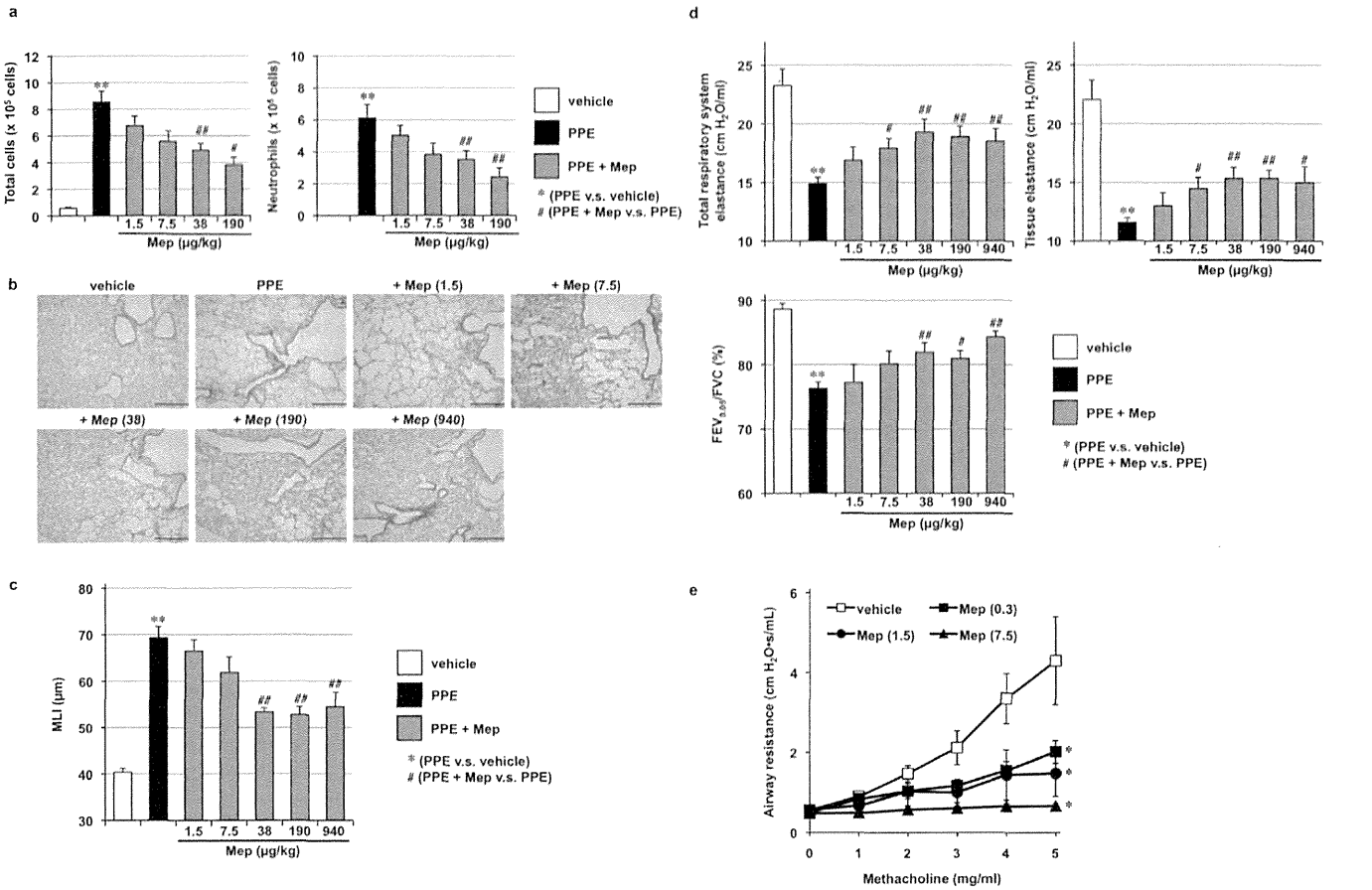

Figure 1 | **Effect of intratracheal administration of mepenzolate on PPE-induced pulmonary damage and methacholine-induced airway constriction.** Mice were treated with or without (vehicle) PPE (15 U/kg) once only on day 0 (a–d). The indicated doses (μg/kg) of mepenzolate (Mep) were administered intratracheally once only (a) or once daily for 12 days (from day 0 to day 11) (b–d). Twenty-four hours after the PPE administration, BALF was prepared and the total cell number and the number of neutrophils were determined as described in the Materials and Methods (a). Sections of pulmonary tissue were prepared on day 14 and subjected to histopathological examination (H & E staining) (scale bar, 500 μm) (b). Airspace size was estimated by determining the MLI as described in the Materials and Methods (c). Total respiratory system elastance, tissue elastance, and $FEV_{0.05}$/FVC were determined on day 14 as described in the Materials and Methods (d). Indicated doses (μg/kg) of mepenzolate (Mep) were administered intratracheally. After 1 h, mice were exposed to nebulized methacholine 5 times and airway resistance was determined after each methacholine challenge as described in the Materials and Methods (e). Values represent mean ± S.E.M. (n = 3–10). * or $^{\#}$ $P < 0.05$; ** or $^{\#\#}$ $P < 0.01$.

administration via the intratracheal route (Fig. 4g), but not for orally or intravenously administered drug (data not shown). The results in Fig. 4g also showed that most of intratracheally administered mepenzolate disappeared from the lung within 30 min.

Effect of intrarectally administered mepenzolate on pulmonary damage and airway resistance. It has been reported that, compared to the oral route of administration, the intrarectal route for some drugs results in a much higher uptake efficiency into the circulation due to the circumvention of drug inactivation within the gastrointestinal tract and the first-pass effect, or the higher efficiency of absorption via the rectum compared with the small intestine[20,21]. For these reasons, we examined the effect of intrarectally administered mepenzolate on PPE-induced pulmonary damage and airway resistance. As shown in Fig. 5a–c, intrarectally administered mepenzolate showed a protective effect against PPE-induced pulmonary damage at doses of 1.5 or 7.5 mg/kg, which are much lower than that required in the case of oral administration (Fig. 2a–c). Similar results were observed with respect to the PPE-induced alteration of lung mechanics and respiratory dysfunction; however, the amelioration of respiratory function by intrarectally administered mepenzolate was not statistically significant (Fig. 5d). As shown in Fig. 5e, intrarectally administered mepenzolate suppressed the methacholine-induced increase in airway resistance at lower doses to that seen in response to oral administration of the drug (Fig. 2e).

We also determined the plasma level of mepenzolate after the intrarectal administration of this drug. The dose-response and time-course profiles (Fig. 5f and g) revealed that the absorption into the circulation of intrarectally administered mepenzolate is much more efficient and rapid than that seen with orally administered drug (Fig. 4c and d). The results in Fig. 5 thus suggest that the intrarectal route of mepenzolate administration is more effective than the oral route due to the lower effective doses required.

We also examined the effect of different routes of mepenzolate administration on CS-induced lung inflammatory responses. As shown in Fig. 6a, the total number of leucocytes and the individual number of macrophages in BALF increased after the CS treatment and this increase was suppressed by the simultaneous intratracheal administration of mepenzolate (38 or 190 μg/kg). Similar suppression was observed with oral, intravenous or intrarectal administration of mepenzolate (Fig. 6b–d), however, the oral administration required much higher dose of mepenzolate than the intratracheal administration (Fig. 6a, b). Furthermore, the extent of suppression was not so apparent with the intravenous or intrarectal administration as the intratracheal administration and the suppression of