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Human pharmacology of naproxen sodium

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ADAPT, Alzheimer's Disease Anti-Inflammatory Prevention Trial; AA, arachidonic acid; COX, cyclooxygenase; LPS, lipopolysaccharide; PG, prostaglandin; TX, thromboxane; tNSAIDs, traditional nonsteroidal antiinflammatory drugs.
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Abstract

We compared the variability in degree and recovery from steady-state inhibition of cyclooxygenase (COX)-1 and COX-2 *ex vivo* and *in vivo* and platelet aggregation by naproxen sodium 220mg BID *versus* 440mg BID and low-dose aspirin in healthy subjects. Six healthy subjects received consecutively naproxen sodium 220mg BID, 440mg BID and aspirin (100mg daily) for 6 days, separated by washout periods of two weeks. COX-1 and COX-2 inhibition was determined using *ex vivo* and *in vivo* indices of enzymatic activity: i) the measurement of serum thromboxane (TX)B₂ levels and whole blood lipopolysaccharide-stimulated prostaglandin (PG)E₂ levels, markers of COX-1 in platelets and COX-2 in monocytes, respectively, ii) the measurement of urinary 11-dehydro-TXB₂ and 2,3-dinor-6-keto-PGF₁α levels, markers of systemic TXA₂ biosynthesis (mostly COX-1 derived) and prostacyclin biosynthesis (mostly COX-2 derived), respectively. Arachidonic acid (AA)-induced platelet aggregation was also studied. The maximal inhibition of platelet COX-1 (95.9±5.1 and 99.2±0.4%) and AA-induced platelet aggregation (92±3.5 and 93.7±1.5%) obtained at 2 h after dosing with naproxen sodium 220 and 440mg BID, respectively, was indistinguishable from aspirin but at 12 and 24 h after dosing, we detected marked variability which was higher with naproxen sodium 220mg than 440mg BID. Assessment of the ratio of inhibition of urinary 11-dehydro-TXB₂ *versus* 2,3-dinor-6-keto-PGF₁α showed that the treatments caused a more profound inhibition of TXA₂ than prostacyclin biosynthesis *in vivo* throughout dosing interval. In conclusion, neither of the 2 naproxen doses mimed the persistent and complete inhibition of platelet COX-1 activity obtained by aspirin but marked heterogeneity was mitigated by the higher dose of the drug.
Introduction

Recent lines of evidence sustain that aspirin cardioprotection is the result of an almost complete and persistent suppression of platelet thromboxane(TXA) biosynthesis throughout dosing interval, for irreversible inhibition of platelet cyclooxygenase(COX)-1 activity (Patrignani et al., 1982; Patrono et al., 2005). In fact, even tiny concentrations of TXA can cause platelet activation (Maree et al., 2005; Pulcinelli et al., 2005; Minuz et al., 2006; Sciulli et al., 2006). All other reversible COX inhibitors, i.e. traditional nonsteroidal antiinflammatory drugs(tNSAIDs) and NSAIDs selective for COX-2(coxibs), cause an incomplete and intermittent inhibition of platelet COX-1 insufficient to afford cardioprotection (Patrano et al., 2001; Patrono et al., 2005). In a recent meta-analysis of randomized clinical trials, it has been reported that high dose of the tNSAID naproxen – with a balanced inhibitory effect on COX-1 and COX-2 (Capone et al., 2007) – confers a smaller cardiovascular risk than high dose regimens of some tNSAIDs, such as diclofenac and ibuprofen (Kearney et al., 2006). The meta-analysis of Kearney et al. could not establish whether high-dose naproxen confers a small cardiovascular benefit because of the substantial statistical uncertainty surrounding the point estimate of rate ratio versus placebo. However, it is reasonable to assume that the sustained inhibition of platelet COX-1 ex vivo, in some but not all individuals, by naproxen administration at high doses BID, continuously and regularly (Capone et al., 2004), as it occurs inside the rigour of randomized clinical trials, might be associated with a modest benefit. Differently from aspirin, the persistence of the inhibitory effect on platelet COX-1 by naproxen can be affected in real-life for reduced compliance. This is consistent with the uncertainty of the results of epidemiological studies, showing that naproxen is neutral or somewhat cardioprotective (Hernandez-Diaz et al., 2006; McGgettigan and Henry, 2006). The possible small benefit of naproxen might be undermined by the occurrence of intra- and intersubject variability in degree and duration of COX-1 and −2
inhibition which might lead to a time-dependent divergence in the consequences of suppressing one versus the other COX enzyme (Grosser et al., 2006). Genetic sources of variance, such as polymorphisms detected in COX-1 and cytochrome P450(CYP) 2C9 (the principal metabolizing enzyme of a wide range of coxibs and tNSAIDs, such as naproxen) have been suggested to participate in marked variation in individual response to the COX-2 inhibitors, rofecoxib and celecoxib in healthy subjects (Fries et al., 2006).

Different formulations of naproxen - which differ in the pattern of absorption - are available. Naproxen sodium – characterized by a more rapid absorption from the gastrointestinal tract (Goodman & Gilman’s, 2006) - has recently been in the spotlight because it was administered in the prematurely terminated placebo-controlled trial Alzheimer's Disease Anti-Inflammatory Prevention Trial (ADAPT Trial, 2006). The trial involved three treatment arms: low-dose naproxen sodium (220 mg BID), celecoxib (200 mg BID) and placebo, acting as a control. The termination of both the celecoxib and naproxen arms of the ADAPT trial "reflected the ADAPT investigators' reluctance to imply, by continuing the trial, that naproxen was safer than celecoxib when ADAPT data did not support this conclusion" (ADAPT Trial, 2006).

Data of human pharmacology of low-dose naproxen are not available. Thus, in the present study we explored the variability in degree and recovery from steady-state inhibition of COX-1 and COX-2 both ex vivo and in vivo by 2 therapeutic doses of naproxen sodium(220 and 440 mgBID) versus aspirin(100 mg daily) in healthy subjects. These effects were compared with the impact on platelet function by the different therapies.
Methods

Study design, treatments and assessment

The study protocol was approved by the Ethic Committee of “G. d’Annunzio” University. Written informed consent was obtained from 6 healthy subjects. The subjects were between 23 and 30 years of age and within 30% of ideal body weight and had an unremarkable medical history, physical examination, and routine haematological and biochemical screen. Smokers and subjects with a bleeding disorder, an allergy to aspirin or any other NSAID, or a history of any gastrointestinal or cerebrovascular disease were excluded. Subjects abstained from the use of aspirin and other NSAIDs for at least two weeks before enrollment. The 6 healthy subjects received consecutively naproxen sodium (Roche) 220mg BID, naproxen sodium 440mg BID and aspirin (Bayer) 100 mg daily for 6 days, separated by washout periods of at least two weeks. Blood samples were collected before dosing and on the 6th day after the last dose of the different treatments (at 1, 2, 5, 8, 12 and 24 h after dosing) to assess the inhibition of serum TXB2 (a capacity index of platelet COX-1 activity) (Patrono et al., 1980), lipopolysaccharide (LPS)-induced prostaglandin (PG) E2 production (a capacity index of monocyte COX-2 activity) (Patrignani et al., 1994) and platelet aggregation induced by arachidonic acid (AA) (2 mM) in platelet-rich plasma (Pedersen and FitzGerald, 1985). Urinary samples were collected before treatment (from 8pm to 8am) and on the 6th day after the last dose of the different treatments at 8 am-12 am, 12 am-4 pm, 4 pm-8 pm and 8 pm-8 am to evaluate the urinary excretion of 11-dehydro-TXB2 (a major enzymatic metabolite of TXB2 that is an index of systemic TXA2 biosynthesis in vivo mainly platelet COX-1 derived) (Ciabattoni et al., 1989; Catella and FitzGerald, 1987) and of 2,3-dinor-6-keto PGF1α (a major enzymatic metabolite of prostacyclin that is an index of systemic prostacyclin biosynthesis, mainly vascular COX-2 derived) (FitzGerald et al., 1983; Catella-Lawson et al., 1999; McAdam et al., 1999). Immunoreactive TXB2, PGE2, 11-dehydro-TXB2 and 2,3-dinor-6-keto
PGF$_{1\alpha}$ were measured (Patrignani et al., 1994; Ciabattoni et al., 1987; Minuz et al., 1988). Blood concentrations of naproxen were also evaluated (Santini et al., 1996; Slattery and Levy, 1979).

**Platelet TXB$_2$ production in whole blood**
Duplicate whole blood samples (3 ml) were collected by venipuncture into glass vacutainers containing no anticoagulant and immediately allowed to clot for 1 h at 37°C and serum was collected after centrifugation at 3000 rpm for 10 minutes and stored at –80°C until assayed for TXB$_2$ (Patrignani et al., 1980).

**LPS-stimulated PGE$_2$ production in whole blood**
Duplicate 1-ml aliquots of heparinized (10 U/ml) blood samples were incubated in polypropylene tubes (containing 50 µg of dry aspirin) for 24 h, at 37°C, in the absence or the presence of LPS, (Escherichia coli 026:B6; Sigma Chemical Co., St Louis, MO) 10 µg/ml. Plasma was separated by centrifugation (at 2000 rpm for 10 minutes) and kept at –80°C until assayed for PGE$_2$ (Patrignani et al., 1994).

**In vitro study**
Naproxen sodium (0.02-90 µM), dissolved in saline, was incubated with 1-ml aliquots of human whole blood withdrawn from the same subjects in the absence and in the presence of 10 IU/ml of sodium heparin for 1 h or 24 h with LPS (10 µg/ml), respectively. Serum and plasma samples were assayed for TXB$_2$ and PGE$_2$, respectively (Patrignani et al., 1980; Patrignani et al., 1994).

**Eicosanoid analyses**
Urinary 11-dehydro-TXB$_2$ and 2,3-dinor-6-keto-PGF$_{1\alpha}$, plasma PGE$_2$ and serum TXB$_2$ were assessed by previously described and validated radioimmunoassays (Patrignani et al., 1980; Patrignani et al., 1994; Ciabattoni et al., 1987; Minuz et al., 1988).
Naproxen blood levels

Aliquots of 5µl of serum samples were added to 195 µl of methanol/water(50:50,v/v) and injected directly into a Nova-Pak C18 column(Waters, Mildfort, MA) of a Beckman System Gold HPLC (Santini et al., 1996; Slattery and Levy, 1979). The mobile phase consisted of acetonitrile:acetic acid(100:0.1,v/v) and water:acetic acid(100:0.1,v/v), as follows: 60 and 40%, respectively, at a flow rate of 1 ml/min. Absorbance was assessed at 250nm. Naproxen eluted with a retention time of 5.5 min.

Statistical Analysis

The data were expressed as mean ± standard deviation(SD). The primary end-point of the present study was the assessment of serum TXB2 levels ex vivo; the secondary end-points were the assessment of turbidometric platelet aggregation induced by AA, the urinary excretion of 11-dehydro-TXB2 and 2,3-dinor-6-keto PGF1α and LPS-induced PGE2 production ex vivo. The primary hypothesis was that the administration of naproxen 200 mg BID would cause a lower inhibition of platelet COX-1 activity versus naproxen 400 mg BID and aspirin 100 mg daily, as assessed by the measurement of serum TXB2 on day 6 of therapy. It was anticipated on the basis of previous studies that a sample size of 6 (6 per treatment) would afford a power in excess of 90 percent to detect a difference of 20 percent or greater in serum TXB2 measurements with two-tailed tests of the hypothesis associated with a type I error rate of less than 0.05 for all the main effects (Catella-Lawson et al., 2001). Values were compared by means of an ANOVA model for repeated measures, using the PROC MIXED of SAS. Due to marked heterogeneity in response to naproxen, primarily at 12 and 24 h after dosing, data did not pass normality test(by the method Kolmogorov and Smirnov) in some occasions, thus, we analysed the data also by nonparametric tests, i.e. Friedman test and Wilcoxon matched pairs test. A probability value of P<0.05 was considered to be statistically significant. Concentration-response curves were fitted and IC50(drug concentration required
for obtaining 50% of inhibition) values were analyzed with PRISM (GraphPad, San Diego, CA).
Results

The primary objective of the study was to compare the degree of steady-state inhibition and time-dependent recovery of platelet COX-1 activity *ex vivo* by 2 analgesic doses of naproxen sodium, i.e. 220 and 440 mg BID, *versus* a cardioprotective dose of aspirin, i.e. 100 mg daily. Serum TXB$_2$ levels assessed in the 6 subjects, on 3 consecutive free-drug occasions, i.e. before treatment with naproxen sodium 220 mg BID and 440 mg BID or aspirin 100 mg daily, were not significantly different (277±127, 322±197 and 323±151 ng/ml, respectively). Two h after the last administration of naproxen sodium 220 mg and 440 mg, serum TXB$_2$ levels were comparably reduced by 95.9±5.1 and 99.2±0.4%, respectively (P<0.01 *versus* pre-drug values). These values were not significantly different from the average of inhibition detected at 1 and 24 h after the last dose of aspirin, i.e. 99.1±0.9 and 98.7±1%, respectively (Figure 1A, Table). We set the lowest limit of inhibition of platelet COX-1 activity by aspirin at 97% (indicated by the broken line, in Figure 1A), as estimated by mean minus 2SD of values measured at 1 and 24 h after dosing.

Differently from aspirin, the suppression of platelet COX-1 activity by naproxen sodium recovered in a time- and dose-dependent fashion (Figure 1A, Table). In fact, at 12 and 24 h after dosing with naproxen sodium 220 mg BID, the degree of inhibition of serum TXB$_2$ was significantly (P<0.01) lower than that detected at the corresponding times after naproxen 440 mg BID and after aspirin as well (Figure 1A and Table). The inhibition of serum TXB$_2$ by the administration of naproxen sodium 440 mg BID was significantly (P<0.05) different from aspirin only at 12 h after dosing but the use of a nonparametric test showed a statistically significant divergence at 24 h as well (Figure 1A and Table).

In Figure 1 B, the values of serum TXB$_2$ detected at the different times after dosing with the 3 treatments were reported. Differently from aspirin, marked heterogeneity in serum TXB$_2$ generation was detected after dosing with naproxen 220 and 440 mg BID. The frequency of
samples with serum TXB₂ levels higher than 10 ng/ml [corresponding to the upper extreme value of TXA₂ generated in whole blood of healthy subjects with complete inhibition of platelet COX-1 activity by aspirin (Sciulli et al., 2006)] increased in a time-dependent fashion. After naproxen 220 and 440 mg BID, serum TXB₂ values started to move away from aspirin response, in a statistically significant fashion, at 5 and 12 h after dosing, respectively (Figure 1B).

In order to verify whether time-dependent recovery of platelet COX-1 activity from steady-state inhibition by naproxen translated into a functional effect, we studied platelet aggregation induced by AA. As shown in Figure 1C, aspirin caused a statistically significant (P < 0.01 versus pre-drug values) reduction of AA-induced platelet aggregation at 1 h which persisted up to 24 h after dosing. None of subjects responded to AA after dosing with aspirin 100 mg for 6 days. In contrast, at 12 and 24 h after naproxen sodium 220 mg and at 24 h after naproxen sodium 440 mg, platelet function was not significantly reduced versus pre-drug values. The inhibition of AA-induced platelet aggregation recorded at 24 h after naproxen sodium 220 mg BID, but not after naproxen sodium 440 mg BID, was significantly (P < 0.05) different from aspirin (Figure 1C). At 24 h after naproxen 220 mg, naproxen 440 mg and aspirin, the number of subjects who responded to AA with a complete aggregation in platelet-rich plasma was 4/6, 1/6 and 0/6 subjects, respectively. Interestingly, full platelet aggregation was detected in platelet-rich plasma samples obtained from whole blood that generated TXB₂ concentrations ≥ 50 ng/ml when allowed to clot for 1 h at 37°C.

Then we verified whether the intersubject variability in the response to naproxen was driven by fluctuations of circulating drug levels. As shown in Figure 2A, at each time studied after naproxen 440 mg BID, circulating drug levels were significantly higher than those detected after naproxen 220 mg BID. Individual circulating concentrations and the corresponding degree of COX-1 inhibition measured ex vivo at each time-point were reported on the same
graph depicting the sigmoidal dose-response curve obtained in vitro (Figure 2B). Naproxen inhibited platelet COX-1 activity in vitro in a concentration-dependent fashion, with an IC₅₀ value of 5.8 µg/ml and IC₉₇ value (97% is the lowest limit of platelet COX-1 inhibition by aspirin) of 70 µg/ml (Figure 2B). As shown in the same figure, the pharmacokinetic/pharmacodynamic relationship after dosing with naproxen fitted the concentration-response curve for inhibition of platelet COX-1 obtained in vitro. However, marked heterogeneity in drug response was detected at lower circulating drug levels.

We assessed the impact of chronic dosing with naproxen sodium 220 and 440 mg BID and aspirin 100 mg daily on urinary 11-dehydro-TXB₂, a biomarker of systemic biosynthesis of TXA₂ in vivo - prominently of platelet origin (Catella and FitzGerald, 1987). At pre-drug on 3 different occasions, i.e. before naproxen sodium 220 mg, 440 mg and aspirin, overnight urinary excretion of 11-dehydro-TXB₂ did not differ, in a statistically significant fashion (400±138, 431±108 and 428±114 pg/mg creatinine, respectively). On day 6, after the last administration of aspirin and naproxen sodium 220 and 440 mg, systemic TXA₂ biosynthesis was profoundly and persistently depressed versus overnight pre-drug levels (P<0.01). As shown in Figure 3A, in urine samples collected from 12 to 24 h after dosing with naproxen 220 mg BID, the degree of inhibition of 11-dehydro-TXB₂ was significantly (P<0.01) lower than that detected at 0-4, 4-8, 8-12 and 12-24 after the last administration of naproxen 440 mg BID. The degree of inhibition of the urinary excretion of 11-dehydro-TXB₂ by naproxen sodium 440 mg - recorded at each period of collection - was slightly higher versus the other treatments but it was significantly (P<0.01) different only versus the inhibition detected in the urine samples collected from 12 to 24 h after dosing with naproxen sodium 220 mg, and from 4 to 12 h after dosing with aspirin (Figure 3A). Thus, similarly to the results obtained ex vivo, naproxen dose elevation homogenized the inhibitory
effect on TXA2 generation in vivo. Differently from the results obtained ex vivo, it appeared to be a dose-dependence for inhibition of urinary 11-dehydro-TXB2 by naproxen.

In the same urine collections, we assessed the levels of 2,3-dinor-6-keto-PGFiα, an index of systemic prostacyclin biosynthesis (Catella-Lawson et al., 1999; McAdam et al., 1999). At pre-drug on 3 different occasions, i.e. before naproxen sodium 220 mg, 440 mg and aspirin, overnight urinary 2,3-dinor-6-keto-PGFiα did not differ, in a statistically significant fashion (83±25, 100±44 and 79±20 pg/mg creatinine, respectively). Aspirin did not significantly affect systemic prostacyclin biosynthesis (Figure 3B). The urinary excretion of the prostacyclin metabolite was significantly reduced by the treatment with naproxen sodium 220 and 440 mg (P<0.01 versus overnight pre-drug values). Although the average degree of inhibition of the urinary excretion of 2,3-dinor-6-keto-PGFiα after dosing with naproxen sodium 440 mg was higher versus 220 mg BID, the differences between the 2 treatments were statistically significant only in urine samples collected from 8 to 12 h. However, the proportion of urine samples with degree of inhibition of prostacyclin biosynthesis > 50% detected in the 24 h period was significantly (P<0.01) higher in subjects treated with naproxen 440 mg (18/24) than with 220 mg (8/24) (Figure 3B).

The assessment of LPS-induced whole blood PGE2 generation, a marker to predict drug effects (analgesia) in humans, was performed to verify whether naproxen sodium 220 and 440 mg BID were comparable doses for efficacy. In fact, it has been reported that IC80 evaluated in vitro correlates directly with the analgesic plasma concentrations of different COX inhibitors (Huntjens et al., 2005). Naproxen inhibited LPS-induced PGE2 generation in vitro in a concentration-dependent manner with IC50 and IC80 values of 15 and 30 µg/ml, respectively (Figure 2B). As shown in Figure 2A, at both naproxen doses, blood levels were almost always > IC80 for COX-2 inhibition in vitro throughout the 12 h of dosing interval. This was confirmed by the results obtained ex vivo showing that the proportion of samples
with PGE2 levels reduced at clinically relevant ranges (i.e. >80%) were 18/24 and 24/24 after naproxen sodium 220 mg and 440 mg, respectively (Figure 4).

We verified the occurrence of time-dependent divergence of COX selectivity achieved *ex vivo* and *in vivo* by the 2 naproxen doses. The degree of selectivity for COX-2 by naproxen *in vitro* - a chemical property of the drug - expressed as ratio of IC50 values showed that the drug is 2.6-fold more potent for COX-1 than COX-2 (Figure 2B). However, pharmacokinetic and pharmacodynamic variations between individuals would be expected to affect the degree of COX selectivity actually attained in humans, which can be described by the ratio of COX-1 inhibition *versus* COX-2 inhibition at any given plasma concentration. When the degree of selectivity attained in subjects was estimated using this measure, naproxen sodium at 220 mg BID and 440 mg BID inhibited COX-1 and COX-2 *ex vivo* similarly throughout the 24 h. In fact, the ratio of COX-1 inhibition *versus* COX-2 inhibition *ex vivo* was constantly at around 1 (Figure 5A). Differently, low-dose aspirin was selective for COX-1 *ex vivo* (Figure 5A). The actual COX selectivity achieved *in vivo* was determined by estimating the ratio of urinary 11-dehydro-TXB2 inhibition (mostly COX-1 derived) (Catella and FitzGerald, 1987) *versus* 2,3-dinor-6-keto-PGF1α inhibition (mostly COX-2 derived) (Catella-Lawson et al., 1999; McAdam et al., 1999). Using this measure, we found that all treatments were associated with a ratio > 1 suggesting a more profound inhibitory effect towards TXA2 than prostacyclin biosynthesis *in vivo* (Figure 5B). However, the highest ratios were detected after dosing with aspirin which is consistent with its preferential, even if not exclusive, selectivity towards platelet COX-1, at low doses. We found that the ratio was significantly (P<0.05) higher in urine collected from 4 to 24 h *versus* the first 4 h because urinary 11-dehydro-TXB2 was persistently reduced by aspirin while prostacyclin metabolite excretion was reversibly reduced in some subjects. In urine samples collected after dosing with naproxen 220 and 440 mg BID, inhibitory ratio of TXA2 and prostacyclin biosynthesis *in vivo* was always > 1 suggesting that
the 2 treatments were associated with a constant suppression of the 2 mediators throughout
the interval among doses (Figure 5B).
Discussion

Results of human pharmacology have thrown some light on the phenotypes induced by aspirin and selective inhibitors of COX-2 translating into opposite risk of thrombotic events: reduced risk by the former and increased risk by the latter (Rodriguez and Patrignani, 2006). Aspirin affords cardioprotection because of 2 concurrent effects on platelet COX-1, the only COX-isoform expressed in mature platelets (Patrignani et al., 1999): i) complete inhibition and ii) persistence of this effect throughout dosing interval (Patrono et al., 2005). Partial or no reduction of platelet COX-1 by selective COX-2 inhibitors is unable to counter the thrombotic risk associated with profound suppression (>60%) of COX-2-dependent prostacyclin - which acts as a constraint on endogenous mediators of platelet activation, hypertension, atherogenesis and cardiac dysfunction (Grosser et al., 2006).

In the present study, we showed that 97% is the lowest limit of inhibition of platelet COX-1 activity by aspirin. This value corresponds to the mean minus 2SD of serum TXB2 inhibition detected in 40 samples obtained from healthy volunteers treated with aspirin 100 mg daily, participating in clinical studies that we previously performed (Capone et al., 2004; Capone et al., 2005). Importantly, the occurrence of >97% suppression of platelet COX-1 activity, was always associated with levels of serum TXB2 ≤ 10 ng/ml and a complete suppression of AA-induced platelet aggregation (Sciulli et al., 2006 and the present study).

Varied scenarios are associated with different tNSAIDs because they are a cluster of compounds with a wide spectrum of COX selectivity, as assessed in vitro using whole blood assays (Patrone et al., 1980; Patrignani et al., 1994), ranging from drugs with balanced inhibitory effect on COX-1 and COX-2 (e.g. profen and naproxen) to selective inhibitors of COX-2, such as diclofenac (Capone et al., 2007). For the tNSAIDs causing balanced, profound inhibition of the activity of both COX-isoforms, the thrombotic risk associated with the suppression of prostacyclin can be neutralized when platelet COX-1 is inhibited to
functional range, i.e. >97% (Rodriguez and Patrignani, 2006). This effect can be realized at peak plasma concentrations because they are often administered at higher doses than the minimum efficacious dose required for analgesic/antiinflammatory effects (Capone et al., 2007). Although it has been shown that plasma levels corresponding to the IC\textsubscript{80} of exposure-response relationships \textit{in vitro} for inhibition of LPS-stimulated PGE\textsubscript{2} in whole blood are associated with efficacy (Huntjens et al., 2005), still the selection of doses are driven by clinical end-points that do not have the power to detect precisely differences among doses (Patrono et al., 2001).

The reversible nature of the interaction of tNSAIDs with COX-1, leads to a time-dependent recovery of platelet TXA\textsubscript{2} generation from steady-state inhibition. This translates into an intermittent suppression of platelet COX-1 - throughout dosing interval – which is inconsistent with cardioprotection. Time-dependent recovery of platelet COX-1 activity can be restrained by the administration of these drugs at intervals shorter than the pharmacokinetic half-life. This is realized by naproxen -which has a pharmacokinetic half-life >12 h (Goodman & Gilman’s, 2006)- administered at high doses BID. However, we showed that naproxen 500 mg BID suppresses platelet COX-1 at degree and duration comparable to aspirin only in some, but not all, subjects (Capone et al., 2004) which is compatible with no increased risk or a small reduced risk shown in observational studies (Hernandez-Diaz et al., 2006; McGettigan and Henry, 2006). However, the prematurely terminated ADAPT trial (ADAPT Trial, 2006) led some to speculate that the modest cardioprotection detected with naproxen 500 mg BID (Kearney et al., 2006; Hernandez-Diaz et al., 2006; McGettigan and Henry, 2006) might be dissipated at lower doses. To address this issue we performed a clinical study comparing the degree and duration of platelet COX-1 inhibition by naproxen sodium 220 \textit{versus} 440 mg BID and aspirin 100 mg daily. Our results showed that neither of the 2 naproxen doses mimed the inhibition of platelet COX-1 activity achieved by aspirin with the
major differences on the persistence of the inhibitory effect. Importantly, the maximal inhibition of platelet COX-1 and AA-induced platelet aggregation obtained at 2 h after dosing with naproxen sodium 220 and 440 mg BID was indistinguishable, in a statistically significant fashion, but at 12 and 24 h after dosing, we detected marked variability which was higher with naproxen sodium 220 mg BID than 440 mg BID. At these time-points after both naproxen doses, serum TXB2 levels were frequently higher than 10 ng/ml which corresponds to the highest value detectable in healthy subjects when complete inhibition of platelet COX-1 occurs (Sciulli et al., 2006). Importantly, time-dependent recovery of TXA2 biosynthesis from steady-state inhibition by naproxen 220 mg BID was detected both ex vivo and in vivo and was associated with complete restoration of AA-induced platelet aggregation in some individuals. Large size studies will be required to determine the different sources of variance participating in heterogeneity of platelet COX-1 inhibition by naproxen sodium, such as the occurrence of polymorphisms in COX-1 and in CYP2C9, a major pathway of naproxen metabolism (Goodman & Gilman’s, 2006). However, we showed that naproxen blood levels (total: bound and unbound to plasma proteins) increased in a dose-dependent fashion and had comparable coefficients of variation(CV%) at each time after dosing with naproxen 220 and 440 mg. This suggests that variability in total drug levels is not the cause of marked heterogeneity in drug response. Since higher variability in COX-1 inhibition was detected at lower circulating levels, we suppose the occurrence of intersubject variability in unbound fraction of naproxen. Naproxen is extensively(approximately 99.7%) bound to plasma protein (Goodman & Gilman’s, 2006), thus, small intersubject variability in unbound drug concentrations may translate into a detectable effect when they are lower than the minimal effective concentration required for a full pharmacodynamic effect. In fact, variability in platelet COX-1 inhibition increased when circulating blood levels lowered from 70 µg/ml
(Figure 2A), corresponding to the minimal concentration required for complete inhibition by naproxen.

The extensive binding of naproxen to plasma proteins (Goodman & Gilman’s, 2006) might restrict the compound largely to the plasma compartment. This mirrors the finding that naproxen sodium 220 mg caused an inhibition of the systemic biosynthesis of TXA₂ comparable to aspirin 100 mg, a preferential inhibitor of platelet COX-1 (Cipollone et al., 1997) acting mainly in the pre-systemic circulation (Pedersen and FitzGerald, 1984). Differently, naproxen sodium 440 mg BID caused a slightly higher suppression of 11-dehydro-TXB₂. As the metabolite reflects TXA₂ formation from either COX-1 and COX-2 (Cipollone et al., 1997), this raises the possibility of dose-dependent effect on COX-2-derived TXA₂ such as might derive from macrophages and/or vascular cells. This is coherent with the results of dose-dependent inhibition of the urinary excretion of the prostacyclin metabolite by naproxen. The lower inhibition of prostacyclin by naproxen sodium 220 mg may mitigate the possible cardiovascular risk associated with incomplete suppression of platelet COX-1 at dosing interval. This was verified by estimating the ratio of urinary 11-dehydro-TXB₂ inhibition versus 2,3-dinor-6-keto-PGF₁α inhibition (Catella-Lawson et al., 1999; McAdam et al., 1999). We found that the 2 doses of naproxen were associated with a ratio > 1 which suggests that the inhibitory effect was higher versus TXA₂ than prostacyclin in vivo throughout dosing interval.

Altogether, the results of clinical pharmacology of naproxen sodium 220 mg BID are implausible with increased cardiovascular risk associated with its chronic administration. Consistently with our previous work (Capone et al., 2004), we showed that, even within the context of a controlled and well-monitored study, the chronic administration of high dose of naproxen sodium gets into functionally relevant range of inhibition of platelet COX-1 activity ex vivo at the end of the dosing interval, in some but not all subjects. A finding explaining the
fluctuating results found in clinical studies showing that naproxen is neutral or somewhat cardioprotective (Hernandez-Diaz et al., 2006; McGettigan and Henry, 2006). The marked intersubject variability of platelet COX-1 inhibition by the drug can be reduced but not avoided by increasing the dose. Our results - corroborating previous findings of marked interindividual variability in response to COX inhibitors (Fries et al., 2006) - should accelerate the process of development and validation of genetic and/or biochemical markers to identify patients most likely to benefit or suffer from drug exposure.

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Footnotes:

Marta L Capone and Stefania Tacconelli contributed equally.

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Disclosures:

All the authors do not have any conflict of interest.
Legends for figures

Figure 1. Comparison of degree and duration of steady-state inhibition of COX-1 activity and platelet function *ex vivo* by naproxen sodium 220, 440mgBID or low-dose aspirin for 6 days. (A) Inhibition of platelet COX-1 activity *ex vivo*, as assessed by the measurement of serum TXB₂, in 6 healthy subjects. The empty symbols represent the values detected in each individual, while the full symbols represent the mean±SD; §P<0.05 versus naproxen 440 mg BID at corresponding times, *P<0.05 and **P<0.01 versus aspirin at 24 h; broken line indicates inhibition of platelet COX-1 activity by 97%. Using a nonparametric test #P<0.05 versus aspirin 24 h. (B) Serum TXB₂ levels detected in each individual up to 24 h after dosing with the different treatments (empty symbols). The coloured full symbols represent the mean±SD. The broken line indicates the serum TXB₂ value of 10 ng/ml. *P<0.05 and **P<0.01 versus aspirin at 24 h. (C) Inhibition of AA-induced platelet aggregation detected in 6 healthy subjects up to 24 h after dosing with the different treatments. The empty symbols represent the degree of inhibition of platelet function detected in each individual, while the coloured full symbols represent the mean±SD. f P<0.05 versus aspirin at 1 h. *P<0.05 versus aspirin at 24 h.

Figure 2. Circulating concentrations of naproxen following the administration of 220 or 440 mg BID and corresponding inhibition of platelet COX-1 and monocyte COX-2 activities assessed *ex vivo*. (A) Circulating concentrations of naproxen. The empty symbols represent the circulating concentrations of naproxen detected in each individual, while the coloured full symbols represent the mean±SD(red for naproxen 220 mg BID and green for naproxen 440mgBID).*P<0.05 versus naproxen 440 mg BID at 5, 8, 12 and 24h, **P<0.01 versus naproxen 440 mg BID at 2h. The two broken lines represent IC₉₇(drug concentration required to inhibit the activity of COX-1 by 97%)(70µg/ml) and IC₈₀(drug concentration required to inhibit the activity of COX-2 by 80%)(30µg/ml). (B) Circulating
naproxen concentrations and the corresponding degree of COX-1 and COX-2 inhibition measured \textit{ex vivo} at each time-point were reported on the same graph depicting the sigmoidal concentration-response curve of the drug obtained \textit{in vitro}. Red full and empty symbols represent COX-1 inhibition assessed \textit{in vitro} and \textit{ex vivo}, respectively. Blue full and empty symbols represent COX-2 inhibition assessed \textit{in vitro} and \textit{ex vivo}, respectively. Red and blue lines represent the sigmoidal concentration-response curves of naproxen obtained \textit{in vitro} for COX-1 and COX-2, respectively.

**Figure 3. Comparison of degree and duration of steady-state inhibition of urinary thromboxane and prostacyclin metabolites by naproxen sodium 220, 440 mg BID or low-dose aspirin for 6 days.** (A) Inhibition of TXA$_2$ biosynthesis \textit{in vivo}, as assessed by the measurement of the urinary excretion of 11-dehydro-TXB$_2$ in 4 consecutive urinary samples (time of collection after the last dose: 0 to 4 h, 4 to 8 h, 8 to 12 h and 12 to 24 h) of 6 healthy subjects. Empty symbols represent the inhibition of the urinary metabolites detected in each individual while the coloured bars represent the mean±SD. §P<0.01 \textit{versus} naproxen 440 mg BID at 0-4, 4-8, 8-12 and 12-24 h; *P<0.01 \textit{versus} aspirin at 4-8 and 8-12 h and \textit{versus} naproxen 220 mg BID at 12-24h. (B) Inhibition of prostacyclin biosynthesis \textit{in vivo}, as assessed by the measurement of the urinary excretion of 2,3-dinor-6-keto-PGF$_{1\alpha}$ in 4 consecutive urinary samples of 6 healthy subjects after the different treatments for 6 days. Empty symbols represent the inhibition of the urinary metabolites detected in each individual while the coloured bars represent the mean±SD. §P<0.05 \textit{versus} naproxen 440 mg BID at 8-12 h; *P<0.05 and **P<0.01 \textit{versus} aspirin at the corresponding times of collection.

**Figure 4. Comparison of degree and duration of steady-state inhibition of COX-2 activity \textit{ex vivo} by naproxen sodium 220, 440 mg BID or low-dose aspirin for 6 days.** Inhibition of monocyte COX-2 activity \textit{ex vivo}, as assessed by the measurement of PGE$_2$ induced in heparinized whole blood in response to LPS (10 µg/ml) in 6 healthy subjects, at 1,
2, 5, 8, 12 and 24 h after dosing with naproxen sodium 220 mg BID, 440 mg BID or low-dose aspirin (100 mg daily) for 6 consecutive days. The empty symbols represent the degree of inhibition of plasma PGE$_2$ detected in each individual, while the coloured full symbols represent the mean±SD; in red, green and blue are reported the mean values of LPS-induced PGE$_2$ inhibition by naproxen 220 mg BID, 440 mg BID and low-dose aspirin, respectively. **P<0.01 versus aspirin at 1 and 24 h. Using a nonparametric test, §P<0.01 and #P<0.05 versus naproxen 440 mg BID at the corresponding times.

**Figure 5. Time-dependent COX-selectivity achieved ex vivo and in vivo by naproxen sodium 220, 440 mg BID or low-dose aspirin.** (A) COX-selectivity achieved ex vivo was determined by estimating the ratio of serum TXB$_2$ inhibition versus LPS-induced PGE$_2$ inhibition. The red, green and blue empty symbols represent the COX-selectivity achieved ex vivo in each individual, after treatment with naproxen 220, 440 mg BID or low-dose aspirin, respectively. The broken line represents the ratio of 1, indicating a similar inhibition on COX-1 and –2 activities ex vivo. **P<0.01 versus aspirin at 1 and 24 h. (B) COX-selectivity achieved in vivo was determined by estimating the ratio of urinary 11-dehydro-TXB$_2$ (mainly COX-1 derived) inhibition versus 2,3-dinor-6-keto-PGF$_{1\alpha}$ (mainly COX-2 derived) inhibition. The red, green and blue empty symbols represent the COX-selectivity achieved in vivo in each individual, after treatment with naproxen 220, 440 mg BID or low-dose aspirin, respectively. The broken line represents the ratio of 1, indicating a similar inhibition on TXA$_2$ and PGI$_2$ activities in vivo. #P<0.05 versus aspirin at 0-4 h, *P<0.05 and **P<0.01 versus aspirin at the corresponding times of collection.
Table 1. Effects of naproxen 220, 440 mg BID or aspirin 100 mg daily on COX-1 and COX-2 activities *ex vivo*.

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<td>Naproxen 220mg BID</td>
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Values are reported as mean±SD, *P<0.05, ** P<0.01 versus aspirin at 24 h, §P<0.05 (parametric test), ‡P<0.01 and † P<0.05 versus naproxen 440 mg BID at the corresponding times (nonparametric test) #P<0.05 versus aspirin at 24 h (nonparametric test).
**Figure 1**

(A) Serum TXB2 ng/ml

(B) Serum TXB2 % inhibition

(C) AA-induced platelet aggregation % inhibition

- Naproxen 220 mg BID
- Naproxen 440 mg BID
- Aspirin 100 mg daily

Legend:
- *p < 0.05
- **p < 0.01
- §§ p < 0.001
- # p < 0.0001

Day 6

2 5 8 12 24

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

WO 24 h
Figure 2
Figure 3
Figure 4
Figure 5