Department of Anaesthesiology and Intensive Care Medicine
University of Helsinki
Finland

Characterization of inhibition of platelet function by paracetamol and its interaction with diclofenac and parecoxib

Edward Munsterhjelm

Academic Dissertation

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Supervisors

Professor Per H. Rosenberg, MD, PhD
Department of Anaesthesiology and Intensive Care Medicine
University of Helsinki
Helsinki, Finland

Tomi T. Niemi, MD, PhD
Department of Anaesthesiology and Intensive Care Medicine
University of Helsinki
Helsinki, Finland

Reviewers

Docent Riitta Kekomäki
Blood Service
Red Cross Finland
Helsinki, Finland

Docent Timo Salomäki
Department of Anaesthesiology
University of Oulu
Oulu, Finland

Official Opponent

Professor Ola Dale
Department of Circulation and Medical Imaging
Norwegian University of Science and Technology
Trondheim, Norway

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To Nina and Benjamin

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Contents

Abstract

Aim: To characterize the inhibition of platelet function by paracetamol *in vivo* and *in vitro*, and to evaluate the possible interaction of paracetamol and diclofenac or valdecoxib *in vivo*. To assess the analgesic effect of the drugs in an experimental pain model.

Methods: Healthy volunteers received increasing doses of intravenous paracetamol (15, 22.5 and 30 mg kg^{-1}), or the combination of paracetamol 1 g and diclofenac 1.1 mg kg^{-1} or valdecoxib 40 mg (as the pro-drug parecoxib). Inhibition of platelet function was assessed with photometric aggregometry, the platelet function analyzer (PFA-100), and release of thromboxane B_2. Analgesia was assessed with the cold pressor test. The inhibition coefficient of platelet aggregation by paracetamol was determined as well as the nature of interaction between paracetamol and diclofenac by an isobolographic analysis *in vitro*.

Results: Paracetamol inhibited platelet aggregation and TxB_2-release dose-dependently in volunteers and concentration-dependently *in vitro*. The inhibition coefficient was 15.2 mg L^{-1} (95% CI 11.8 – 18.6). Paracetamol augmented the platelet inhibition by diclofenac *in vivo*, and the isobole showed that this interaction is synergistic. Paracetamol showed no interaction with valdecoxib. PFA-100 appeared insensitive in detecting platelet dysfunction by paracetamol, and the cold-pressor test showed no analgesia.

Conclusions: Paracetamol inhibits platelet function *in vivo* and shows synergism when combined with diclofenac. This effect may increase the risk of bleeding in surgical patients with an impaired haemostatic system. The combination of paracetamol and valdecoxib may be useful in patients with low risk for thromboembolism. The PFA-100 seems unsuitable for detection of platelet dysfunction and the cold-pressor test seems unsuitable for detection of analgesia by paracetamol.

List of original publications

This thesis is based on the following publications:

I Munsterhjelm E, Niemi TT, Syrjälä MT, Ylikorkala O, Rosenberg PH. Propacetamol augments inhibition of platelet function by diclofenac in volunteers. Brit J Anaesth 2003; 91: 357-62

II Munsterhjelm E, Niemi TT, Ylikorkala O, Silvanto M, Rosenberg PH. Characterization of inhibition of platelet function by paracetamol and its interaction with diclofenac in vitro. Acta Anaesthesiol Scand 2005; 49: 840-6

III Munsterhjelm E, Munsterhjelm NM, Niemi TT, Ylikorkala O, Neuvonen PJ, Rosenberg PH. Dose-dependent inhibition of platelet function by acetaminophen in healthy volunteers. Anesthesiology 2005; 103: 712-7

IV Munsterhjelm E, Niemi TT, Ylikorkala O, Neuvonen PJ, Rosenberg PH. Influence on platelet aggregation of i.v. parecoxib and acetaminophen in healthy volunteers. Brit J Anaesth 2006 in press.

The publications are referred to in the text by their roman numerals. The articles are reprinted with kind permission of the copyright holders.

Abbreviations and definitions

AA	arachidonic acid
ADP	adenosine diphosphate
ARG	arginine
ASA	acetylsalisylic acid
cAMP	cyclic adenosine diphosphate
CI	confidence interval
CNS	central nervous system
COX	cyclooxygenase
CYP	cytochrome P450
EC_{50}	concentration causing 50% effect
F	(clotting)factor
γ	interaction index
GLU	glutamate
GP	glycoprotein
IC_{50}	concentration causing 50% inhibition
ILE	isoleucine
K_i	inhibition coefficient
NNT	number-needed-to-treat
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PAR	protease activated receptor
PFA-100	platelet function analyzer
PGD_2	prostaglandin D_2
PGDS	prostaglandin D_2 synthase
PGE_2	prostaglandin E_2
PGES	prostaglandin E_2 synthase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PGIS	prostaglandin I_2 synthase
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase Cγ2
PRP	platelet rich plasma
PPP	platelet poor plasma
RNA	ribonucleic acid
TF	tissue factor
TP	thromboxane-prostanoid
TRAP	thrombin receptor activating peptide
TxA_2	thromboxane A_2
TXAS	thromboxane A_2 synthase
TxB_2	thromboxane B_2
TYR	tyrosine
VAS	visual analogue scale
VWF	von Willebrand factor

Introduction

Paracetamol, also known as acetaminophen, has been in clinical use since 1893. Considering that few other drugs with this long a history are still in wide clinical use, it is surprising that the precise mechanism of action of paracetamol is still undetermined. Paracetamol is related to the large group of non-steroidal anti-inflammatory drugs (NSAIDs), but some distinct differences exist. Paracetamol is an effective antipyretic in relatively low doses whereas the analgesic efficacy seems somewhat lower than that of NSAIDs.[1] Paracetamol is virtually devoid of anti-inflammatory effect.[2] Also, the side-effect spectrum of paracetamol differs from that of NSAIDs; the classic gastric and renal toxicity of traditional NSAIDs is in the case of paracetamol substituted by a significant risk for hepatic toxicity associated with over-dosing.[3]

The mechanism of action of NSAIDs is well known.[4] By inhibiting cyclooxygenase (COX), the NSAIDs block the synthesis of prostaglandins, an important class of lipid messenger molecules responsible for a variety of both physiological and pathological functions. Both the therapeutic and side effects of the NSAIDs are related to COX-inhibition, COX-1 is responsible for most physiological syntheses of prostaglandins whereas COX-2 is up-regulated in inflammatory states. Although paracetamol is a weak inhibitor of COX-1 and a very weak inhibitor of COX-2,[5] the therapeutic effects of paracetamol is unrelated to this effect. Ever since the classic experiment of Flower and Vane in 1972, where they showed that prostaglandin synthesis in brain tissue is inhibitable by paracetamol in much lower concentrations than prostaglandin synthesis in peripheral tissue, central prostaglandin inhibition has been considered the main mechanism of action of paracetamol.[6] The discovery by Chandrasekharan and co-workers in 2002 of the COX-3 variant, expressed mainly in cerebral tissue, suggests a possible target for paracetamol.[7]

Thromboxane A_2, synthesized by COX-1 and thromboxane synthase in platelets, is one important messenger in the complex event of blood clotting.[8] By inhibiting thromboxane formation with an NSAID the risk of clinically significant perioperative bleeding will occur, as shown in clinical studies [9] and known to the experienced anaesthesiologist. Paracetamol is considered safe in this respect, although platelet function impairment can be demonstrated upon intravenous administration of paracetamol.[10] The present study was designed to characterize the platelet function impairment by paracetamol alone and in combination with NSAIDs.

Review of the literature

Biosynthesis of prostaglandins

The first step in the biosynthesis of prostaglandins is release of arachidonic acid (AA) from phospholipids in cellular membranes. AA is a 20-carbon polyunsaturated fatty acid, containing four carbon-carbon double bonds in positions 5,8,11 and 14.[11] In cellular membranes AA is bound to a glycerol backbone, from where it can be released through hydrolysis by phospholipase A_2 (PLA_2). This enzyme belongs to a large family, new members of which are continuously being identified. The PLA_2s are mainly classified into three groups: (a) secretory, Ca^{2+}-dependent, low molecular weight PLA_2s; (b) cytosolic Ca^{2+}-dependent, high molecular weight PLA_2s; and (c) Ca^{2+}-independent PLA_2s.[12] Although many details are still unknown, the cytocolic phospholipase $cPLA_{2\alpha}$ appears to play a key role in release of AA in platelets.[13]

Arachidonic acid released from cell membranes is the substrate of the enzyme cyclooxygenase (COX), also referred to as prostaglandin H_2 synthase, which catalyses the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) through the intermediate prostaglandin G_2 (PGG_2). PGH_2 is further converted by specific synthases to its biologically active derivatives: prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A_2 (TxA_2).

Two types of prostaglandin D_2 synthase (PGDS) have been identified.[14] The lipocalin-type PGDS is mainly expressed in the brain and is involved in regulation of sleep and pain responses. The haematopoietic PGDS is found in peripheral tissues and is thought to participate in immune responses and female reproduction.[15]

PGE_2 can be synthesized by several different synthases (PGESs).[16] The membrane bound mPGES-1 is upregulated by inflammatory stimuli whereas mPGES-2 and the cytosolic cPGES are constitutively expressed in many tissues. Besides its role in inflammation and fever, PGE_2 is thought to play a role in kidney function, bone metabolism and reproduction. The cytoprotective effect of PGE_2 in gastric mucosa is of major clinical significance.[17]

$PGF_{2\alpha}$ is synthesized by several different enzymes; from PGD_2 by PGD_2 11-ketoreductase, from PGH_2 by PGH_2 9,11-endoperoxide reductase or from PGE_2 by PGE_2 9-ketoreductase.[18] Among other physiological effects, $PGF_{2\alpha}$ causes constriction of bronchial and vascular smooth muscle.

Prostaglandin I_2 synthase (PGIS), which catalyzes the conversion of PGH_2 to prostacyclin, is a membrane bound enzyme located in the endoplasmic reticulum and belongs to the microsomal P450 superfamily.[19] Prostacyclin is mainly produced by vascular endothelial cells and its main physiological effects are vasodilation, and inhibition of platelet aggregation and adhesion to vascular endothelium.[20] It also inhibits the adhesion of leukocytes to the endothelium and may inhibit their activation during inflammation.

TxA_2 is synthesized by thromboxane A_2 synthase (TXAS), which, in conformity

with PGIS, is bound to endoplasmic reticulum and belongs to the microsomal P450 superfamily.[21] TXAS is mainly active in platelets, but it has been isolated from many different tissues.[22] The major physiological functions of TxA_2 are opposite to those of PGI_2; TxA_2 is a potent vasoconstrictor and trigger of platelet aggregation.

Structure and function of cyclooxygenase

Three isoforms of COX have been identified so far. COX-1, the gene of which was cloned in 1989,[23] is considered to be responsible mainly for the physiological functions of COX since it is constitutively expressed in many tissues.[24] COX-2, on the other hand, is inducible upon inflammatory stimuli, and is considered responsible for prostaglandin production in pathologic states. Although this is probably an oversimplification, the distinction is useful as long as many details are still uncovered. The gene of COX-2 was cloned in 1992.[25] COX-3, the youngest member of the COX family, was discovered in 2002.[7] This isoform is not the product of its own gene, but the result of an alternative splicing of the messenger RNA of the COX-1 gene. COX-3 mRNA is found throughout the rat central nervous system (CNS) with a high density in brain microwessels, suggesting a possible association with vascular tissue.[26] Whether the same is true for the human CNS remains to be elucidated.

COX is a membrane-bound enzyme existing as a dimer of two identical mono-

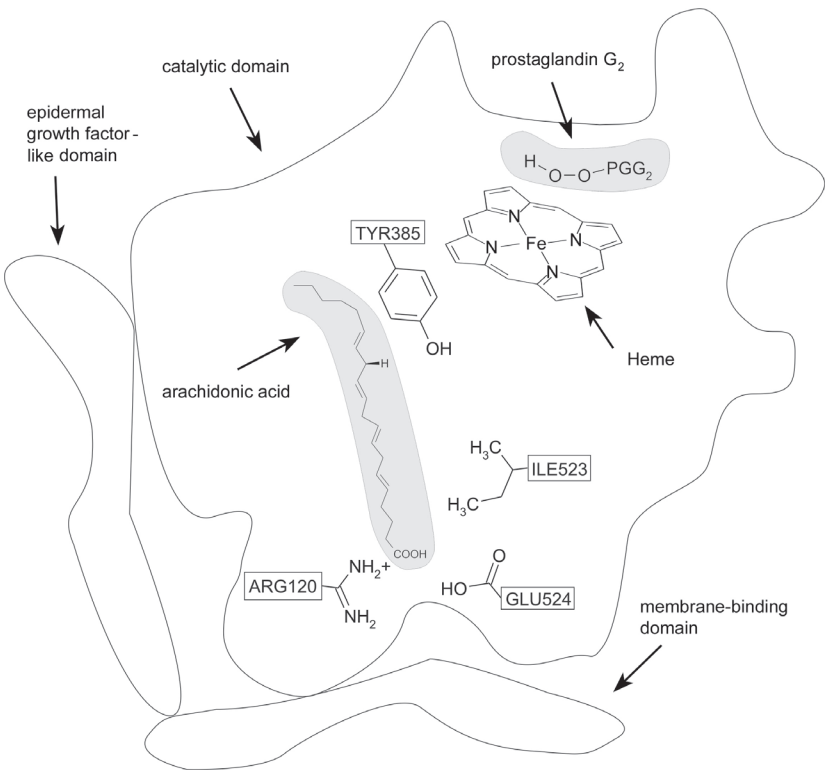

Figure 1. *Structure of the cyclooxygenase-1 monomer*

mers, the crude structure of the COX-1 monomere is shown in figure 1.[27] In addition to the membrane-binding domain, consisting of four helices, the enzyme consists of an epidermal growth factor-like domain, commonly found in many proteins and thought to serve as a structural building block,[28] and a catalytic domain containing the cyclooxygenase and peroxidase active sites. The cyclooxygenase active site resides at the end of a long, hydrophobic channel which penetrates from the membrane binding surface into the core of the catalytic domain.[27] In the channel only two polar residues are found, arginine 120 and glutamate 524.[28] The cyclooxygenase reaction is catalysed by a tyrosine residue, Tyr 385 (COX-1 numbering), located at the top of the channel. The peroxidase active site is located on the protein surface in a shallow cleft, defined by four helices. This site contains a heme cofactor, being responsible for the peroxidase catalytic activity.

These two catalytic centres are spatially adjacent and the enzymatic reactions are also mechanistically connected.[27] The chain of reactions starts with the heme group of the peroxidase active site being oxidized to an oxyferryl radical intermediate (Intermediate I or Compound I) by its substrate PGG_2 or some other peroxide. Intermediate I is then reduced in two steps: first to the oxyferryl heme Intermediate II and further back to the resting ferric state (Fig. 2).[29] The reduction of Intermediate I to Intermediate II oxidizes the adjacent Tyr 385 to a tyrosyl radical, which is required for initiation of the cyclooxygenase reaction.[30] Two different models for the interrelation between reactions have been proposed. The branched chain mechanism states that one cycle in the peroxidase reaction may facilitate

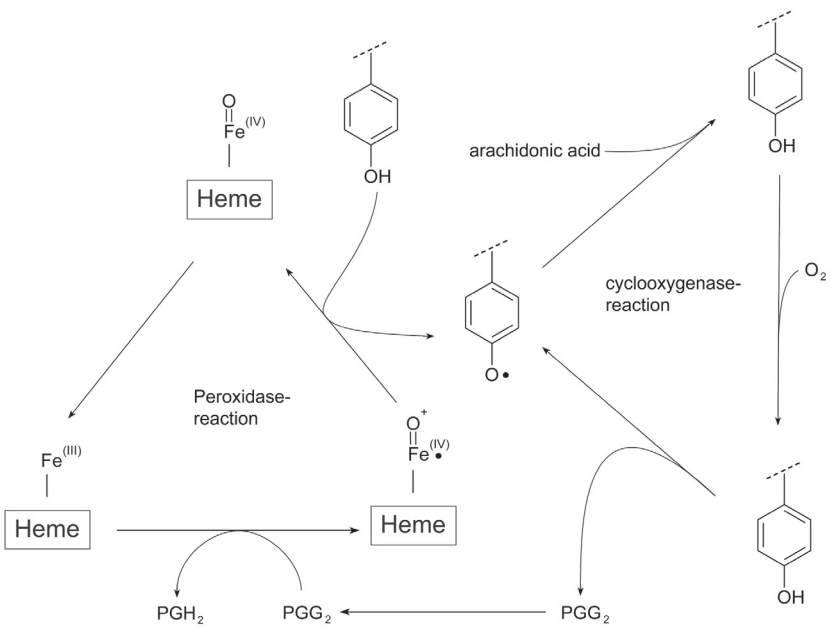

Figure 2. *Function of cyclooxygenase*

multiple turnovers in the cyclooxygenase reaction, since the tyrosyl radical is regenerated at the end of the cyclooxygenase catalytic cycle. The alternative model, the tightly coupled mechanism, states that the tyrosyl radical needs to be reoxidated by Compound I in every cycle, and therefore one cycle in the peroxidase reaction is needed to initiate every turnover in the cyclooxygenase reaction.[31] The former mechanism appears to be the prevailing in the literature.

The hydrophobic structure of the channel leading to the cyclooxygenase reaction catalytic centre is essential for appropriate positioning of arachidonic acid. Many weak bonds are formed between AA and amino acid residues lining the channel.[32] During the cyclooxygenase cycle two molecules of oxygen are added to AA and a new bond is formed between carbons 8 and 12 (Fig. 3).[33] The newly formed PGG_2 molecule then diffuses to the peroxidase site of the same or adjacent COX complexes for reduction to its corresponding alcohol PGH_2 (Fig. 3).

The amino acid sequence differ between COX-1 and COX-2, approximately 60% being identical. However, the sequence near the catalytic domain is highly conserved,[34] and the catalytic mechanism is identical in the two isoforms. The amino acid numbering differs somewhat between isoforms but, for clarity, the COX-1 numbering is used. The hydrophobic channel leading to the cyclooxygenase reaction catalytic centre is somewhat less narrow in COX-2. The isoleucine residue in position 523 in COX-1 is in COX-2 substituted with a valine, which exposes a side pocket in the channel near the catalytic centre.[35] Some differences also occur at the mouth of the channel.[34]

The precise structure of COX-3 is still undetermined. In fact, its existence in humans has been questioned.[36]

Little is known about how the reactions of the COX complex are regulated. Availability of AA is probably of major significance. Based on mathematical simulations, a threshold switch mechanism has been proposed.[37] In this model, a certain threshold concentration of AA is required for propagation of the reaction. Below threshold AA concentration, PGG_2 is quickly depleted and the reaction comes to a halt. In this way, minimal amounts of prostaglandins are produced under resting conditions whereas rapid synthesis occurs upon increased liberation of AA. Another factor considered significant is self-inac-

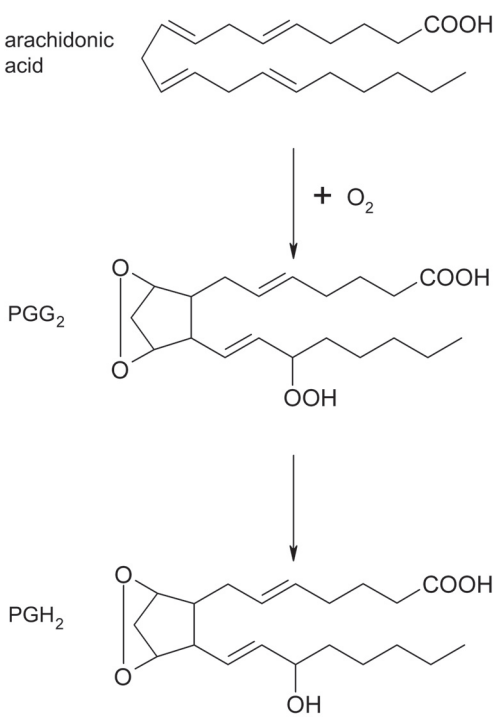

Figure 3. *Synthesis of prostaglandin*

tivation of the enzyme. Some aspects of this suicide activity have been uncovered. The peroxidase reaction is thought to self-inactivate by a mechanism where Intermediate II is rendered inactive through oxidation to Intermediate III, which does not participate in the normal catalytic cycle.[38] The cyclooxygenase reaction appears to self-inactivate through a mechanism different from that of the peroxidase reaction,[39] but the details are not known.

The activity of COX is also dependent on the redox state of the environment; a certain amount of peroxides is needed to initiate the chain of reactions. Since the peroxidase reaction is not very substrate-specific,[40] a variety of peroxide may initiate the chain of reactions. Lowering the peroxide tone in the environment with glutathione peroxidase suppresses the cyclooxygenase activity.[41] The two main COX isoforms, COX-1 and COX-2, appear to differ with respect to their sensitivity to peroxide tone.

Pharmacologic inhibition of COX isoforms

Non-selective inhibitors

The non-steroidal anti-inflammatory drugs (NSAIDs) constitute a large group of structurally different pharmacological substances able to inhibit the action of COX.[4] Traditional NSAIDs, such as indomethacin, ibuprofen, diclofenac, naproxen and acetylsalicylic acid (aspirin), inhibit both COX-1 and COX-2.[5] NSAIDs inhibit COX by blocking the hydrophobic channel leading to the cyclooxygenase active site.[42] Aspirin differs from other NSAIDs through its ability to irreversibly inhibit COX by acetylating serine 530 in the channel.[43] All other NSAIDs are competitive inhibitors and form non-covalent bonds to different amino acid residues in the channel. Based on X-ray crystal structure models, acidic NSAIDs appear positioned with their carboxyl group towards the mouth of the channel and the nonpolar groups towards the catalytic centre.[28] Arginine 120 is important, since its positively charged side chain binds to the negatively charged carboxyl group of acidic NSAIDs,[44] although affinity differs between NSAIDs.[45] Diclofenac is less dependent on interaction with Arg 120 than indomethacin. The carboxyl group of diclofenac probably binds to tyrosine 355 near the mouth of the channel [46] or through an inversed orientation to Ser 530 and Tyr 385 near the catalytic centre.[47]

The kinetics of the reaction between NSAIDs and COX is, however, more complex than the simple interaction between a receptor and an antagonist. Several NSAIDs, for example indomethacin [48] and diclofenac,[49,50] exhibit a time-dependent inhibition in addition to the normal concentration dependent inhibition of COX. A two-step kinetic model has been proposed.[51] The initial step is rapid and easily reversible, the second step is slow and poorly reversible. In the case of indomethacin, a possible mechanism is insertion of a methyl group of the drug into a hydrophobic pocket in the side of the channel. [51]

COX-2 selective inhibitors

Based on the structural differences between COX-1 and COX-2, highly selective COX-2 NSAIDs, coxibs, have been developed. Although many COX-2-selective inhibitors have been synthesized, five coxibs have been assessed in clinical trials. They are

(in order of increasing COX-2 selectivity): celecoxib, valdecoxib, etoricoxib, rofecoxib and lumiracoxib.[52] In contrast to traditional NSAIDs, which are fairly linear molecules, coxibs have a bulkier, tricyclic structure.[53] The phenylsulphonamide/phenylsulphone group common to all coxibs but lumiracoxib enters the side pocket in the cyclooxygenase catalytic channel, made accessible in COX-2 by valine instead of isoleucine in position 523.[54,35] Changing valine 523 to isoleucine by site-directed mutagenesis in COX-2 reverses the selectivity of inhibitors.[34] The adjacent histidine 513 in COX-1 is substituted with arginine in COX-2, but this does not appear to affect the affinity of coxibs.[55] More detailed studies suggest a time-dependent kinetic mechanism for selective inhibition,[56] resembling that of time-dependent non-selective inhibitors. According to this model, the last, poorly reversible step in the reaction would represent entrance of the phenylsulphonamide group into the side pocket. In contrast to non-selective NSAIDs, arginine 120 does not appear important for binding of coxibs to COX-2. Arg-120 mutations rather display increased sensitivity to certain inhibitors.[57]

Paracetamol

In their classic experiment in 1972, Flower and Vane showed that prostaglandin synthesis in brain tissue is inhibitable by paracetamol in much lower concentrations than prostaglandin synthesis in peripheral tissue.[6] From this experiment it has been concluded that paracetamol acts by inhibiting COX in the CNS. The newly discovered COX-3 appears more sensitive to the action of paracetamol than COX-1 and COX-2.[7] In peripheral tissues, COX-1 appears slightly more sensitive than COX-2.[5]

Analgesic effect of paracetamol

Postoperative pain

Paracetamol was introduced in medicine in 1893, but did not gain popularity until in the 1950s when paracetamol was recognized as the major active metabolite of the nephrotoxic analgesic phenacetin.[58] Paracetamol differs from NSAIDs through its weak anti-inflammatory activity, although it has potent antipyretic and analgesic functions.[2]

The effect of oral paracetamol for postoperative pain has been assessed by Barden and co-workers in a meta-analysis including 4186 patients.[59] The numbers-needed-to-treat (NNTs) with 95% confidence intervals (CI) for at least 50% pain relief following a single dose of paracetamol were: 325 mg NNT 3.8 (2.2-13.3), 500 mg NNT 3.5 (2.7-4.8), 600/650 mg NNT 4.6 (3.9-5.5), 975/1000 mg NNT 3.8 (3.4-4.4), and 1500 mg 3.7 (2.3-9.5). Rectal and parenteral paracetamol have been shown effective in a meta-analysis by Rømsing and co-workers.[60] Parenteral paracetamol can be administered as the prodrug propacetamol, which plasma esterases rapidly hydrolyse into equal amounts of paracetamol and diethylglycine,[61] or as a novel intravenous paracetamol solution. Propacetamol 2 g is bioequivalent to intravenous paracetamol 1 g.[62] Most reports on the analgesic effect of intravenous paracetamol have been performed with propacetamol, the conventional dose being 2 g in adults. Several recent randomized placebo-controlled studies not included in the meta-analysis by Rømsing and co-workers also address this question; propacetamol has been shown effective for example after dental surgery (n=31 in the propacetamol group)[63] and spinal fusion surgery

(n=21 in the propacetamol group),[64] but has not proved satisfactory after cardiac surgery (n=40 in the propacetamol group) [65] or hepatic resection (n=40 in the propacetamol group).[66] In a large study (n=275 in the propacetamol group) on a mixed surgical material Aubrun and co-workers showed a clear morphine-sparing effect of propacetamol, but did not find any significant reduction of morphine-related side effects.[67] In their recent study, Sinatra and co-workers defined the morphine sparing effect of both propacetamol (29%) and intravenous paracetamol (33%).[68]

Osteoarthritis

The analgesic effect of paracetamol has also been assessed in other painful conditions than the postoperative one. The effect of paracetamol in osteoarthritis (OA) is somewhat controversial. In a meta-analysis including 10 trials with a total of 1712 patients with OA in the knee, hip or multiple joints, paracetamol proved effective, although less effective than NSAIDs.[69] Two recent randomized, placebo-controlled studies show conflicting results. In their large study (n=405 in the paracetamol group) Miceli-Richard and co-workers found equal effect of paracetamol 4 g/day and placebo in OA of the knee.[70] On the other hand, in a crossover multicenter trial (n=239 in the paracetamol – placebo or placebo – paracetamol groups) Pincus and co-workers found paracetamol 4 g/day superior to placebo, although less effective than celecoxib.[71]

Experimental pain models

A variety of experimental pain models have been developed to study analgesics in healthy volunteers. Stimulation of the skin has been achieved by mechanical, thermal, electrical, and chemical stimulation. Muscle pain can be induced by ischemia, pressure, electrical stimulation or intramuscular injections of algesic substances.[72] To assess the degree of pain achieved, either psychophysical or electrophysical methods can be used. The visual analogue scale (VAS) is a widely used psychophysical method, electrophysiological methods include the withdrawal reflex and evoked brain potentials.

The cold pressor test has been used to study the effect of several opioids such as morphine,[73] oxycodone,[74] codeine,[75] and alfentanil,[76] or NSAIDs such as ibuprofen[73] and indomethacin,[77] or antidepressants such as imipramine [78] and venlafaxine,[79] as well as α_2-agonists such as dexmedetomidine [80] and clonidine.[81] The cold pressor test generally works well with opioids and α_2-agonists whereas no effect was detected in response to NSAIDs and antidepressants. Pain intensity in the cold pressor test has been shown to correlate with the need for analgesia after oral surgery.[82] Paracetamol has shown an analgesic effect with several experimental methods: electrical stimulation,[83] laser induced pain [84] and the cold pressor test.[85]

Analgesic effect of NSAIDs

The analgesic effect of NSAIDs when treating postoperative pain is well documented in several meta-analyses.[86,87] Coxibs are also effective in postoperative

pain.[88] The usefulness of NSAIDs in osteoarthritis is also very well documented. The large meta-analysis by Bjordal and co-workers including 10 845 patients with knee osteoarthritis is one recent example.[89] Coxibs are also effective in this disorder.[90]

Combination of paracetamol and NSAIDs

Paracetamol and NSAIDs have been combined to improve postoperative analgesia. Hyllested and co-workers assessed the effect of this combination in a qualitative review in 2002.[86] They concluded that the combination seems more effective than either drug alone, but that data are sparse. Only a few studies addressing this question have been published in recent years. Hiller and co-workers found no difference between propacetamol, diclofenac or their combination after tonsillectomy,[91] but the number of patients was small (21-25 per group) and no placebo group was included. Viitanen and co-workers found a reduced need for analgesia after discharge of children undergoing adenoidectomy in the group receiving a combination of paracetamol and ibuprofen compared to either drug alone, although the opioid sparing effect versus placebo was equal in all treatment groups.[92] Dahl and co-workers found ibuprofen comparable to a combination of paracetamol and ibuprofen after orthopaedic surgery,[93] but small groups and lack of placebo group were potential methodological problems. In a recent study, Hiller and co-workers evaluated the effect of rectal paracetamol 60 mg kg^{-1}, intravenous ketoprofen 2 mg kg^{-1} or their combination in pediatric patients.[94] They found the combination superior to paracetamol but not to ketoprofen. Unfortunately, no placebo group was included.

Very little documentation is available regarding the combination of coxibs and paracetamol. Issioui and co-workers combined celecoxib with paracetamol after otolaryngologic surgery and found the combination superior to either drug alone.[95] On the contrary, Pickering and co-workers reported a negative result with the combination of rofecoxib and paracetamol in comparison with paracetamol alone after tonsillectomy in children.[96]

Pharmacokinetic properties of paracetamol, diclofenac and parecoxib

Paracetamol

Paracetamol can be administered through the rectal, oral or intravenous route. Absorption through the two former routes is influenced by a variety of factors such as pharmaceutical formulation, fed or fasting state, rate of gastric emptying, and position of the patient. As a result, enteral dosing tend to cause highly variable plasma concentrations.[97, 98] The half-life of paracetamol in plasma is about 2 hours, different studies displaying slightly varying results.[99] Paracetamol is metabolized in the liver, mainly through conjugation with glucuronic or sulfuric acid. A small proportion of the drug undergoes oxidation through the cytochrome P450-system (CYP) to form the highly reactive intermediate N-acetyl-benzquinoneimine.[58] This intermediate is normally neutralized by glutathione, but if a large dose is ingested hepatic glutathione stores may be depleted and hepatic necrosis may result.

Diclofenac

Diclofenac can be administered by the oral or parenteral route. After administration through the oral route it is generally rapidly absorbed.[100] Its half-life in plasma is approximately 1 hour, and metabolism is through CYP2C9.[101]

Parecoxib

Parecoxib is an injectable pro-drug of valdecoxib, and so far the only coxib for parenteral use. Parecoxib is converted to valdecoxib by hepatic hydrolysis within 30 min after intravenous administration.[102] The half-life of valdecoxib in plasma is relatively long, more than 7 hours, and metabolism is mainly through cytochrome P450-independent glucuronidation.[103]

Platelet function and haemostasis

The cell-based model of haemostasis

Injury to the vascular wall exposes extravascular tissues to plasma and platelets initiating the complex process of coagulation. In 1964 the traditional cascade model was proposed, recognizing the coagulation process as a series of proteolytic reactions.[104,105] The coagulation cascade was divided into the intrinsic and the extrinsic pathways based on measurements *in vitro*. Although the picture has changed in recent years, certain features of the cascade model are still valid. Circulating coagulation factors are inactive and activated by proteolytic cleavage. The active forms of factors VII, IX, X and II (prothrombin) are serine proteases that activate other coagulation factors. Apart from prothrombin, all factors have to form a complex with their non-enzymatic cofactors; tissue factor (TF), FVIII, and FV, respectively, to be enzymatically active.[106] The new cell based model of haemostasis, proposed in 2001 by Hoffman and Monroe, emphasizes the importance of interaction between coagulation factors and platelets *in vivo*.[107]

The initiation phase

TF is a membrane-bound protein expressed in many tissues, such as brain, lung, skin, and mucosal epithelium. Fibroblasts in blood vessel walls also express large amounts of TF.[108] Upon vessel wall injury, TF is exposed and initiates coagulation by binding activated factor VII (FVIIa). This complex then activates factors FIX and FX.[109] Factor Xa activates FV,[110] and together FXa and FVa can produce small amounts of thrombin.[111] This chain of events is referred to as initiation of coagulation.

In parallel with initiation of coagulation, platelet activation occurs. When exposed to subendothelial matrix, platelets adhere to collagen and von Willebrand factor (VWF). Synthesized, stored, and secreted by endothelial cells, VWF is a polypeptide polymer to which platelets adhere through its glycoprotein (GP) Ib-IX-V complex.[112] The GPIb-IX-V complex is composed of four transmembrane subunits: GPIbα, GPIbβ, GPIX, and GPV. The GP Ibα and GPIbβ subunits are covalently linked by a disulfide bond whereas the other subunits are linked together by noncovalent binding. The VWF binding domain resides in the GPIbα subunit.[113] Platelet adhesion directly to collagen is through two major receptors; GPIa-IIa (also referred to as integrin $\alpha_2\beta_1$) and GP VI.[114] Upon binding to their ligands, all three receptors interact in activating the platelet.

Many details still remain to be elucidated about the intracellular signalling pathways activated by the receptors. In general, all receptors activate different protein kinases, such as Src and Syk, which in turn activate phospholipase Cγ2 (PLC).[115-117] PLC participates in intracellular signal transduction in many cell types by catalyzing the formation of two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, from phospholipids in the cellular membrane.[118] Inositol 1,4,5-trisphosphate mediates the release of Ca^{2+} from the platelet dense tubular system whereas diacylglycerol, together with Ca^{2+}, activates protein kinase C (PKC). PKC is an essential step towards platelet degranulation and aggregation.[119] Local thrombin production by the FXa-FVa-complex also contributes to platelet activation.

The amplification phase

The next phase of the haemostatic process comprises amplification of the coagulation cascade, and platelet shape change, secretion, and aggregation. In the resting state platelets are oval discs, but when activated they become spherical and start forming finger-like projections, filopodia. Next, platelets flatten and start spreading over the surface to which they adhere.[120] In order to attract more platelets to the site of injury, platelets start secreting a variety of substances acting in an autocrine and paracrine manner. Platelets contain two different types of secretory granule, alpha and dense granules. The alpha granules contain a variety of large proteins such as adhesive glycoproteins, haemostasis factors and cofactors, growth factors, and protease inhibitors whereas the dense granules contain high concentrations of small pro-aggregatory molecules, serotonin and adenosine diphosphate (ADP).[121] Another important pro-aggregatory molecule, not stored in granules but synthesized on demand, is TxA_2. This molecule is synthesized by the PLA_2-COX-TXAS-pathway, described in detail previously. COX-1 is the only active COX-isoform in platelets.[122]

ADP released during platelet degranulation, TxA_2 produced by the platelet, and thrombin produced by the coagulation system, all bind to their own receptors on the surface of the platelet. These receptors belong to the seven-transmembrane receptor superfamily, with a common architecture including seven membrane spanning helices and a G-protein coupled signal transduction system.[123] Platelets express two ADP receptors, $P2Y_1$ and $P2Y_{12}$, with slightly different functions.[124] The $P2Y_1$ receptor activates PLC through G_q,[125] and the $P2Y_{12}$ receptor inhibits the synthesis of cyclic adenosine monophosphate (cAMP) through G_{i2}, a member of the G_i protein family (Fig 4).[126] The thromboxane-prostanoid receptors (TP receptors), to which TxA_2 binds, are coupled to G_q and $G_{12/13}$.[127,128] The $G_{12/13}$ protein is thought to mediate platelet shape change, a process independent of Ca^{2+} signalling and PKC activation,[129] through phosphorylation of myosin light chains by the intracellular GTPase Rho.[130] Two thrombin receptors are expressed by the platelet, protease activated receptor 1 (PAR1) and PAR4.[131,132] The PAR family differs slightly from the other members of the seven-transmembrane receptor family, since thrombin activates the PAR by cleaving the receptor at its amino-terminal extracellular end, after which the cleaved peptide activates the receptor.[133] Signalling through the thrombin receptors

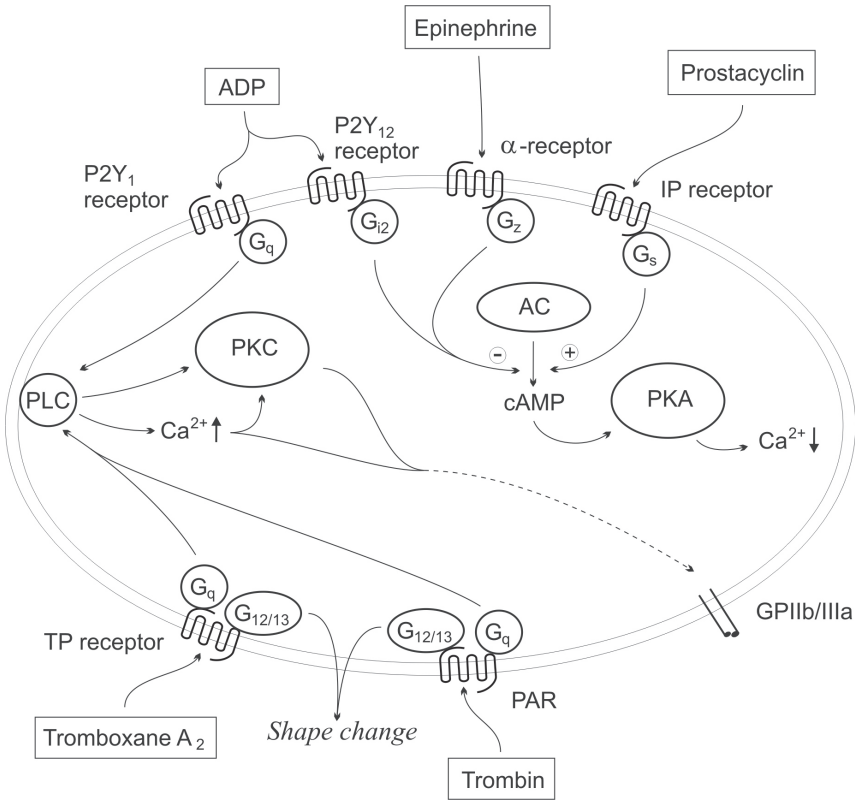

Figure 4. ***Platelet intracellular signalling***

are coupled to G_q and $G_{12/13}$.[134-136] In addition to the PARs, ADP-, and TP-receptors, platelets also express α-adrenergic seven transmembrane receptors coupled to G_z, another member of the G_i family (Fig 4).[137,138]

Platelet aggregation requires simultaneous signalling through several pathways. Concomitant signals through G_q and G_i, or through $G_{12/13}$ and G_i cause Ca^{2+} mobilisation and aggregation.[139] Aggregation may, however, also result from G-protein-independent signalling. Stimulation of the GPIb-IX-V complex with a VWF analogue may induce aggregation,[140] as may high concentrations of collagen.[141] Anyhow, amplification of the signal through release of ADP and TxA_2 is essential for haemostasis, since patients with defective receptors display chronic bleeding disorders.[142,143]

A key event in platelet aggregation is activation of the GPIIb/IIIa complex (also referred to as integrin $\alpha_{IIb}\beta_3$). This complex is the major platelet receptor; as many as 80,000 copies reside on the platelet surface.[144] The GPIIb/IIIa complex have binding sites for several adhesive macromolecules, but most important are fibrinogen and VWF.[145] In the resting state, platelets have low affinity for fibrinogen and VWF, but upon activation the GPIIb/IIIa complex undergoes both conformational changes and

receptor clustering.[146,147] Intracellular Ca^{2+} is a key signal in activating the GPIIb/IIIa complex. Signalling through both the G-protein coupled pathways and the GPIb-IX-V complex induces an increase in intracellular Ca^{2+} concentration, and Ca^{2+} chelation prevents GPIIb/IIIa activation through both pathways.[148,140] The GPIIb/IIIa complex itself exhibits intracellular signalling properties, called outside-in signalling, launching a positive feedback loop including PKC and further Ca^{2+} mobilisation [149] that leads to an oscillating cytosolic Ca^{2+} concentration, a pattern typical for intracellular signalling.[150] The activated GPIIb/IIIa complex binds fibrinogen and VWF, which function as bridges between platelets and facilitate aggregation.[151]

In parallel with platelet aggregation, amplification of the coagulation cascade occurs on the platelet surface. Platelet α-granules contain FV in a partially activated form, rapidly activated by FXa or thrombin.[152] Together the factors Va and Xa form the "prothrombinase" complex, responsible for thrombin production.[153] Thrombin also cleaves FVIII,[154] and FVIIIa subsequently forms a complex with factor IXa, activated by the initial TF-FVIIa complex. Since the FIXa-FVIIIa complex, the "tenase" complex, catalyses the activation of FX at a rate 50-fold compared to that of the TF-FVIIa complex,[155] the amplification loop is complete.

The propagation phase

When the "tenase" and "prothrombinase" complexes, i.e. the FIXa-FVIIIa and FXa-FVa complexes, have assembled on the surface of the platelet, large-scale thrombin production is initiated. The thrombin now produced rapidly cleaves circulating, soluble fibrinogen that polymerizes and forms an insoluble web supporting the growing thrombus.[156] Clot strength is further enhanced by crosslinking of fibrin by FXIIIa.[157]

The termination phase

Without a tight regulation, the haemostatic process would continue until all fibrinogen is consumed. In order to restrict the clotting to only the site of bleeding, the coagulation cascade outside the surface of the platelet is rapidly terminated by physiological inhibitors, such as activated protein C, protein S, antithrombin III, and the tissue factor pathway inhibitor.

Platelet function is inhibited by intact vascular endothelium by several mechanisms. Most important is to constitute a physical barrier between the bloodstream and the TF-bearing cells in the subendothelium. Endothelial cells also secrete several vasoactive substances; PGI_2 and nitric oxide (NO) are potent inhibitors of platelet function. PGI_2, synthesized by the PLA_2-COX-PGIS pathways described previously, binds to G_s-coupled platelet surface receptors. G_s counteracts the proaggregatory G_i by stimulating the synthesis of intracellular cAMP, which in turn lowers intracellular Ca^{2+} concentration by activating protein kinase A (PKA, Fig. 4).[158,159] COX-2 is responsible for PGI_2 synthesis in vascular endothelium.[160] Intracellular Ca^{2+} concentration also decreases in response to NO, but partly through a different mechanism; NO is a potent stimulator of platelet guanylyl cyclase, responsible for production of cGMP.[161] cGMP in turn activates protein kinase G.[162]

Influence on platelet function of paracetamol and NSAIDs

NSAIDs

By inhibiting COX-1, as described in detail previously, the NSAIDs shut down the production of TxA_2 in the platelet. The platelet-inhibiting effect of NSAIDs is well documented,[163,164] and low dose aspirin is widely used to prevent arteriothrombotic events.[165] The COX-2 selectivity of the coxibs makes them unable to influence platelet function.[166,167]

Paracetamol

Paracetamol is usually considered not to influence platelet function *in vivo*, based on studies on oral paracetamol.[168,169] *In vitro*, however, paracetamol has been shown to inhibit platelet aggregation and production of TxB_2 in several studies.[170, 171, 172] Our research group has also shown that a high dose of propacetamol inhibits platelet function *in vivo*.[10] The effect on platelet aggregation of the combination of paracetamol and NSAIDs or coxibs has not been studied.

Platelet function tests

The effect of drugs on platelet function has been evaluated with a wide variety of methods, focusing on different stages of the haemostatic process. Platelet count is a routine method, performed with standard cell counters. Although widely available, this method gives no information about platelet function. The Ivy bleeding time, described more than 70 years ago,[173] evaluates haemostasis by measuring the time until cessation of bleeding after a skin cut. This method is highly user dependent, and correlates poorly with clinical bleeding.[174] Obviously, this method is also influenced by other aspects of haemostasis than platelet function.

Platelet activation can be assessed with flow cytometry, using an antibody directed towards some marker displayed on the platelet surface during activation.[175] Platelet adhesion can be assessed with perfusion chambers, where platelets are flowing over a thrombogenic surface.[176] This method allows the study of different surfaces at different shear rates, but requires a specialized laboratory. The gold standard for assessment of platelet aggregation is photometric aggregometry based on the method of Born.[177] This method measures the increase in light transmission through a suspension of platelets during aggregation. This method is fairly laborious since it requires the preparation of platelet-rich plasma or washed platelets, but on the other hand it offers the possibility to study aggregation triggered through many different pathways. Platelet secretion can be assessed by measuring the concentration of the substance of interest with an immunologic or some other suitable method.

The platelet function analyser (PFA-100) assesses the combination of platelet adhesion and aggregation under high shear rates.[178] In this method anticoagulated blood is drawn under vacuum through a membrane coated with collagen and ADP or epinephrine.[179] The time before membrane occlusion is recorded. PFA-100 has been used to evaluate the response to acetylsalicylic acid treatment, and a dose-dependent increase in collagen/epinephrine closure time has been established.[180]

Aims of the study

The main goal of this thesis was to describe the inhibition of platelet function by paracetamol. The specific questions were as follows:

1. Does paracetamol exhibit a dose-dependent inhibition of platelet function *in vivo* (III)?

2. Can the inhibition of platelet function by paracetamol be characterized by pharmacodynamic models *in vitro* (II)?

3. Do diclofenac and paracetamol interact in inhibiting platelet function *in vivo* (I)?

4. Is the interaction between paracetamol and diclofenac synergistic, additive or subadditive (II)?

5. Do valdecoxib and paracetamol interact in inhibiting platelet function *in vivo* (IV)?

Methods

Study designs

Studies I, III and IV were double-blinded, randomized and placebo-controlled, carried out in healthy, non-smoking, male volunteers applying a cross-over design. Study II was carried out *in vitro*, blood was donated by healthy, non-smoking donors. All studies were approved by the Ethics Committee for Studies in Healthy Subjects and Primary Care in the Hospital District of Helsinki and Uusimaa, and studies I, III-IV by the National Agency for Medicines in Finland.

Studies comprised 10 (I), 15 (III), and 18 (IV) volunteers, and 22 donors (II), all of whom gave written informed consent. In studies III and IV abnormal plasma alanine transaminase or aspartate aminotransferase activities were an exclusion criterion. The use of acetylsalicylic acid was forbidden for ten days and that of other drugs affecting platelet function for one week prior to each experiment (I, III, IV) or prior to blood donation (II). Study characteristics are summarized in Table 1.

Sampling and drug infusions

After 3 hours of fasting, a venous blood sample (approximately 35 ml) was drawn from an antecubital vein through a 20-gauge needle (PrecisionGlide™, Becton Dickinsson) (I-III) or a 17-gauge cannula (Venflon™, Becton Dickinson, Franklin Lakes, New Jersey, USA) (IV) into polypropylene tubes (Vacuette®, Greiner bio-one, Austria) containing 3.2% buffered citrate, and into plastic EDTA-tubes (5.9 mg K2EDTA, VenoSafe™, Terumo Europe, Haasrode, Belgum) (III, IV). After the baseline sample, a dorsal vein of the contralateral hand was cannulated with a 20-gauge cannula and the volunteers received the drugs studied: In study I a 30-minute infusion of either diclofenac (Voltaren®, Novartis, Finland) 1.1 mg kg^{-1}, a combination of diclofenac 1.1 mg kg^{-1} and propacetamol (Pro-Dafalgan®, Bristol-Myers Squibb, France) 30 mg kg^{1}, or placebo (NaCl 0.9%); in study III a 10-minute infusion of paracetamol (Perfalgan®, Bristol-Myers Squibb, New York, NY) 15 mg kg^{-1}, 22.5 mg kg^{-1}, 30 mg kg^{-1} or placebo (NaCl 0.9%, Braun, Kronberg, Germany); and in study IV a slow infusion of 0.9% NaCl was started and parecoxib 40 mg (Dynastat®, Pfizer, New York, USA) or placebo (NaCl 0.9%) was given as a 1-minute bolus. In study II a maximum of 200 ml blood was drawn on one or two occasions.

The second sample was drawn 5 min (I), 10 min (III), or 50 min (IV) after drug administration. In studies I and III the third sample was drawn 90 min after drug administration. In study IV paracetamol 1 g (Perfalgan®) or placebo (NaCl 0.9%) was administered as a 10-minute infusion approximately half an hour after the second sample, and the third sample was drawn 10 minutes later. In study I a fourth sample was drawn after 22-24 h. The interval between experiments in the same volunteer was at least one week.

Table 1. *Study characteristics.*

Study	Design	N	Drugs
I	volunteers	10	paracetamol 15 mg kg^{-1} (as propacetamol 30 mg kg^{-1})+ diclofenac 1.1 mg kg^{-1}, diclofenac 1.1 mg kg^{-1} or placebo
II	in vitro	22	paracetamol 10 - 80 mg L^{-1} and/or diclofenac 0.1 - 0.8 mg L^{-1}
III	volunteers	15	paracetamol 15, 22.5 or 30 mg kg^{-1} or placebo
IV	volunteers	18	paracetamol (1 g) + parecoxib (40 mg), paracetamol (1 g) or placebo

Cold pressor test

In studies I and II, a cold pressor test was performed immediately after every blood sample, except after 22-24 h. The volunteer immersed his non-dominant hand, halfway to the elbow, into an ice bath and estimated the pain intensity on a 10 cm VAS at 30 s and 60 s (I). Alternatively the times elapsed before the first sensation of pain, and the sensation of strong pain, were recorded (III). In study I the volunteer was instructed to withdraw his hand earlier, if the pain became unbearable. The interval between the cold pressor test and the next blood sampling was at least 50 minutes.

Laboratory tests

Platelet aggregation

Platelet aggregation was measured with a four-channel photometric aggregometer (Packs-4, Helena Laboratories, USA). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifuging at +20°C at 160 g for 9 min and at 3000 g for 5 minutes, respectively. Platelet count in PRP was adjusted to 300 x 10^9 L^{-1} ± 10% by diluting with autologous PPP. During continuous stirring (1000 rpm) at 37°C, a 270 μL-sample of PRP was analysed. Aggregation was induced by adding 30 μL of an agonist; ADP to a final concentration of 3 or 1.5 μM, AA to a final concentration of 1000, 750 or 500 μM, collagen to a final concentration of 50 mg L^{-1}, thrombin receptor activating peptide (TRAP, SFLLRN) to a final concentration of 10 μM, or epinephrine to a final concentration of 5 μM. All concentrations of all agonists were not used in all the studies. The reagents were purchased from Sigma-Aldrich (St Louis, USA) and Bachem (Weil am Rhein, Germany). The aggregation was allowed to proceed for 300 s, after which plasma

for thromboxane B_2 (TxB_2) determination was prepared and the area under the curve of the aggregometry was recorded.
In study II, paracetamol (Perfalgan®) at a final concentration of 10, 20, 40 or 80 mg L^{-1} (66, 132, 265 or 529 μM) or diclofenac (Voltaren®) at a final concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.8 mg L^{-1} was added to PRP, prior to aggregometry. Drugs were added alone or in combination. Acetylsalicylic acid (100 μM) was used as a positive control.

PFA-100

In studies I and II the PFA-100 (Dade Behring, USA) was used to determine closure times of 900 μL-samples of citrated whole blood. After collection, all samples were incubated at room temperature for 30 min to 2 h. In study II the plasma volume was calculated based on the haematocrit and paracetamol was added at a final plasma concentration of 20, 40 or 80 mg L^{-1} (132, 265 or 529 μM) prior to analysis. 100 μM of acetylsalicylic acid was used as a positive control. Cartridges containing collagen/epinephrine or collagen/ADP membranes were used. The upper detection limit of the closure time is 300 s. When exceeded, the result was considered 300 s to allow statistical analysis.

Thromboxane B_2 concentration

TxB_2 is the stable metabolite of TxA_2, released during aggregation. TxB_2 concentrations in PRP triggered with ADP 3 μM or AA 1000 μM were determined with a radioimmunoassay.[181] The intra-assay coefficient of variation was 17% (n=10).

Paracetamol concentration

Blood samples were centrifuged at 3000 g for 10 min and plasma was stored at -20°C. Paracetamol concentration was determined using high performance liquid chromatography.[182] The limit of quantification was 0.1 mg L^{-1}, the day-to-day coefficients of variation were 4.2% at 2.5 mg L^{-1} and 4.1% at 14.4 mg L^{-1} (n=4).

Schild-plot and isobolographic analysis

In study II the effect of paracetamol and interaction of paracetamol and diclofenac were analysed with a Schild-plot and an isobolographic analysis, respectively.

Schild-plot

After addition of 0-120 mg L^{-1} of paracetamol to PRP, aggregation was induced with 100-3000 μM of AA to achieve near half-maximal responses. In every experiment (n=7) plain PRP, 3-4 different concentrations of paracetamol and 100 μM of acetylsalicylic acid were used. Aggregation in plain PRP induced with 1000 μM of AA was considered 100% aggregation and acetylsalicylic acid-treated plasma 0% aggregation. The concentrations of AA causing a 50% aggregation (EC_{50}) were determined with non-linear regression based on the Hill equation,[183] and the ratio of EC_{50} in paracetamol-treated and plain PRP (agonist ratio) was calculated. *Log (agonist ratio –1)* was plotted versus *log (paracetamol concentration)*. The apparent inhibition coefficients (K_i) were determined by linear regression with slope = 1. Actual slopes were calculated with linear regression.

Isobolographic analysis

Paracetamol (0-120 mg L^{-1}), diclofenac (0-2 mg L^{-1}) or both were added to PRP and aggregation was induced with 1000 μM of AA. Drug concentrations causing 50% inhibition of aggregation (IC_{50}) were determined as above and the interaction index (γ) was calculated:

$$\gamma = a/A+b/B$$

where *A* and *B* are the IC_{50} of paracetamol and diclofenac alone, and *a* and *b* are the IC_{50} of the same drugs when added in combination.[184] IC_{50} of paracetamol and diclofenac (i.e. *A* and *B* in the formula) were first determined independently. IC_{50} of diclofenac (i.e. *b* in the formula) in PRP containing approximately 0.25 IC_{50}, 0.5 IC_{50} or 0.75 IC_{50} of paracetamol (i.e. *a* in the formula) was then determined (0.75 IC_{50} was omitted in one experiment because of shortage of PRP).

Statistics

The studies I, III and IV were designed to discover a difference in platelet aggregation greater than one standard deviation (α-error = 5%) between the groups of interest.[185] The Bonferroni correction was applied when appropriate. A difference smaller than one standard deviation was considered of minor clinical significance. Study power was 80%, except in study IV, where the power was increased to 95% since no difference was anticipated.

Data was tested for normality with the Kolmogorow-Smirnov test. Non-normally distributed data in three or four groups was analyzed with Friedman's test (repeated measures ANOVA on ranks), and pairwise comparison was with the Wilcoxon matched pairs signed rank sum test (with Bonferroni correction when appropriate). In study II the Student-Newman-Keuls method was applied. From normally distributed data confidence intervals were calculated using the appropriate t-distribution. Statistical testing was done with SigmaStat for Windows Version 2.03 (SPSS Inc. Chicago, IL, US).

Results

Inhibition of platelet function by paracetamol (II, III)

Paracetamol *in vitro* showed a concentration-dependent inhibition of platelet aggregation triggered with AA 1000 μM (II, Fig. 1). Paracetamol 80 mg L^{-1} caused complete inhibition, as did acetylsalicylic acid 100 μM. The concentration-inhibition curve was sigmoidal when triggered with AA whereas a linear curve was displayed with ADP as a trigger (Fig. 5). With ADP the inhibitory effect of both paracetamol and acetylsalicylic acid was less pronounced.

In vivo, paracetamol exhibited a similar dose-dependent inhibition of platelet aggregation triggered with AA, ADP or epinephrine (III, Table 2). Ten minutes after infusion, all doses of paracetamol (15, 22.5 and 30 mg kg^{-1}) caused a significant inhibition of platelet aggregation triggered with AA. Inhibition was most pronounced with AA 500 μM, with AA 1000 μM the inhibition was minimal, although still statistically significant. Aggregation triggered with ADP or epinephrine was less sensitive to inhibition by paracetamol, at 10 minutes a significant inhibition was achieved only with paracetamol 30 mg kg^{-1}. Aggregation in response to all triggers was recovering at 90 min after infusion. Plasma paracetamol concentrations were determined after paracetamol administration (III, Table 1).

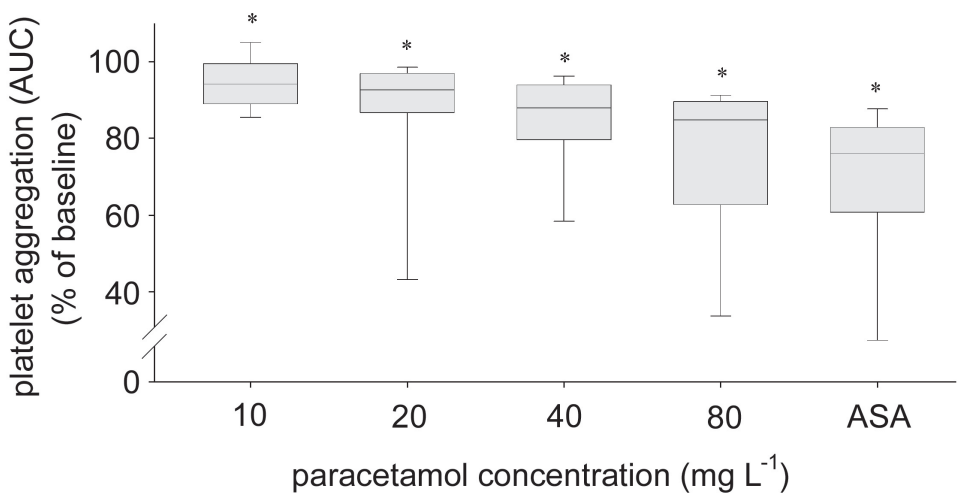

Figure 5. *Effect of paracetamol on platelet aggregation triggered with ADP (3mM). Acetylsalicylic acid (ASA) was used as a positive control. The box-plots show medians, 25th/75th and 5th/95th percentiles, n=9. P < 0.001, all concentrations; *P < 0.05 vs. baseline (no drugs added). Statistical tests are: Friedman's test (all concentrations) and the Student-Newman-Keuls method (pairwise comparison).*

Paracetamol inhibited TxB_2 release during aggregation both in vitro (II, Table 2) and in vivo (III, Table 3). In both systems aggregation triggered with AA released much larger amounts of TxB_2, and paracetamol inhibited TxB_2 release in response to AA less potently than in response to ADP, especially *in vitro*. Although paracetamol significantly inhibited TxB_2 release, the inhibition by paracetamol was less strong than that by diclofenac. Five minutes after diclofenac, TxB_2 release in response to ADP was only 3.3% of baseline release whereas it was 24% of baseline 10 minutes after paracetamol 30 mg kg^{-1}.

The PFA-100 closure time in collagen/epinephrine cartridges was significantly prolonged after the addition of acetylsalicylic acid (II, Table 3), but paracetamol caused a significantly prolonged closure time only at high concentrations.

A more detailed characterization of the inhibition of platelet aggregation by paracetamol with the Schild-plot showed a mean apparent K_i of 15.2 mg L^{-1}, with a 95% confidence interval of 11.8 – 18.6 mg L^{-1} (II, Fig 2, n=7). Regression lines were very close to linearity and the slope close to unity in the concentration range used; mean actual slope was 0.98 with a 95% confidence interval of 0.82 – 1.15.

Interaction of paracetamol and diclofenac (I, II)

The anti-aggregatory effect of diclofenac was augmented by paracetamol *in vitro* (II, Table 4). *In vivo*, diclofenac 1.1 mg kg^{-1} fully inhibited platelet aggregation immediately after administration, and no interaction was detected at this stage when diclofenac was combined with propacetamol 30 mg kg^{-1} (corresponding to paracetamol 15mg kg^{-1}) (I, Fig. 1, Table 1). Ninety minutes after drug infusion the effect of diclofenac had decreased, but in combination with propacetamol platelet aggregation was still almost fully inhibited (I, Fig. 1, Table 1). When aggregation was triggered with AA the difference between diclofenac and the combination was statistically significant (I, Fig 1). When aggregation was triggered with ADP or TRAP the over-all differences were much smaller, and no significant differences were detected between diclofenac and the combination (I, Table 1).

Release of TxB_2 during aggregation showed a similar pattern (I, Table 2). At 90 minutes, release of TxB_2 was significantly lower in the combination group than in the diclofenac group, when triggered with both AA and ADP. On the contrary, the PFA-100 failed to detect any difference between groups at 90 minutes, although closure times were significantly different from those of placebo in both treatment groups 10 minutes as well as 90 minutes after drug infusion (I, Table 3). On the next day, the inhibitory effect of both diclofenac and its combination with propacetamol had disappeared.

A more detailed characterization of the interaction *in vitro*, using an isobolographic analysis, showed that the interaction was synergistic (II, Fig. 3). Mean apparent γ was 0.86 with a 95% confidence interval of 0.74 – 0.99 (n=5).

Interaction of paracetamol and parecoxib (IV)

Parecoxib showed no inhibition ($P = 0.36$) whereas paracetamol 1 g showed a clear

inhibition of platelet aggregation triggered with AA 500 μM (P = 0.006, IV, Fig 1). No statistical difference occurred between the combination and paracetamol only (P = 0.85), although aggregation varied in five volunteers. Mean plasma paracetamol concentrations were similar in both groups; 12.7 mg L^{-1} (95% CI 11.6 – 13.8) in the paracetamol group, and 12.6 mg L^{-1} (95% CI 11.6– 13.7) in the combination group. Those three volunteers showing more inhibition by the combination had plasma paracetamol concentrations of 11.9, 9.7, and 14.2 mg L^{-1} after the combination and 16.8, 11.5, and 12.8 mg L^{-1} after paracetamol alone, respectively, whereas those two showing a clear inhibition only by paracetamol alone had plasma paracetamol concentrations of 14.9 and 10.9 mg L^{-1} after the combination and 14.6 and 13.8 mg L^{-1} after paracetamol alone.

To further elucidate any possible interaction between paracetamol and parecoxib, AA EC_{50}-values were determined *in vitro* after adding paracetamol 20 mg L^{-1}, before and after administration of parecoxib 40 mg. The selected volunteers, marked with closed circles in IV, figure 1, included all those with varying inhibition by paracetamol and the combination. Mean EC_{50} was 843 μM (95% CI: 705 – 981) before and 742 μM (95% CI: 616 – 868) after parecoxib administration. Mean change after parecoxib was -10.8% (95% CI: -23.7 – 2.3).

When triggered with AA 750 μM or 1000 μM, ADP (1.5 μM or 3 μM), or epinephrine (5 μM) platelet aggregation showed no differences between paracetamol 1 g and the combination of paracetamol 1 g and parecoxib 40 mg (IV, Table 1). TxB_2 release during aggregation triggered with ADP was inhibited by paracetamol and inhibition was the same after the combination (IV, Table 2). When triggered with AA, no differences between groups were detected.

Analgesic effect of paracetamol and diclofenac (I, III)

The cold pressor test showed a large inter- and intra-individual variation (I, Fig 2 and III, Fig 1). Pain threshold was not elevated by paracetamol, and pain ratings were unaffected by diclofenac 1.1 mg kg^{-1} and the combination of diclofenac 1.1 mg kg^{-1} and paracetamol 15 mg kg^{-1}.

Discussion

This thesis showed that paracetamol inhibits platelet aggregation and TxB_2-release dose-dependently in volunteers and concentration-dependently *in vitro,* determined the K_i value of the inhibition; 15.2 mg L^{-1} (95% CI 11.8 – 18.6), and showed that paracetamol displays synergism when inhibiting platelet aggregation together with diclofenac, but fails to interact with parecoxib. It also assessed the PFA-100 in detecting platelet dysfunction by paracetamol as well as the cold pressor test for measuring analgesia.

Inhibition of platelet function by paracetamol

Traditionally paracetamol is considered not to influence platelet function *in vivo* based on studies on conventional doses (approximately 1 g) of oral paracetamol.[168,169] The results in this thesis contradict the conclusion drawn from those studies. I suggest two main reasons for this contradiction. Firstly, ADP, epinephrine, and collagen were used to trigger aggregation in those previous studies. When platelet aggregation was triggered with ADP or epinephrine in the present study, only the highest dose of intravenous paracetamol, 30 mg kg^{-1} corresponding to a total dose of approximately 2 g, caused a significant inhibition. Secondly, a lower peak plasma paracetamol concentration is achieved with oral than with intravenous administration.[98]

Arachidonic acid triggered aggregation sensitive to paracetamol. The sigmoidal concentration inhibition curve of paracetamol, the linear Schild-plot, and the opposite effects of concentration of arachidonic acid in the assay and dose of paracetamol given to the volunteers, suggest that paracetamol competes with arachidonic acid or an intermediate compound in the TxA_2-synthesis pathway. The reason for this conclusion is derived from classic receptor theory, the mathematical considerations of which are shown in detail in the appendix. When plotted on a semi logarithmic scale with agonist concentration on the abscissa and response (0-100%) on the ordinate, the response to receptor stimulation by an agonist is a sigmoidal curve, increasing from 0% to 100%, described by the Hill equation:

$$\text{Effect} = E_0 + (E_{max} - E_0)\, C^\gamma / (C_{50}{}^\gamma + C^\gamma)$$

When adding a competitive inhibitor, the curve will move to the right, i.e. a 100% response can be achieved, but a higher concentration of agonist will be needed. In the present study AA was the agonist, paracetamol the inhibitor and platelet aggregation the response. Since the concentration of AA was kept constant in study II, figure 1, the Hill equation can be applied with only minor modifications:

$$\text{Effect} = I_0 - (I_0 - I_{max})\, C^\gamma / (C_{50}{}^\gamma + C^\gamma)$$

The result will be the same sigmoidal curve, but now it will decrease from the maximal effect towards the minimum upon

an increasing concentration of inhibitor. The sigmoidal shape in study II, figure 1, suggests that the modified Hill equation above can be implied. On the contrary, when ADP was used as an agonist in figure 5 the curve appears linear and the modified Hill equation above seems unsuitable. This is quite logical, since ADP binds to its own receptors, which paracetamol is not known to affect.

To further characterize the inhibition by paracetamol, the *inhibition coefficient* (K_i) can be determined. Since paracetamol itself does not elicit any response in platelets, K_i could not be determined directly. A possible technique for direct determination of dissociation constants would be the use of radiolabeled ligands, but since this technique was not available I turned to an old technique described by Arunlakshana and Schild in 1959 [186] (appendix).

The Schild-plot, used in study II, figure 2, to characterize the inhibition of platelet function by paracetamol, allows only a linear plot with unit slope, i.e. the sigmoidal concentration-response curve is equally displaced to the right by the same proportional increase in inhibitor concentration. This is true only for competitive inhibition, when the agonist can easily displace the inhibitor. If the inhibitor-receptor complex dissociates very slowly or the inhibitor binds to another site altogether, the agonist will not be able to displace the inhibitor, the concentration-response curve will be gradually flattened rather than displaced to the right, and the Schild-plot will be non-linear, or the slope will be different from unity, or both. In this case, the apparent K_i will have no meaning.[187] Since the Schild-plots appeared linear and the mean actual slope was very close to unity (0.98 with a 95% confidence interval of 0.82 – 1.15), it can be concluded that paracetamol is a competitive inhibitor in the concentration range tested. When paracetamol was given intravenously, the inhibitory effect of paracetamol also appeared fully reversible by an increased concentration of arachidonic acid in the assay, a fact that fits well with a competitive mechanism.

The mechanism by which paracetamol inhibits COX is not fully clear, but some clues are found in the literature. Ouellet and Percival[188] found that paracetamol acts as a reducing agent for COX and that paracetamol protects the enzyme from inactivation by H_2O_2, Boutaud and co-workers[189] found that the inhibitory effect of paracetamol can be reversed by adding PGG_2 to the assay, and Catella-Lawson and co-workers[190] found that paracetamol, in contrast to ibuprofen, does not prevent the anti-aggregatory effect of aspirin. Taken together these findings suggest that paracetamol inhibits the peroxidase rather that the cyclooxygenase reaction of COX by a competitive mechanism.

In platelets the same mechanism would be expected, although a downstream inhibition by TXAS cannot be excluded based on the current data or the literature.

Interaction between paracetamol and diclofenac or parecoxib

The antiaggregatory effect of analgesic doses of diclofenac is well known[163] and was confirmed in this study. As with paracetamol only, aggregation triggered with arachidonic acid was the most sensitive to inhibition. Diclofenac fully inhibited

platelet COX immediately after the infusion and at this stage no additional effect of paracetamol could be detected. At 90-min-sampling the antiaggregatory effect was decreasing because of the relatively short half-life of diclofenac in plasma, 1.1 h,[100] and the inhibitory effect was significantly augmented by paracetamol. Based on the findings in volunteers (I) the nature of this interaction could not be established. A pharmacokinetic interaction through protein binding or metabolism would have been possible, although less probable. In contrast to diclofenac, paracetamol is not highly protein-bound at therapeutic concentrations, and the drugs have different metabolic pathways in the liver.[58] Since the same interaction was observed *in vitro*, I conclude that the interaction is pharmacodynamic at the level of COX inhibition. By an isobolographic analysis an interaction can be characterised as synergistic, additive or subadditive.[184] The $\gamma < 1$ in study II suggests a synergistic inhibition. This fits well with slightly different mechanisms of COX-inhibition by paracetamol and the traditional NSAID diclofenac; the former inhibits the peroxidase reaction of COX, the latter inhibits the cyclooxygenase reaction of COX.

In contrast to diclofenac, valdecoxib has low affinity for COX-1,[191] and has no antiaggregatory properties.[192] Therefore, platelet function was unaltered 50 minutes after parecoxib administration, when the peak valdecoxib concentration had been reached with high probability. Paracetamol 1 g administered after parecoxib treatment caused an immediate antiaggregatory effect when aggregation was triggered with AA acid 500 μM. This effect was not statistically different from the effect of paracetamol without prior parecoxib. AA 1000 μM and 750 μM, on the other hand, triggered aggregation insensitive to both paracetamol alone and the combination. Lowering the concentration of AA in the assay, however, not only increases the sensitivity to the drugs, but also increases the amount of random variation as the individual aggregation threshold is approached. A high degree of both inter- and intra-subject variability in aggregation triggered with arachidonic acid has been previously reported.[193] Part of the variation in the results can be explained by random variation in paracetamol concentrations between experiments; two volunteers with a different degree of aggregation after paracetamol and combination showed a plasma paracetamol concentration more than one standard deviation higher in the non-aggregating sample. The interpretation that the variation is by random rather than an effect of parecoxib is further supported by the lack of difference between arachidonic acid EC_{50}-values before and after parecoxib administration.

Other triggers of aggregation

In addition to arachidonic acid, platelet aggregation was also triggered with ADP, epinephrine, and TRAP in order to stimulate the P2Y-, α-, and PAR-receptors, respectively. All these triggers showed a clearly lower sensitivity than AA to inhibition by paracetamol. This was quite expected, since no other target for paracetamol than COX have been suggested in platelets. Paracetamol would therefore only inhibit the AA-dependent part of platelet aggregation triggered by ADP,

epinephrine and TRAP. It is known that aggregation in response to these triggers is not dependent the AA pathway, although AA is released.[194] Release of AA by ADP requires both the $P2Y_1$ and the $P2Y_{12}$-receptor, as well as outside-in signalling through the GPIIb/IIIa complex.[195] Epinephrine is a weak trigger of platelet aggregation, mostly used to enhance aggregation by other triggers. In the case of thrombin, especially the PAR4 receptor appears important, since this receptor causes a long lasting Ca^{2+} influx that activates PLA_2.[196] PAR1 has, on the other hand, a higher affinity for thrombin, and is more important in inducing aggregation.[197]

Release of thromboxane B_2 from activated platelets

Another approach to evaluate COX inhibition is to measure release of the stable end-product of the COX-TXAS pathway, TxB_2. TxA_2 which is synthesized by TXAS is unstable and rapidly decomposes into TxB_2. The measurements of TxB_2 concentrations confirmed the results of the photometric aggregometry. Paracetamol dose-dependently inhibited TxB_2 release, the combination of paracetamol and diclofenac inhibited TxB_2 release more than either drug alone, and inhibition of TxB_2 release by the combination of paracetamol and parecoxib was not different from that by paracetamol alone.

The results of TxB_2 concentration measurements also confirm the fact that paracetamol is a weak inhibitor of COX.[5] Paracetamol 15 mg kg^{-1}, 22.5 mg kg^{-1} and 30 mg kg^{-1} caused a 35.1%, 64.9% and 76.8% reduction in TxB_2 release, respectively, compared to a 96.6% reduction after diclofenac 1.1 mg kg^{-1} in response to 3 μM of ADP.

PFA-100

The collagen/epinephrine closure time was prolonged after administration of both diclofenac and the combination of diclofenac and propacetamol, but showed no difference between the two groups. *In vitro*, PFA-100 was sensitive only to high concentrations of paracetamol. This is in accordance with the relatively low sensitivity to paracetamol of aggregation triggered with epinephrine. The high shear stress conditions in the PFA-100 probably also makes it less sensitive to COX-inhibition; shear-induced platelet aggregation is relatively independent of TxA_2.[198]

Analgesic effect of paracetamol, diclofenac and the combination

In contrast to clinical observations, the cold pressor test detected no analgesic effect of paracetamol or diclofenac. The short acting pain in the cold pressor test may explain the lack of an analgesic effect. Although the major mechanism of action of NSAIDs is peripheral, there is evidence in favour of a central mechanism involving spinal COX-2 inhibition.[199] The site of action of paracetamol is considered to be the central nervous system, and paracetamol has been shown to reduce central hyperalgesia induced by electrical stimulation.[200] Both spinal COX-2 upregulation and central hyperalgesia would probably need a noxious stimulus of longer duration

than the cold pressor test to evolve. Why 1 g of oral paracetamol showed an analgesic effect in this pain model in a previous study remains, however, unclear.[85]

Clinical considerations

The plasma concentration of paracetamol required for maximal analgesia is not known, but antipyretic properties of paracetamol are evident in the plasma concentration range of 10-20 mg L^{-1}.[201] The 95% confidence interval of K_i for paracetamol-induced platelet aggregation (11.8 – 18.6 mg L^{-1}) roughly equals the antipyretic concentration range. Twice the stimulus is therefore needed to elicit a normal platelet aggregation via the COX-TXAS pathway in the presence of an antipyretic concentration of paracetamol. These *in vitro* observations were confirmed in volunteers by significant inhibition of platelet aggregation by antipyretic plasma paracetamol concentrations.

It has been suggested that analgesia may require higher concentrations than antipyresis in adults, but this topic is controversial since few studies have measured both plasma paracetamol concentrations and analgesia. Beck and co-workers found that 20 mg kg^{-1} of rectal paracetamol caused concentrations partly below 10 mg L^{-1} whereas 40 mg kg^{-1} caused concentrations in the antipyretic range in adults.[202] In that particular study morphine consumption was, however, not reduced by the larger dose of paracetamol. Hahn and co-workers used intravenous paracetamol 5, 10, and 20 mg kg^{-1}, but were unable to detect any differences in alfentanil consumption after 1-3 h.[203] In children paracetamol showed a linearly increasing morphine-sparing effect when administered rectally in doses up to 60 mg kg^{-1}.[204] In an experimental study using transcutaneous electrical stimulation, 2 g of intravenous paracetamol was analgesically more effective than 1 g.[205] The same effect was observed after tooth extraction in a recent study.[206] In a clinical trial 1 g of intravenous paracetamol was more effective than the same dose given orally, suggesting that a high peak plasma concentration might be important, since the site of action of paracetamol is in the central nervous system.[207] Increasing the dose of paracetamol may therefore increase the analgesic effect, but our results indicate that an increasing inhibition of platelet function will follow.

What is the clinical relevance of the platelet function impairment observed in the present study? A precise answer to this question would require large clinical trials, and therefore the clinical implications remain speculative. A few studies have estimated the correlation between platelet function tests and surgical blood loss with conflicting results. Decreased platelet aggregation triggered with TRAP was associated with excessive postoperative blood loss,[208] and aggregation in response to collagen correlated inversely with postoperative blood drainage and need for blood transfusions after cardiopulmonary bypass.[209] In another study, however, collagen-induced platelet aggregation was similar in patients experiencing normal and excessive blood loss undergoing non-cardiac surgery.[210] Irani and co-workers found no correlation between perioperative blood loss during cardiopulmonary bypass surgery and platelet aggregation triggered with collagen, AA or ADP [211] whereas Greilich and co-workers found that a reduction in platelet force devel-

opment correlated inversely with blood drainage during 24 h after the same procedure.[212] PFA-100 does not seem suitable for predicting blood loss associated with cardiopulmonary bypass surgery. [213,214]

In contrast to paracetamol, traditional NSAIDs have been shown to increase the risk of perioperative haemorrhagia suggesting that the impairment of platelet aggregation observed with photometric aggregometry in the present study may be clinically relevant. In a systemic review on NSAIDs and bleeding after tonsillectomy, an increased risk for reoperation after use of NSAIDs was found.[9] Likewise, pre-treatment with ibuprofen appears to increase perioperative blood loss during total hip replacement [215] and low-dose aspirin has the same effect when given before transurethral prostatectomy.[216] Treatment with the COX-2 selective rofecoxib, on the other hand, showed a smaller intraoperative blood loss than treatment with diclofenac.[217] Since paracetamol inhibits TxA_2 synthesis less than traditional NSAIDs, surgical bleeding attributable to paracetamol seems unlikely in an otherwise healthy haemostatic system. The situation may change, however, if drugs are combined.

The combination of paracetamol and a traditional NSAID is useful for postoperative analgesia,[86] but this combination results in a synergistic inhibition of platelet function. This may suggest that the combination increases the risk of hemorrhagic events. Surgical patients today may receive other treatments that affect the haemostatic system, such as low molecular weight heparin, FXa antagonists or oral anticoagulants, or platelet inhibitors, such as clopidogrel or GPIIb/IIIa inhibitors. In these patients even a weak antiagreggatory effect of paracetamol may be clinically significant.

The clinical implications of reduced platelet aggregation could, however, also be advantageous. Low-dose aspirin has been shown to reduce the incidence of deep-vein thrombosis in patients undergoing surgery for hip fracture as well as the incidence of death from pulmonary embolism.[218] The high COX-2 selectivity of the coxibs seems, in fact, disadvantageous in certain circumstances. In contrast to the TxA_2 formation in platelets, PGI_2 in endothelium is synthesized from arachidonic acid by COX-2.[160] The use of coxibs therefore leads to a proaggregatory state, and an increased risk for thromboembolic events have been associated with these drugs in large randomized clinical trials.[219, 220] Patients undergoing cardiovascular surgery, for instance, should not use coxibs,[221] but valdecoxib appears safe after non-cardiac surgery.[222] Our results indicate that the combination of parecoxib and paracetamol causes a mild degree of COX-1 inhibition. It can be speculated that this combination proves useful in patients with a low risk of thrombotic events susceptible to hemorrhagic complications of nonselective NSAIDs.

Conclusions

1. Paracetamol exhibits a dose-dependent inhibition of platelet aggregation in response to arachidonic acid and of TxB_2 release from activated platelets when administered intravenously. Although statistically significant, the inhibition was weaker than that of diclofenac.

2. The inhibition of platelet function by paracetamol appears to be a competitive inhibition of COX based on a Schild-plot *in vitro*. A downstream inhibition of TXAS cannot be excluded, however.

3. Paracetamol augments the platelet inhibition by diclofenac in volunteers.

4. The interaction between paracetamol and diclofenac appears to be synergistic based on an isobolographic analysis *in vitro*.

5. Paracetamol does not interact with valdecoxib in inhibiting platelet function *in vivo*.

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Appendix. Receptor ligand interactions

Any drug (D) binding to its receptor (R) follows the simple law of mass action:

$$[D][R] \underset{k_2}{\overset{k_1}{\leftrightarrow}} [DR]$$

where [D] is the drug concentration, [R] is the amount of free receptors, [DR] is the amount of occupied receptors, k_1 is the rate constant for the drug binding to its receptor, and k_2 is the rate constant for drug-receptor dissociation.[1] The law of mass action can be described mathematically:

$$[D][R]/[DR] = k_1/k_2 = K_D \quad \text{(eq. 1)}$$

where K_D is known as the *dissociation constant*. If the drug concentration in eq. 1 is adjusted to achieve an equilibrium where 50 % of receptors are occupied, it follows that [R] = [DR] and K_D = [D], i.e. K_D equals the drug concentration of a half-maximal receptor occupation. In general, the *receptor occupancy* (O) would be the fraction of occupied receptors:[2]

$$O = [DR]/([DR] + [R]) \quad \text{(eq. 2).}$$

If we rearrange eq. 1, we get:

$$[R] = [DR]\ K_D/[D] \quad \text{(eq. 3)}$$

and when placed into eq. 2 it follows that:

$$O = [DR]/([DR] + [DR]\ K_D/[D]) \quad \text{(eq. 4).}$$

Simple rearrangment yields:

$$O = [D]/([D] + K_D) \quad \text{(eq. 5).}$$

If O is plotted versus [D], the result will be a hyperbola, asymptotically approaching 1, and K_D is the concentration where O = 0.5, as stated above.[2] If [D] is plotted on a semi-logarithmic scale, the plot will be the familiar sigmoid curve.

If instead of receptor occupation, biological effect of the drug is concidered, the relationship is slightly more complex. It is described by the Hill equation:[1]

$$\text{Effect} = E_0 + (E_{max} - E_0)\ C^\gamma\ /(C_{50}{}^\gamma + C^\gamma) \quad \text{(eq. 6)}$$

where E_0 is the baseline effect without any drug, E_{max} the maximum effect possible, C the drug concentration, C_{50} the drug concentration eliciting a half-maximal effect, and γ the Hill coefficient. If one considers the simplified case where no baseline effect is present, E_0 = 0, and denotes the full effect E_{max} = 1, the Hill equation can be simplified to:

$$\text{Effect} = C^\gamma\ /(C^\gamma + C_{50}{}^\gamma) \quad \text{(eq. 7).}$$

When comparing equations 5 and 7, the similarity is obvious, except the exponent γ in the latter. The exponent, the Hill coefficient, is γ = 1 in most cases where the drug itself has no influence on receptor affinity (for instance via a regulatory receptor site).[2] If γ = 1 equations 5 and 7 will be fully congruent, and the

concentration-effect relationship will be described by the same sigmoidal curve on a semi-logaritmic scale.

The Schild-plot, described by Arunlakshana and Schild in 1959, is derived from the equations above.[3] According to the law of mass action, an inhibitor (I) will bind to its receptor in accordance with equation 1:

$$[I][R]/[IR] = K_{DI} \quad \text{(eq. 8)}.$$

Equation 2, describing receptor occupancy of the agonist (D), will be modified to:

$$O = [DR]/([DR] + [R] + [IR]) \quad \text{(eq. 9)}$$

taking into account that part of the receptors will be occupied by the inhibitor and therefore unavailable for agonist binding. From equation 8 it follows that:

$$[IR] = [I][R]/K_{DI} \quad \text{(eq. 10)}$$

and if equation 10 is inserted into equation 9 we get:

$$O = [DR]/([DR] + [R] + [I][R]/K_{DI}) \quad \text{(eq. 11)}$$

into which equation 3 can be inserted and by rearrangement it follows that:

$$O = [D]/([D] + K_D(1+[I]/K_{DI})) \quad \text{(eq. 12)}$$

which in fact is a generalization of equation 5. If no inhibitor is added, i.e. [I] = 0, equation 5 will result. Graphically equation 12 will yeld the same sigmoidal curve as equation 5, but when increasing concentrations of inhibitor are added, the curve will be displaced to the right. This is quite intuitive, when considering the definition of a competitive antagonist; by increasing the concentration of the agonist the initial receptor occupancy will eventually be achieved. Mathematically this can be expressed as an equal receptor occupancy:

$$[D]/([D] + K_D) = [D]_I/([D]_I + K_D(1+[I]/K_{DI})) \quad \text{(eq. 13)}$$

where [D] is the agonist concentration in the absence and $[D]_I$ in the presence of inhibitor. Now, if we define the *agonist ratio* = $[D]_I/[D]$ it follows that:

$$[D]_I = [D]\textit{agonist ratio} \quad \text{(eq. 14)}$$

and if equation 14 is inserted into equation 13 we get:

$$[D]/([D] + KD) = [D]\textit{agonist ratio}/([D]\textit{agonist ratio} + KD(1+[I]/KDI)) \quad \text{(eq. 15)}$$

which by rearrangement yields:

$$\textit{agonist ratio} - 1 = [I]/K_{DI} \quad \text{(eq. 16)}.$$

If we draw the logarithm of both sides it follows that:

$$\log(\textit{agonist ratio} - 1) = \log([I]) - \log K_{DI} \quad \text{(eq. 17)}$$

which by definition is the same as:

$$\log(\textit{agonist ratio} - 1) = \log([I]) + pK_{DI} \quad \text{(eq. 18)}.$$

Equation 18 transforms into a simple linear equation (y = x+b) if plotted on a

logaritmic scale; log(*agonist ratio* –1) versus log([I]) yields a straight line intersecting the x-axis at pK_{DI}.

So far I have described the interplay between an agonist and an inhibitor relative to receptor occupancy. These equations easily transform into a description of the agonist/inhibitor concentrations versus biological effect. As I showed above (equations 5 and 7), the Hill equation is a generalization of the law of mass action, introducing the Hill coefficient (γ). In the simplified case, when $\gamma = 1$, the two equations are congruent and equation 18 turns into:

$$\log (\textit{agonist ratio} - 1) = \log([I]) + pK_i$$
(eq. 19).

The distinction between K_{DI} and K_i is important. The former is the dissociation constant of the inhibitor-receptor complex, denoting the inhibitor concentration at which half the receptors are occupied whereas the latter is the inhibition coefficient, denoting the inhibitor concentration at which half the biological effect is blocked. Often K_i is markedly bigger than K_{DI}, since the cell carries a vast extra receptor capacity. Equation 19 represents the Schild plot used in study II, Figure 2.

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