THE DEVELOPMENT OF COX2 INHIBITORS

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Aspirin, arguably the world's favourite drug, has been around since the late nineteenth century, but it wasn't until the late 1970s that its ability to inhibit prostaglandin production by the cyclooxygenase enzyme was identified as the basis of its therapeutic action. Early hints of a second form of the cyclooxygenase that was differentially sensitive to other aspirin-like drugs ultimately ushered in an exciting era of drug discovery, culminating in the introduction of an entirely new generation of anti-inflammatories. This article reviews the story of this discovery and looks at the future of cyclooxygenase pharmacology.

CASE HISTORY

ISOZYME (ISOENZYME)
One of several forms of an enzyme in an individual or population that catalyse the same reaction but differ from each other in such properties as substrate affinity and maximum rates of enzyme–substrate reaction.

ANTIPYRETIC
Describes the fever-suppressive activity of a drug.

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The league table listing the 'top 20' drugs includes rofecoxib (Vioxx) and celecoxib (Celebrex), two inhibitors of the prostaglandin-forming cyclooxygenase (COX) (FIG. 1), which between them commanded sales in excess of US $4 billion in the year 2000 (REF. 1). This statistic might seem surprising — after all, the therapeutic use of COX inhibitors has a venerable history dating back to the introduction of aspirin in 1898 (or even earlier if the use of salicylate-containing plant extracts is included) and, since then, the field has a record of almost continuous development. The 1940s saw the introduction of phenylbutazone, the fenamates appeared in the 1950s, indomethacin in the 1960s, the proprionates in the 1970s and the oxicams in the 1980s. With such a long record of drug discovery in the area and such a vast range of drugs to choose from, one might be forgiven for thinking that the wellspring of chemical innovation that nurtured the field so efficiently over the past century must have long since run dry — or at least begun to falter — and that the current market would have little room for new versions of what would seem to be a rather tired and well-worn formula.

However, the discovery in the early 1990s of a second COX ISOZYME revitalized the field and stimulated a hunt for new and selective isoform inhibitors. This culminated in the introduction of Vioxx and Celebrex in Europe and North America within a mere ten years, and, at the same time, brought a fresh perspective on the unusual therapeutic profile of several existing non-steroidal anti-inflammatory drugs (NSAIDs). But we are getting ahead of ourselves — how did this idea of the second isoform come about in the first place and what are the therapeutic advantages of these new inhibitors over the many older drugs which, after all, have seen valiant clinical service over the decades? To answer these questions, we must return to the 1970s when much of the seminal work on COX inhibition was published.

Early work

Although the NSAIDs do not reverse the course of systemic diseases such as arthritis, they form the mainstay symptomatic treatment of many inflammatory disorders and soft-tissue injuries. Approximately 50 NSAID preparations are listed in *Monthly Index of Medical Specialities* and, as a class, these are among the most commonly prescribed drugs. In the United Kingdom, for example, recent data (1999) indicate that 18.5 million prescriptions are written for NSAIDs each year at a cost of £170 million — and this figure doesn't take into account the over-the-counter sales, which are considerable. Aspirin itself is still consumed in prodigious amounts around the world and new uses are continually being found for this drug.

NSAIDs are sometimes known as the aspirin-like drugs because they have an activity profile that is broadly similar to that of aspirin. That is, they all possess

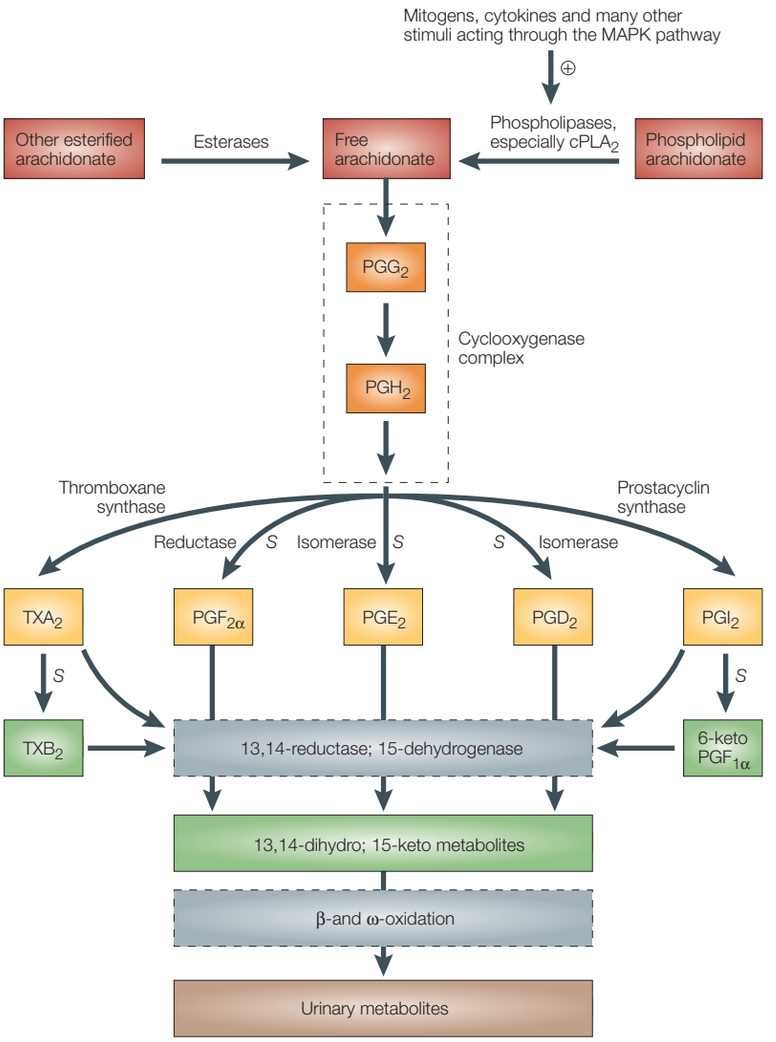

Figure 1 | **An overview of prostaglandin synthesis and metabolism.** In theory, free fatty acids such as arachidonate can be formed from several sources, although phospholipid-bound arachidonate is probably the most significant pool. Phospholipases, especially cytosolic phospholipase A_2 ($cPLA_2$), are highly-regulated enzymes (by the MAP kinase (MAPK) pathway) that liberate arachidonate, which is then transformed by the cyclooxygenase (COX) complex. The mechanism of this reaction is complex — 2 moles of molecular oxygen are introduced sequentially by a lipoxygenase reaction, followed by a COX reaction. This generates PGG_2, a 15-hydro-peroxide prostaglandin that is reduced to PGH_2, the corresponding hydroxy product. Both of these intermediates are short-lived but may have independent biological activity. However, in most cases it is not yet clear whether this independent activity is significant *in vivo*. A further battery of enzymes transform PGH_2 into a variety of products. Some of these enzymes, such as thromboxane (TX) synthase and prostacyclin (PGI_2) synthase, show marked tissue localization (for example, TX synthase in platelets and PGI_2 synthase in vascular endothelium). Other enzymes, such as the endoperoxide reductases and isomerases, are quite widely distributed, although in some cases they can be induced following inflammatory stimuli. *In vitro* at least, the endoperoxides PGG_2 and PGH_2 can also decay spontaneously (*S*) to $PGF_{2\alpha}$, PGE_2 and PGD_2. The evanescent products, TXA_2 and PGI_2, decay spontaneously to their respective inactive metabolites, TXB_2 and 6-keto $PGF_{1\alpha}$. The biological activity of the other prostaglandins is curtailed following uptake into cells, by a series of metabolic enzymes that are present in some tissues (for example, the lung) at high concentrations. Inactive metabolites of these prostanoids undergo carbon chain shortening (especially in the liver) prior to secretion in the urine, in which estimates of total body prostaglandin turnover can be made by selectively monitoring these products. The most significant products from the point of view of this review are PGE_2, because of its importance in inflammation, fever and pain; PGI_2 because of its anti-aggregatory action and possible role in hyperalgesia, and TXA_2 because of its important role in platelet aggregation. Colour code: red, precursors; orange, intermediates; yellow, 'primary' prostaglandins that mediate most of the biological activity of this system; green, inactive or largely inactive metabolites; brown, end metabolites that are excreted primarily in the urine.

analgesic, anti-inflammatory and ANTIPYRETIC properties to some degree, and produce characteristic side effects, including gastric intolerance and depression of blood clotting through inhibitory action on platelet function. As a group, the NSAIDs are structurally diverse, with most (but not all) being carboxylic acids (FIG. 2). The main question, from the pharmacologist's point of view, was how these apparently disparate therapeutic and side effects were mechanistically linked.

There were several early suggestions (REFS 2,3), but the real breakthrough came in 1971. Vane tells us[4] that the idea that the aspirin-like drugs blocked the conversion of substrate arachidonic acid to prostaglandins came to him while reviewing experiments in which aspirin blocked the release of 'rabbit aorta contracting substance' (RCS) from guinea-pig and dog lung. Believing that RCS was an intermediate in prostaglandin synthesis, he wrote "…a logical corollary was that aspirin might well be blocking the synthesis of prostaglandins". A quartet of papers appeared that year from Vane and members of his group, showing that aspirin itself, indomethacin and (less effectively) sodium salicylate blocked prostaglandin synthesis in a cell-free system[5] and in isolated perfused spleen of dogs[6]. In humans, therapeutic doses of aspirin taken by volunteers reduced prostaglandin generation by aggregating platelets *ex vivo*[7] or in seminal plasma samples collected during the course of the treatment[8]. The overall message was clear — at least some NSAIDs were able to prevent the generation of prostaglandins by direct action on the COX enzyme, and did so in humans in clinical doses. But how was this linked to their therapeutic actions?

The late 1960s and early 1970s had seen an explosion of interest in the biology of the prostaglandins. It had already been shown that prostaglandins are generated during platelet aggregation to produce fever, HYPERALGESIA and inflammation (reviewed by Willis[9]). Prostaglandins had also been detected in gastric mucosa and been shown to inhibit ulcer formation in rodents[10]. In other words, the ability of NSAIDs to block COX provided the much sought after link between the therapeutic and side effects of these drugs.

Over the next couple of years, several other important findings emerged. Significantly, it was shown that the entire gamut of NSAIDs inhibit COX at concentrations well within their therapeutic plasma range and that the overall order of potency corresponded to their therapeutic activity[11]. Other types of anti-inflammatories, such as the glucocorticoids and the so-called disease-modifying drugs, were inactive in these cell-free assays, providing further evidence for the specificity of the effect. Using ENANTIOMERIC pairs of NSAIDs, such as naproxen[12], an exquisite correlation was observed between the anti-inflammatory and anti-COX activity, and many more studies confirmed the notion that this is a fundamental mechanism of this class of drug (reviewed in REF. 13).

By 1974, Vane's concept was firmly established. Researchers were quick to seize upon the fact that NSAIDs could be used to probe the functions of prostanoids in physiology and pathology, thereby opening an entirely new chapter in eicosanoid research.

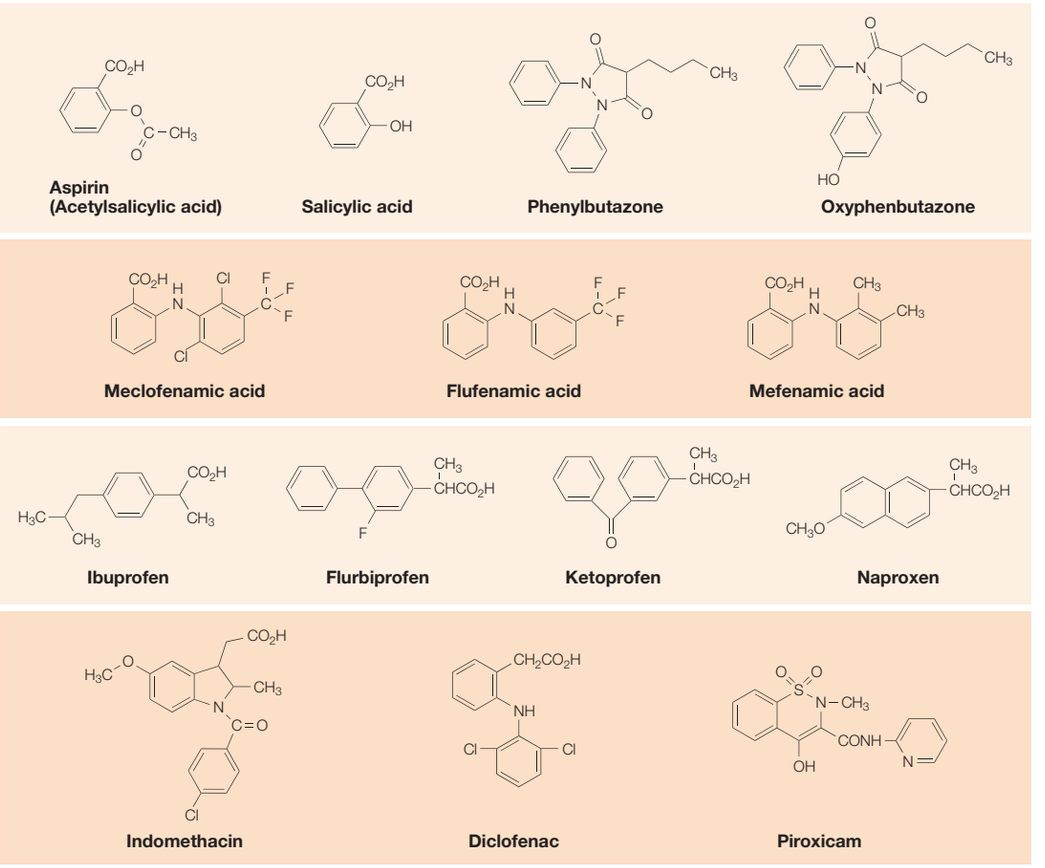

Figure 2 | **Chemical structures of NSAIDs and related compounds.** Structures of some 'classical' NSAIDs, including representative salicylates, pyrazolones, fenamates, proprionates, oxicams and indomethacin. Note the general presence of a carboxylic-acid moiety.

Parenthetically, one might add that the pharmaceutical industry now possessed, probably for the first time, a simple and robust *in vitro* technique to screen compounds for putative anti-inflammatory activity. This in itself was a significant advance, and the number of chemical abstracts dealing with potential inhibitors of the COX enzyme rose markedly, with more than 2,500 per year recorded within a decade of these ideas taking hold.

But right from the beginning of the story, several anomalies were noted. The most relevant concerns paracetamol (acetaminophen) — another hugely popular drug that was also introduced in the 1890s (although it was not in common use until some 40 years later). As for all other aspirin-like drugs, paracetamol possessed antipyretic and analgesic activity but, unlike most, had little anti-inflammatory activity and caused virtually no gastric or platelet side effects. Apparently in accord with its therapeutic profile, paracetamol was found to have a different pattern of inhibitory activity, being more effective against brain COX than enzyme prepared from peripheral tissues such as the spleen[14]. At the time, this was put forward as a putative explanation for the selectivity of its therapeutic action, and the idea that there are several forms of the enzyme was formulated. Wide variations in the inhibitory potency of indomethacin against COX enzymes prepared from a range of tissues was subsequently reported[15] and the isoenzyme idea was further elaborated in several reviews[13,16].

The development of the field

Despite the early indications for alternative forms of COX, little concrete evidence emerged to support this idea for several years. The structural features of COX, which has dual hydroperoxidase and cyclooxygenase activity[17], were not well understood. With the notable exception of bovine or ovine seminal vesicles, COX is usually expressed in tissues in low abundance. As a dimeric membrane-bound protein, it posed many challenges to purification and was not sequenced until 1988 (REFS 18,19). Other factors complicating the interpretation of potency differences included the wide variations in assay conditions that were used by different groups — which is still a problem today — as well as differences in the kinetics of the COX inhibitors, some of which produced 'competitive reversible' inhibition, whereas others exhibited unusual inhibitory effects such as the 'competitive non-reversible' mechanism that is observed in the case of indomethacin[20]. The discovery[21,22] that, alone among the group, aspirin

HYPERALGESIA
An abnormal state of increased sensitivity to painful stimuli.

ENANTIOMERS
A pair of compounds whose molecular structures are mirror images of each other.

irreversibly acetylated a serine residue (Ser530) within the COX active site to produce its effects, reinforced the opinion held by many workers at that time that the NSAIDs were a chemically heterogeneous group of drugs with widely differing modes of inhibitory action. In short, it seemed at least possible that some of these differences in inhibitor potency could be the result of factors other than the presence of isozymes.

Meanwhile, other apparently unrelated investigations were to provide the backdrop for future dramatic revelations. Using a model of rabbit kidney inflammation induced by ligation of the urethra, Needleman's group observed[23] that the affected, but not the CONTRALATERAL, organ unexpectedly developed an enormous capacity to generate prostaglandins. In the following year[24], the group showed that this increase was due to *de novo* synthesis of fresh enzyme, although no suggestion was made at this time that the new enzyme was a variant form. Nevertheless, this theme was pursued by the Needleman group over the next few years and, in 1982, a paper appeared that suggested the presence of two distinct forms of COX in brain tissue with differing sensitivities to indomethacin[25]. Other studies in gastrointestinal tissue were also supportive of the selectivity of action of NSAIDs in different tissues[26].

The problem was taken up again by several laboratories towards the end of the 1980s. Treatment of vascular smooth muscle cells with epidermal growth factor (EGF)[27] or fibroblasts, and monocytes with pro-inflammatory stimuli such as interleukin-1[28,29] or lipopolysaccharide[30], induced *COX* mRNA and *de novo* synthesis of enzyme which, when partially sequenced, seemed to be identical to the bovine seminal vesicle COX. In one paper[31], Needleman's group wrote "Clearly, those putative enzyme pools may arise as different gene products, possibly through the expression of different COX genes…". Interestingly, glucocorticoids inhibited the induction of this new COX protein without altering the amount of enzyme that was constitutively present in cells[32]. Elsewhere, further evidence emerged indicating different forms of the enzyme. For example, Wong and Richards, using immunological techniques, reported two isoforms in the rat ovary, one of which was regulated by hormones[33].

The discovery of COX2

Paradoxically, the next significant step forward came in 1991 from workers in an entirely different field. While investigating the expression of early-response genes in fibroblasts transformed with Rous sarcoma virus, Simmons and his colleagues[34] identified a novel mRNA transcript that coded for a protein that had a high sequence similarity, but was not identical, to the seminal vesicle COX enzyme. The suggestion was that a COX isozyme had been discovered. Independent and simultaneous confirmation of this exciting finding came from the laboratory of Herschman and colleagues, who discovered a novel cDNA species encoding a protein with a predicted structure similar to COX1 while studying phorbol-ester-induced genes in Swiss 3T3 cells[35]. The same laboratory subsequently showed that this gene product was indeed a novel COX[36] and that its induction was inhibited by dexamethasone[37]. Similarly indicative findings were also reported in mouse fibroblasts[38], cultured rat mesangial cells[39], RAW 264.7 cells[40], rat alveolar macrophages[41,42], the ovary[43,44] and other cell types[45].

But was this enzyme physiologically relevant? Needleman's group conclusively identified their inflammation-inducible form of COX as the species that both Simmons and Herschman had cloned[32]. As the evidence for the relevance of the two enzymes mounted, they were renamed COX1, referring to the original enzyme isolated from seminal vesicles and subsequently found to be distributed almost ubiquitously, and COX2, denoting the 'inducible' form of the enzyme (although it was expressed basally in the brain[46]). The two genes had a different chromosomal organization in rodents[47] and humans[48], and *COX1*/*COX2* mRNA was differentially expressed[49] in human tissues. Promoter analysis confirmed a fundamental difference between the two isozymes, with the *COX2* promoter containing elements strongly reminiscent of those genes that are switched on during cellular stress and switched off by glucocorticoids[50], whereas *COX1* had the appearance of a 'HOUSEKEEPING' GENE. Histological and other studies confirmed this apparent division of labour between the two enzymes, and COX1 seemed to be the predominant isoform in healthy gastrointestinal tissue from rat, dog and monkey[51].

These facts begged a key question — if COX2 was the predominant inflammatory species, shouldn't this be the target for NSAIDs? If this was so, the corollary was surely that COX2 inhibition was the true therapeutic modality of NSAIDs, whereas COX1 inhibition might be the cause of side effects such as gastric irritation and depression of platelet aggregation. This was the notion put forward by more than one group[52–54], which came to be known in a rather Orwellian way as the 'COX2-bad:COX1-good' hypothesis. If these propositions were true, then a selective COX2 inhibitor would be an ideal drug, possessing the anti-inflammatory action but lacking the gastric and other side effects. But would it be possible to find or design such a drug?

Some encouragement for this idea could be derived from publications of the day[52,53]. Most of the non-steroidal drugs available at that time actually inhibited both enzymes to a greater or lesser degree, but some selectivity of action was seen with experimental drugs such as 6-MNA and BF389. But other data that subsequently emerged from transgenic approaches were less encouraging. *Cox1*-null (REF. 55) and *Cox2*-null (REF. 56) mice did not behave exactly as expected — Cox1-deficient mice did not have spontaneous stomach ulcers and, whereas the ulceration caused by indomethacin in these animals was less than in the wild types, it was still very much in evidence. Again contrary to theoretical predictions, homozygous mutant mice lacking Cox2 showed a normal (albeit acute) inflammatory response when treated with several agonists, apparently contradicting the idea that Cox2 was the predominant enzyme in

CONTRALATERAL
On or affecting the opposite side.

HOUSEKEEPING GENE
A gene that is usually expressed at a fairly constant rate in cells as it subserves some constant physiological requirement. By comparison, an inducible gene is one that normally appears at a very specific time and in response to a specific stimulus.

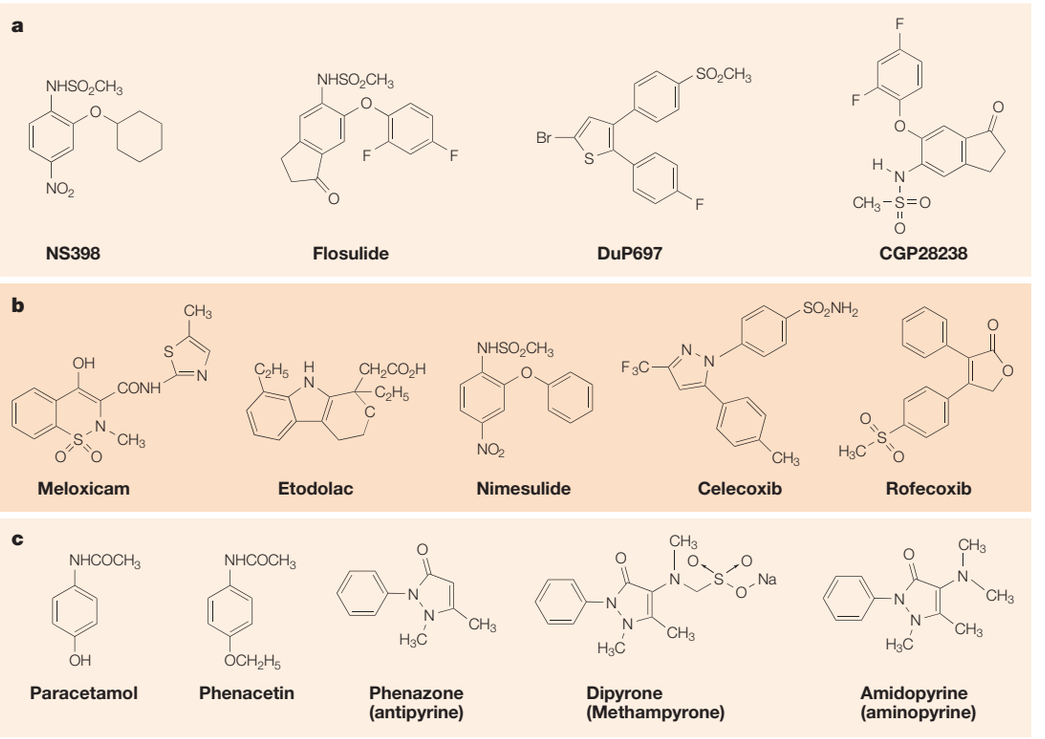

Figure 3 | **Chemical structures of NSAIDs and related compounds. a** | Structures of DuP697, NS398 and other similar compounds. **b** | Selective COX2 inhibitors that were discovered as a result of a search for selective isoform inhibitors (celecoxib and rofecoxib) or that were 'revealed' as being COX2 selective (meloxicam, etodolac and nimesulide). **c** | Structures of some compounds that are more effective inhibitors of COX3 according to Simmons[124].

inflammation[56]. From the medicinal chemist's point of view, there was also another depressing fact — the sequence of the catalytic domains of the two isozymes was so similar that the prospect of finding a specific inhibitor seemed remote. Fortunately, this perception was soon to be changed in a rather dramatic way.

'Sleepers'

DuP697 was a drug reported in 1990 by the Dupont Company to be an effective anti-inflammatory agent with reduced ulcerogenic properties[57]. Significantly, DuP697 showed only feeble activity in *in vitro* assays of COX using seminal vesicle or rat kidney preparations (known to predominantly contain COX1), but was more effective against rat brain prostanoid synthesis. The authors originally attributed the gastrointestinal safety of this compound to its chemical structure (a non-acidic thiophene) (FIG. 3a), which was presumed to have different pharmacokinetic properties to other COX inhibitors, most of which were carboxylic acids. Reports of other compounds with similar properties were published later, including some experimental compounds such as NS398, flosulide and CGP28238 (REF. 58) (FIG. 3a). However, in light of the discovery of COX2, it was not long before alert pharmacologists realized that these drugs might have this unusual behaviour because they acted predominately on the COX2 isozyme.

The moment of this Damascene revelation cannot be pinpointed precisely but, in the context of future drug development, two 'prostaglandin' meetings in 1992 (in Keystone, Colorado, in January, and Montreal in July) seem to have been of particular significance. At the first meeting, the Dupont Group presented evidence that gastric tolerance to DuP697 might be accounted for by differential inhibition of COX enzymes. At the second meeting, workers from Taisho described similar data with another structurally unrelated compound, NS398 (REFS 59,60). These meetings were attended by many industrial scientists, and it seems clear from published accounts that these events initiated — or at least greatly accelerated — many in-house 'COX2' programmes. It is also clear that the structures of DuP697 and NS398 were crucial starting points in the hunt for new selective inhibitors. Two companies who capitalized on these leads were Searle Monsanto (now Pharmacia) and Merck. The former focused on sulphonamide-substituted 1,5-diaryl pyrazole compounds, whereas Merck scientists settled on a series of methylsulphonylphenyl compounds.

While medicinal chemistry programmes were being developed, the field continued to produce data that were, on the whole, supportive to the COX1/COX2 concept. For example, COX2 was found in human SYNOVIAL TISSUE taken from patients with rheumatoid arthritis, and the 'inducibility' of this enzyme by cytokines was

SYNOVIAL TISSUE
The tissues that surround the joints.

confirmed[61,62]. Support for the COX1/COX2 concept also came from pharmacological studies. Masferrer *et al.*[63] showed that the onset of inflammation in the rat air pouch correlated with the appearance of COX2 in the lesion, and that NS398 blocked the production of prostaglandins at this site without causing intestinal lesions or affecting gastric prostaglandin synthesis. Similar results were seen in various inflammatory models with an early Searle Monsanto selective inhibitor SC58125 (REFS 64,65). A number of other useful, selective COX2 inhibitors were soon described in the literature — SC558 (a celecoxib prototype) showed good efficacy in rodent models of inflammation, fever and pain, whereas the closely related SC560, a selective COX1 inhibitor, was ineffective[66]. Celecoxib itself (then known as SC58635) reversed CARRAGEENAN-induced hyperalgesia and local prostaglandin production[67] in rats, and a related compound was active intrathecally[68]. Recombinant COX1 and COX2 had by now been prepared, and the selectivity of DuP697 and NS398 had been confirmed with *in vitro* assay systems using these enzymes[69,70].

The solution of the crystal structures of COX1 in 1994 (REF. 71) and COX2 in 1996 (REF. 72) made a substantial impact on the field. In the former paper, attention was drawn to the crucial roles of arginine 120 (Arg120, which interacted with the carboxyl group of both substrate and inhibitors) and tyrosine 355 (Tyr355) in determining the stereospecificity of NSAID binding (FIGS 4, 5). In the latter paper, the structure of COX2 was determined in the presence of several inhibitors, enabling the authors to deduce the conformational and other changes required for selective inhibition. Despite the great similarity in the sequence data, detailed examination of the structure of the catalytic sites revealed the substrate binding 'channel' in the two enzymes to be quite different. A single amino-acid change, from the comparatively bulky isoleucine (Ile) in COX1 to valine at position 523 in COX2 (equivalent to position 509 in COX1), and the conformational changes that this produced resulted in enhanced access to a 'side pocket' that allowed the binding of COX2-specific inhibitors by providing a docking site for the bulky phenylsulphonamide residue of drugs such as SC558 (FIG. 4). In an elegant demonstration of how crucial this single change was, Gierse *et al.*[73] showed that mutation of this residue in COX2 back to Ile largely prevented selective inhibitors such as SC58125, SC236 and NS398 from working. Further work on the structural basis for inhibition of both isoforms delineated the role of Arg106 in the binding of substrate and certain NSAIDs[74], as well as the role of Tyr355 located at the entrance of the active site of COX2 (REF. 75).

These structural data also helped explain differences in the inhibitory kinetics of COX1 and COX2 with drugs such as DuP697 and NS398 (REF. 76). There are, as stated above, several distinct mechanisms by which COX1 inhibitors can inhibit the enzyme, but many are of the competitive reversible type. By contrast, the COX2 inhibitors are irreversible, time-dependent (in the context of enzyme kinetics) inhibitors, partly as a result of the binding of the sulphonamide (or related) moiety into the enzyme 'side pocket'. In an analysis of the kinetic behaviour of several COX inhibitors, Gierse *et al.*[77] subsequently discerned four separate modes of enzyme inhibition, ranging from the simple competitive inhibition of drugs such as ibuprofen, through the 'weak binding, time-dependent' mechanism of naproxen and the oxicams and the 'tight binding, time-dependent' inhibition of indomethacin , to the covalent modification produced by aspirin.

The development of the 'coxibs'

Encouraged by the 'concept testing' experiments with selective inhibitors, and armed with several solid leads and a clear idea of the nature of the binding site, development of this field was rapid. Celecoxib arose from the Searle Monsanto programme and showed marked selectivity for COX2 *in vitro*[78]. Preclinical studies with this compound revealed that the drug had good efficacy in rodent models of inflammation, fever and pain. Early human studies confirmed its effectiveness in the treatment of osteoarthritis, rheumatoid arthritis and post-surgical pain when tested in comparison with a placebo or with comparator NSAIDs such as naproxen, ibuprofen and diclofenac. Crucially, there was also confirmation of the reduced incidence of platelet and gastrointestinal side effects (reviewed by Lefkowith[79]), and celecoxib was subsequently licensed in the United Kingdom for osteoarthritis and rheumatoid arthritis in 2000.

From Merck's methylsulphonylphenyl series came MK0966, later named rofecoxib. Once again, a series of preclinical studies confirmed the efficacy and gastrointestinal safety of this compound in rodent models of inflammation, and early human studies established its clinical utility in fever and pain (dental, DYSMENORRHOEA and post-operative models) when compared with standard NSAIDs such as ibuprofen or naproxen (reviewed by Morrison[80]). In osteoarthritis and rheumatoid arthritis, rofecoxib proved superior to placebo and comparable in efficacy to standard doses of other NSAIDs such as diclofenac. Rofecoxib was also found to have greater gastrointestinal safety and not to affect platelet aggregation — it was licensed in the United Kingdom for osteoarthritis in 1999 and rheumatoid arthritis in 2001.

But it was not just the quest for new drugs that was stimulated by the discovery of COX2. The enolcarboxamide meloxicam (Boehringer Ingleheim), as well as the tetrahydropyranoindole etodolac (Wyeth/Shire), were drugs already under development at the time that the COX2 story 'broke', whereas a sulphonanilide drug, nimesulide (Helsinn), had been marketed in Europe since 1985 (FIG. 3b). In each case, clinical and experimental evidence already indicated that these agents were different from the other NSAIDs, especially in terms of their good gastrointestinal tolerance. Pharmacologists could now view these anomalous data with a vision greatly sharpened by the emerging COX2 concept, and these drugs turned out to be effective COX2 inhibitors[81–83]. In an authoritative survey, Warner *et al.*[84] found, for example, that meloxicam and etodolac showed almost the same order of selectivity for COX1/COX2 as some of the newer agents.

CARRAGEENAN
A sulphated cell-wall polysaccharide that is found in certain red algae, which contains repeating sulphated disaccharides of galactose, and sometimes anhydrogalactose, and is used to induce an inflammatory lesion when injected into experimental animals.

DYSMENORRHOEA
Painful menstruation, often associated with nausea, vomiting, headache and faintness. It is thought to be related to excessive prostaglandin production.

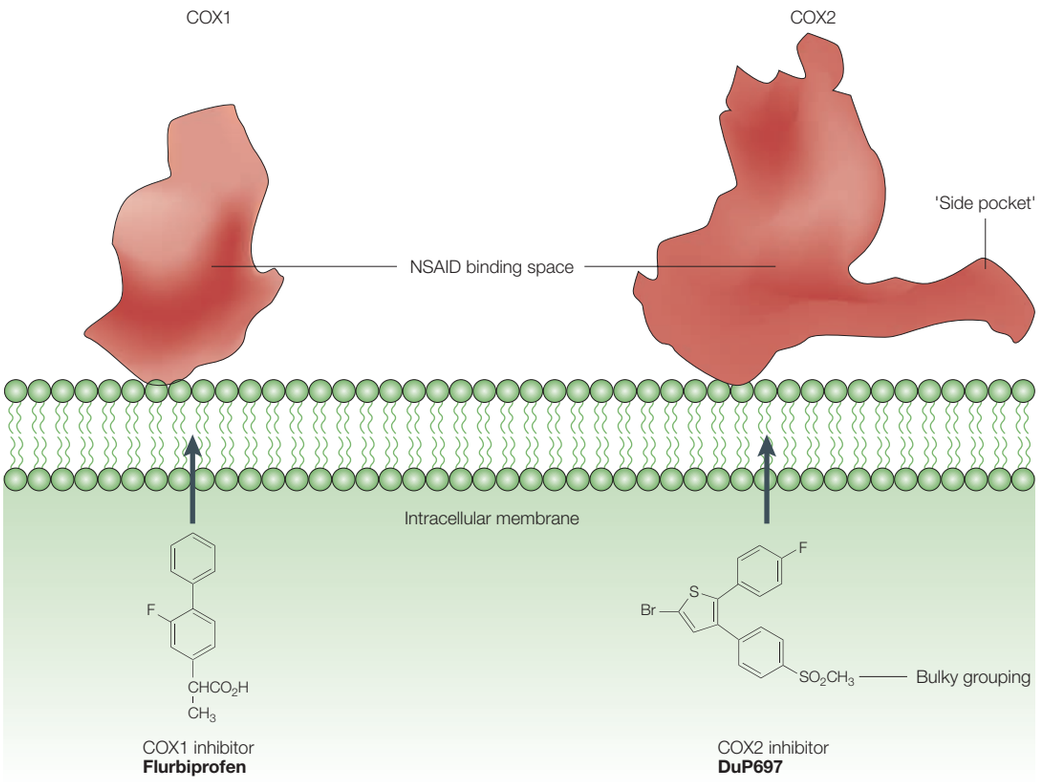

Figure 4 | **Comparison of the NSAID binding sites of COX1 and COX2 after Browner.** Schematic cartoon (modified from REF. 142), showing the differences in the NSAID binding sites of COX1 and COX2. Note that the COX2 binding site is more accommodating and is characterized by a 'side pocket' that can accommodate bulky groups such as the methyl sulphonyl moiety of DuP697. COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug.

The COX1/COX2 concept today

How does this very influential idea stack up today in the light of the results that have been published on the actions of the coxibs and other selective inhibitors of the COX2 isoform? Like all ideas, it has seen some modifications over the passage of time. At the tissue level there is an awareness that COX2 is more widespread in 'normal' tissue than was at first suspected and that it is not necessarily restricted to inflammatory sites. Rat brain has a large amount of the enzyme under 'resting' conditions[85], with further COX2 induced at separate sites following the administration of lipopolysaccharide[86]. Certain other tissues around the body, including the MACULA DENSA region of the kidney[87], are also rich sources of the enzyme under 'normal' conditions and, probably because of this, COX2 inhibitors can cause hypertension and fluid retention in some patients[88]. Conversely, it has become apparent that COX1 can also be regulated under some circumstances[89].

The central idea that it is the inhibition of COX2 that is responsible for the therapeutic actions of the drugs, whereas COX1 inhibition is responsible for the side effects, is still valid, although there are some caveats. For example, there have also been well-founded reports that COX2 is present in the marginal tissue of healing ulcers and that COX2 products might contribute to the resolution of inflammation in the gastrointestinal tract[90–93] and elsewhere[94]. This implies that inhibition of the healing response in various tissues might be an unwelcome side effect of these drugs. Unexpectedly, celecoxib (as well as ibuprofen, a mixed inhibitor) reduced the generation of the protective eicosanoid prostacyclin in humans, giving rise to a concern about unwanted cardiovascular side effects[95]. Taking another tack entirely, Wallace and his colleagues[96] have argued convincingly that inhibition of one isoform in the gastrointestinal tract can be compensated for if the other isoform is not inhibited, and that it is only mixed inhibitors of the COX isoforms that give rise to gastrointestinal damage. Interestingly, the administration of COX1 inhibitors leads to the rapid induction of COX2 in the gastric mucosa (presumably a protective response), perhaps explaining why both need to be inhibited to produce damage[97].

Despite all these qualifications, the hypothesis has taken us a long way forward. It has led to the discovery of a family of drugs that are better tolerated than the older NSAIDs, our knowledge of the action of COX inhibitors has been greatly fortified and the advent of COX2-selective drugs has enabled us to discern the role of this enzyme in physiological or pathological processes. There have, of course, been new technical and other problems to solve. The question of the correct way of assessing and expressing the selectivity of

MACULA DENSA
Part of the juxtaglomerular apparatus of the kidney that is important in sensing changes in blood pressure.

COX1/COX2 inhibitors is one that has exercised many investigators and been responsible for the appearance of much confusing data in the literature. Novel COX assay systems had to be devised and the potential pitfalls, such as variations in the amount of substrate, time of pre-incubation and other factors that are likely to influence the action of putative inhibitors, have been highlighted by several authors[98,99]. Many members of the scientific and clinical community have now settled on the 'whole blood assay'[100] that was originally described by Patrignani *et al.* in 1994 (REF. 101) as a standard technique. No assay is without its irritations, but this method has the great advantage of measuring the relative inhibitory activity in one sample of blood from the relevant species (usually human) and of taking into account factors such as plasma protein binding, which are usually ignored. The new data have also forced us to review the way in which we classify NSAIDs. There have been various suggestions[102,103] for new nomenclature or classification of these drugs. Using a modification of Patrignani's method, Warner *et al.*[84] produced a definitive survey of most of the COX1/COX2 inhibitors available in 1999 and proposed an extremely useful classification system that has been widely adopted (TABLE 1).

From the clinical point of view, the results of two major prospective studies — the VIGOR (Vioxx Gastrointestinal Outcomes Study)[104] and the CLASS (Celecoxib Long Term Arthritis Safety Study)[105] — compared gastrointestinal safety records of rofecoxib with those of naproxen, and celecoxib to ibuprofen and diclofenac. Despite some similarities, the two trials were executed somewhat differently. In the former study using a cohort of rheumatoid arthritis patients, the concomitant use of other NSAIDs was not allowed, but the osteoarthritis cohort in the CLASS study were allowed to take low-dose aspirin in addition to celecoxib. In the completed VIGOR study, the incidence of PUBs (perforations, ulcers and bleeding) in subjects taking rofecoxib was less than half that in those using naproxen. Data from the first six months of the CLASS study did not show any difference in the incidence of the primary end point (the appearance of complicated ulcers), although the incidence of the secondary end point (symptomatic ulcers) was significantly reduced when compared with ibuprofen, but not when compared with diclofenac. One-year data from the CLASS study showed some benefit compared with ibuprofen, but not when aspirin was allowed. Interestingly, patients entered into the VIGOR study showed a slightly higher incidence of cardiovascular events, indicating that the loss of the antiplatelet (COX1) effect of NSAIDs might be clinically disadvantageous to a small proportion of patients, even though concomitant therapy vitiates the beneficial gastrointestinal profile. Overall, the results of these two long-term studies might be said to support the COX1/COX2 concept while at the same time highlighting potential cardiovascular risks to some patients. The clinical implications of this and other aspects of the pharmacology of COX2 inhibitors have been well reviewed by FitzGerald and Patrono[106].

Lessons for drug discovery

So what lessons can we draw from this story? There are many ways of discovering new drugs, but it is clear that the rapid and successful discovery and marketing of celecoxib and rofecoxib owed little to the high-tech approach that most feel is the way forward for the pharmaceutical industry in this post-genomic era. It can be argued, with some justice, that this was a unique case, in that several potential lead compounds were already apparent from the literature. But at the very least, the episode shows that highly successful drugs can still be discovered (and substantial sales achieved) by organizations that are flexible in their approach to finding new medicines. It stresses the key role of the skilled and alert scientist backed by management with the vision and resources to exploit such findings as they arise.

The manner in which COX2 was identified also reminds us that we seldom know where the cutting edge of our field is at any one time. It highlights the extraordinary value of the anomalous observation and warns us to be wary of ignoring compounds that have therapeutic profiles that do not quite 'fit'. Molecular biology was key in the discovery and characterization of the COX2 isozyme, but the outcome of the initial transgenic 'hypothesis testing' studies were, perhaps, not so clear, and those who rely heavily on transgenic disease models would be right to regard this as a cautionary tale. The other point that emerges clearly is the significance of structural data, with its important lesson that even small and apparently unimportant changes in sequence might make a huge difference to the pharmacology of the enzyme.

Table 1 | **A classification of NSAIDs according to Warner *et al.*[84]**

Class	Properties	Examples
Group 1	NSAIDs that can completely inhibit both COX1 and COX2 but have little selectivity	Aspirin, diclofenac, fenoprofen, flurbiprofen, indomethacin, ibuprofen, ketoprofen, mefenamic acid, naproxen, piroxicam, sulindac sulphide
Group 2	NSAIDs that inhibit COX2 with a 5–50-fold selectivity	Celecoxib, etodolac, meloxicam
Group 3	NSAIDs that inhibit COX2 with a >50-fold selectivity	Rofecoxib
Group 4	NSAIDs that are weak inhibitors of both isoforms	5-amino salicylic acid, diflunisal, sodium salicylate, nabumetone, sulphasalazine

Adapted from REF. 84. COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug.

IC_{50} VALUE
The concentration of a drug or substance that inhibits a particular effect by 50%.

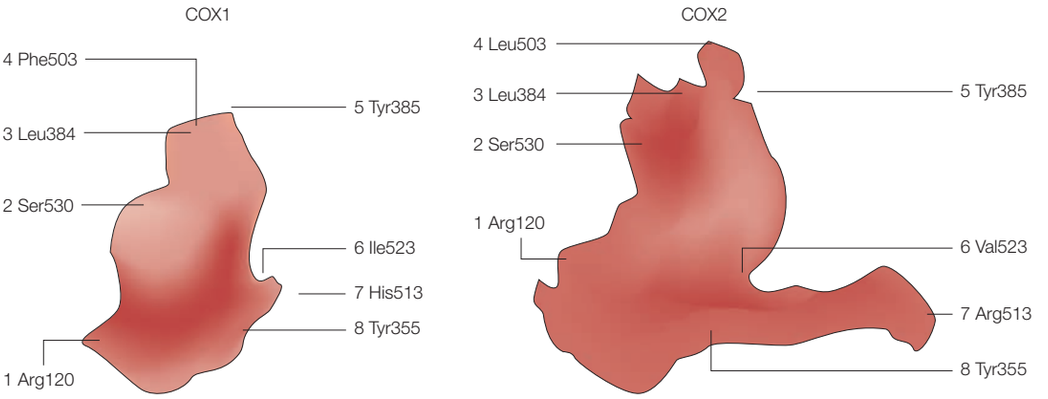

Figure 5 | **Some key residues in COX1 and COX2.** The left-hand panel shows a schematic diagram of the NSAID-binding site in COX1. The highly conserved residues Arg120 and Tyr355 (1 and 8) stabilize the carboxylate group that is present in most NSAIDs, whereas the aromatic ring structures are accommodated within the largely hydrophobic binding channel and often abut onto the highly conserved Tyr385 (5). Tyr385 is close to the peroxidase site that forms a tyrosyl radical that is crucial to the introduction of molecular oxygen into the arachidonic acid substrate. Ser530 (2) is the residue that is acetylated by aspirin. Note the presence of the relatively bulky Ile523 (6) and the presence of Leu384 (3) in proximity to Phe503 (4), and the presence of His513 (7). The right-hand panel depicts the COX2 binding site. The highly conserved residues Arg120 and Tyr355 (1 and 8) are present as before, as is Tyr385 (5) and Ser530 (2). However, residue 4 now becomes Leu503 which, being less bulky, is not packed as tightly. This allows expansion of the available space at the top of the channel. Residue 6 becomes Val523 in COX2, which allows opening of the side pocket. The side pocket can completely accommodate the sulphonamide or analogous group of the COX2 inhibitors that are stabilized by hydrogen bonding with residue 7, which becomes Arg513 in COX2. Overall, the available space in the COX2 binding pocket is more than 25% greater than in the COX1 binding site. This is mainly due to the side pocket and the increase in available space at the top of the channel. Adapted from REFS 72, 143. For clarity, an equivalent numbering system has been used for both COX enzymes. COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug.

The future of COX pharmacology

Are there any more 'anomalous' COX inhibitors out there and can we expect any further surprises from the COX system? Perhaps. We do not have to look far for drugs with unusual inhibitory mechanisms. In fact, the oldest NSAID of all must surely fall into this category. Clinically, salicylic acid is almost equipotent with aspirin as an anti-inflammatory drug but shows up with much lower IC_{50} VALUES in all *in vitro* assays. The discovery of COX2 has not helped in this instance, as the drug is a weak inhibitor of both isoforms *in vitro*. A key observation relating to this matter was published in 1972 by Hamberg[107], who measured the excretion of prostaglandin metabolites in volunteers taking indomethacin, aspirin or salicylic acid. Indomethacin was the most potent, but both aspirin and salicylate (4 × 600 mg per day) had a similar maximum effect on prostaglandin output. Although the numbers were small, there was a tantalizing hint of a latency of effect with the latter drug, indicating a mode of action other than simple COX inhibition.

Several attempts have been made to solve the discrepancy between the potency of salicylic acid *in vitro* and *in vivo*. Early studies focused on the possibility that salicylate metabolites, such as the dihydroxy gentisic acid, were active inhibitors of COX[13] — a notion that has received recent support[108]. A further suggestion that has been widely canvassed is that salicylate inhibits transcription of COX genes by preventing phosphorylation of IκBα (inhibitor α of nuclear factor-κB (NF-κB)), thereby inhibiting NF-κB activation[109,110], or by inhibiting *COX2* gene transcription[111]. Tumour necrosis factor (TNF)-stimulated inducible nitric oxide synthase (iNOS) gene expression and adhesion molecule expression in HUVECs[109] has been reported to be inhibited by both aspirin and sodium salicylate[112]. However, this is by no means universally agreed as a potential mechanism for controlling COX[113]. Effects on other signalling systems, such as extracellular signal-regulated kinase and c-JUN N-terminal kinase in human neutrophils and fibroblasts have also been claimed[114,115], as well as other adenosine-dependent effects of these drugs that are apparently maintained in Nfκb-null mice[116,117]. What this all means mechanistically and whether any of these studies are truly relevant to therapeutic dosing is still not entirely clear. Ironically, it seems that although being the simplest from a structural viewpoint, the salicylates might turn out to be among the most complex drugs of all. The matter is still very relevant, for there is sound evidence from contemporary double-blind clinical trials that willow-bark extracts can ameliorate the pain of osteoarthritis[118], and this effect is almost certainly due to conjugated salicylate in the preparation. It is an area that will certainly repay a further inspection.

Ironically, the discovery of COX2 also failed to directly solve the paracetamol mystery. As an inhibitor, this drug is weakly active against both purified preparations of COX1 and COX2, but the concentrations that are required for inhibitory activity are higher than one would expect to achieve *in vivo*. Some exciting findings from Simmons' laboratory[119,120] sparked speculations[120–123] that there might be other forms of the enzyme yet to be discovered. Simmons' team observed

APOPTOTIC
A term used to describe the state of programmed cell death.

Table 2 | **The COX family: a summary of properties**

Gene	Gene product	Tissue expression	Functions	Inhibitors	Comments
COX1	COX1	Constitutively expressed in most tissues	Platelet aggregation, GI protection, some pain, production of vascular prostacyclin	Most 'classical' NSAIDs; some selective inhibitors	First COX to be identified
COX1	COX3	Brain, heart and aorta; constitutive?	Pain perception?	Paracetamol, diclofenac, ibuprofen, dipyrone, phenacetin, antipyrine	Few details presently known
COX1	pCOX1a	Brain	?	NA	Not catalytically active
COX1	pCOX1b	Brain	?	NA	Not catalytically active
COX2	COX2	Induced in many tissues by many stimuli including growth factors, cytokines, oxidative stress, brain hypoxia or seizures, and other forms of injury or stress. Constitutively present in brain, kidney and elsewhere	Inflammation, fever, some pain, parturition and renal function	Many NSAIDs, COX2-selective drugs such as the coxibs and others	
COX2	COX?	J774 cells during apoptosis	?	Paracetamol	Only studied in one system to date

Modified from REF. 143. COX, cyclooxygenase; GI, gastrointestinal; NA, not applicable; NSAID, non-steroidal anti-inflammatory drug.

the appearance of (what seem to be) isoforms of COX2 that are significantly more sensitive to paracetamol than lipopolysaccharide-induced COX2 in APOPTOTIC J774.2 cell lines, and have argued that this enzyme can exist in two catalytically distinct states.

Simmons' group have now progressed even further with this idea. While analysing COX expression in canine cerebral cortex, Simmons and colleagues noticed two distinct RNA species that hybridized with *COX1* cDNA on Northern blots. They went on to describe the cloning, structure and expression of a variant form of COX1 — dubbed COX3 — together with two other species, partial (p) COX1a and pCOX1b[124]. All of these species are derived from COX1 by an unusual splicing mechanism that retains intron 1 in the mature protein. In addition, pCOX1a has a deletion of amino acids 117–336 (corresponding to exons 5–8), whereas pCOX1b is identical to pCOX1a except that it lacks intron 1. All three proteins are glycosylated, but only COX3 has catalytic activity (TABLE 2).

COX3 therefore retains the principal features of the general and catalytic structures of COX1 and 2, but the presence of the translated intron 1 adds a further 30 amino acids to the amino terminus which, Simmons speculates, could alter the dimerization, folding or intracellular targeting of the enzyme. Reverse transcriptase polymerase chain reaction (RT-PCR) and other techniques indicate that COX3 is present mainly in the brain (where it is expressed at a level of approximately 5% of COX1), heart and aorta — a distribution that differs from that of COX1.

From the point of view of COX pharmacology, the most fascinating thing about COX3 is its unusual drug sensitivity. Using a canine cortex cDNA library and insect cell-expression systems, Simmons' group produced catalytically active COX3 and tested its sensitivity to several NSAIDs. They found that COX3 had enhanced sensitivity to paracetamol, diclofenac, ibuprofen and aspirin when compared with COX1 or COX2. COX3 was also selectively sensitive to other antipyretic drugs, including antipyrine, aminopyrine, dipyrone and phenacetin (FIG. 3c), which are largely inactive against other COX preparations[13,124].

We must now attempt to reconcile the sensitivity of this enzyme with the analgesic/antipyretic activity of these drugs. There is ample evidence for a role of both COX1 and 2 in various types of pain[64,125,126], but the question of antipyretic action is less clear. COX3 is derived from the same gene as COX1, but it is thought that COX2 is the main enzyme involved in the pathogenesis of fever, as highly selective COX2 inhibitors are effective antipyretics in man[127], whereas *Cox2*-null mice have a reduced pyretic response to lipopolysaccharide[128]. One possibility is that, like COX1, alternative splicing of COX2 can give rise to other species that might also be sensitive to paracetamol. Simmons' previous work on diclofenac-inducible COX2 might point to this as a feasible mechanism.

While this concept is being developed and tested (and indeed other plausible explanations for the paracetamol enigma have been advanced (REFS 129,130)), we might expect further selective COX2 inhibitors to flow from the new medicinal chemistry opportunities that are afforded by the discoveries of the past decade. Already, Pharmacia and Merck have follow-up drugs (etoricoxib and valdecoxib) available in the clinic with apparently improved pharmacokinetics. Elsewhere, there have been reports of interesting new families of inhibitors, several of which are methylsulphones or sulphonamides[131–133]. In other cases, advantage was taken of the unique structural features of the COX2 binding pocket to design 'aspirin-like' molecules (for example, 2-acetoxyphenyl methyl sulphide) that preferentially acetylate the COX2 active site at the Ser516 residue[134,135]. Ester and amide derivatization of indomethacin transforms this mixed COX inhibitor into a highly selective COX2 inhibitor[136,137], an approach that could presumably be used with other NSAIDs.

RT-PCR
(Reverse transcription polymerase chain reaction). A technique that is used to amplify cDNA from an mRNA template using sequence-specific primers.

The story of COX pharmacology has already run for more than a century, but with so many innovative new leads and the recognition that COX2 inhibitors might be useful for treating diseases such as colon cancer[138,139] and Alzheimer's[140,141], we might expect the field to continue to flourish for many years to come. Undoubtedly, the recent discovery of COX3, and the prospect of more potent non-opioid analgesics, perhaps based on the structure of existing COX3 inhibitors, is likely to fuel another explosion of interest in COX pharmacology.

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