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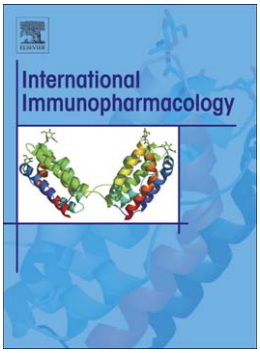

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Inhibition of Arachidonic Acid Metabolism and its Implication on Cell Proliferation and Tumour-angiogenesis

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Abstract Arachidonic acid (AA) and its metabolites have recently generated a heightened interest due to growing evidence of their significant role in cancer biology. Thus, inhibitors of the AA cascade, first and foremost COX inhibitors, which have originally been of interest in the treatment of inflammatory conditions and certain types of cardiovascular disease, are now attracting attention as an arsenal against cancer. An increasing number of investigations support their role in cancer chemoprevention, although the precise molecular mechanisms that link levels of AA, and its metabolites, with cancer progression have still to be elucidated.

This article provides an overview of the AA cascade and focuses on the roles of its inhibitors and their implication in cancer treatment. In particular, emphasis is placed on the inhibition of cell proliferation and neo-angiogenesis through inhibition of the enzymes COX-2, 5-LOX and CYP450. Downstream effects of inhibition of AA metabolites are analysed and the molecular mechanisms of action of a selected number of inhibitors of catalytic pathways reviewed. Lastly, the benefits of dietary omega-3 fatty acids and their mechanisms of action leading to reduced cancer risk and impeded cancer cell growth are mentioned. Finally, a proposal is put forward, suggesting a novel and integrated approach in viewing the molecular mechanisms and complex interactions responsible for the involvement of AA metabolites in carcinogenesis and the protective effects of omega-3 fatty acids in inflammation and tumour prevention.

Keywords Arachidonic Acid, COX inhibitors, LOX inhibitors, CYP450, cancer

INTRODUCTION

Tumourigenesis is a multi-factorial sequential process which usually takes many years to progress. To date, the greatest challenge in cancer prevention and treatment still lies in identifying the multitude of cellular interactions of the complex and partially interconnected pathways critical to malignant cell proliferation, cell survival, tumour metastasis and neo-angiogenesis. Among the vast number of factors involved in tumour progression, arachidonic acid (AA) and its metabolites have recently generated a heightened interest due to growing evidence of their significant role in cancer biology.

As one of the body's essential fatty acids AA is required by the majority of mammals. Its metabolites, collectively termed eicosanoids, are converted from AA by the catalytic activities of three key enzymes, namely cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450). The eicosanoids comprise a number of lipid signalling mediators that play a central role in cellular signalling cascades of physiological and pathophysiological relevance. Although their involvement in the development of human cancer has long been suggested, it is only recently that they have been identified as active carcinogens or tumour promoters, their aberrant or increased expression levels having detrimental effects on cancer development. So far, inhibitors of the AA cascade, first and foremost COX inhibitors, have mainly been of interest in the treatment of inflammatory conditions and certain types of cardiovascular disease. However, an increasing number of investigations support their role in chemoprevention of cancer, although the precise molecular mechanisms that link levels of AA, and its metabolites, with cancer progression have still to be elucidated.

In carcinogenesis, relatively few human cancer risk factors/activators, such as exogenous chemicals, UV light, stress, endogenous enzymes, transcription factors, growth factors and cytokines act purely in either cytotoxic or mitogenic fashion. Instead, the majority seem to drive cell proliferation and metastasis through mechanisms of inflammation. Evidence for the

role of inflammation in cancer comes from a large number of epidemiological observations, indicating that regular and prolonged treatment with a vast number of synthetic anti-inflammatory drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, can reduce the incidence and recurrence of several human cancers by up to 50% [1, 2, 3, 4, 5].

In cancer treatment, inhibition of tumour promotion is key, whether in the form of tumour prevention or inhibition of tumour progression. Angiogenesis, which plays a key role in carcinogenesis, is largely dependent on various exogenous signalling molecules that induce and inhibit neovascularisation. The formation of new blood vessels is critical for cancer progression since the growth potential of cells is limited by availability of nutrients. Furthermore, new tumour vessel growth often coincides with tumour metastasis and is of prognostic significance. Therefore, by targeting initiators, co-carcinogens and tumour promoters, tumour growth could potentially be prevented. Unfortunately, the identification of such agents can be difficult. Moreover, the real challenge does not usually begin until after an appropriate target has been identified and the investigations on the exact roles, molecular mechanisms and signalling pathways reveal complex interdependencies, which raise many more questions.

This article focuses on the roles of inhibitors of the AA cascade and their implication in cancer treatment. In particular, emphasis is placed on the inhibition of cell proliferation and neo-angiogenesis through inhibition of the enzymes COX-2, 5-LOX and CYP450. Downstream effects of inhibition and modulation of AA metabolites are exemplified by reviewing the molecular mechanisms of action of a selected number of inhibitors of the named catalytic pathways. In addition, the protective effects of dietary omega-3 fatty acids and their mechanisms of action leading to reduced cancer risk and impeded cancer cell growth are mentioned. Finally, a proposal is put forward that outlines signalling and cross-talk

between the AA cascade, inflammatory mediators and cell signal transduction pathways, suggesting a novel and integrated approach in viewing the molecular mechanisms and complex interactions responsible for the involvement of AA metabolites in carcinogenesis.

THE ARACHIDONIC ACID CASCADE

AA (cis-,cis-,cis-,cis-5,8,11,14-eicosatetraenoic acid) is a 20-carbon polyunsaturated fatty acid and the central eicosanoid precursor in mammalian cells. Since AA cannot be synthesized *de novo* from animal cells, most of AA in the human body is derived from linoleic acid, which can be obtained only from dietary sources. After biosynthesis of AA from its precursor, it is esterified into the phospholipids of the outer cell membranes. Each membrane phospholipid contains two fatty acids, some of which are the essential fatty acids (EFAs) AA, eicosapentaenoic acid (EPA) or dihomo γ-linolenic acid (DGLA) [6].

The first step in the AA cascade (Figure 1) is cleavage and release of AA from the phospholipid-bound form. It is suggested that this may be achieved with the assistance of at least one of three different enzymes, namely phospholipase A_2 (PLA_2), phospholipase C and phospholipase D [7]. PLA_2, however, is the only phospholipase that seems to be able to release free AA directly in a single-step reaction, by hydrolysing an ester bond at the sn-2 position of phospholipids [8], which is why it features as the main phospholipase of interest in most literature in connection with AA metabolism. Mammalian cells contain several isoforms of the enzyme PLA_2 [9], which receive their stimulatory signals from a vast range of inflammatory signals, cytokines, growth factors and hormones.

The majority of AA metabolites can act both as pro- and anti-inflammatory mediators [10], modulating gene expression, cytokine signalling and other immune regulatory factors.

Figure 1

The AA Metabolic Pathways

Both endogenous and exogenous AA levels have been shown to mediate events critical to cancer development. For example, evidence indicates that free AA can induce apoptosis via conversion of sphingomyelin to ceramide, which triggers the release of pro-apoptotic proteins [11, 12]. As a result, inhibition or modulation of the AA cascade can have anti-inflammatory and anti-carcinogenic effects. However, to better understand the large number of AA derivatives and their specific actions, it is necessary to take a closer look at the three key metabolic pathways responsible, namely the COX, LOX and CYP450 pathways.

The COX Pathway

To date, three isoforms of the membrane bound enzyme COX have been identified, COX-1, COX-2 and COX-3. Although they differ in their pattern of expression and tissue distribution in human cells [13, 14], collectively they are responsible for the stepwise conversion of AA to the three classes of prostanoids. Whilst COX-1 is ubiquitous and produced constitutively in most mammalian cells and tissues to maintain baseline levels of prostaglandins, COX-2 is normally absent. However, at the sites of inflammation COX-2 is found to be readily induced by a variety of stimuli associated with inflammatory responses such as cytokines, growth factors and other tumour promoters [15, 16].

The first step in the COX metabolic pathway (Figure 2) is oxygenation of AA by its cyclooxygenase activity to give PGG_2, followed by rapid conversion of PGG_2 by its peroxidise activity into PGH_2. PGH_2 is an unstable endoperoxide that functions as intermediate for all further synthetic steps in the COX pathway, which are catalyzed by a number of cell-specific isomerases and lead to the formation of the prostaglandins (PGs) prostacyclin D_2 (PGD_2), prostacyclin E_2 (PGE_2), prostacyclin $PGF_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin I_2 (PGI_2) and thromboxane A_2 (TXA_2) [17]. PGs are inflammatory mediators in a number of

conditions and diseases such as inflammation of the skin [18], arthritis [19], and asthma [20]. In the gastrointestinal tract PGs have been found to both have a stimulatory effect as well as elicit a protective function in certain inflammatory conditions. Sudden dramatic increases in mucosal PGs are positively correlated with disease activity of inflammatory bowel disease [21] and experimental colitis [22], whereas base-line expression of PGs generally exert a protective function against gastrointestinal injury [23] and ulcers [24] as well as acute and chronic enterocolitis [25].

Figure 2

It has been extensively documented that overexpression of COX-2 is implicated in various forms of human cancers such as cancer of the lung [26], breast [27], colorectal [28], prostate [29], head and neck [30] and others [31, 32]. In particular, increased COX-2 expression has been brought in connection with tumour metastasis in colon cancer [33] where aberrant COX-2 expression was shown to correlate with carcinogenesis in more than 80% of colorectal cancers [34]. In animal models COX-2 expression was found to be sufficient to induce tumourigenesis [35]. In head and neck cancer, increased expression levels of COX-2 was found to correlate with the extent of lymph node metastasis and tumour vascularisation, the latter being clearly correlated to PGE_2 biosynthesis and vascular endothelial growth factor (VEGF) expression levels [36, 37]. Corresponding findings were reported for COX involvement in angiogenic signalling in non-small cell lung cancers [38]. Indeed, raised PGE_2 expression was shown to trigger β-catenin signalling via the Wnt pathway, thereby activating the proto-oncogenes c-myc and c-jun as well as cyclin D1 expression [39, 40]. In addition, in gastric tumours, increased PGE_2 levels were found to be correlated with tumour invasion, lymph node metastasis and carcinogenesis and are believed to affect VEGF signalling as a

result of increased matrix metalloproteinase 9 (MMP-9) activity [41, 42]. Furthermore, raised COX-2 expression was shown to contribute towards astrocytic carcinogenesis in gliomas, by promoting new blood vessel formation in connection with increased inducible nitric oxide synthase (iNOS) and VEGF signalling [43]. In vitro studies have reported that fibroblasts derived from COX-2 knockout mice displayed an up to 94% reduction in their ability to produce VEGF in comparison to wild-type fibroblasts [44].

Rather intriguingly, since COX inhibition has been brought in connection with increased COX mRNA expression, it seems that one or more COX-produced metabolites of AA must act in a negative feedback mechanism on COX [45]. Finally, studies investigating the nature of regulatory factors controlling COX-2 expression identified reactive oxygen species (ROS)-mediated nuclear factor-κB (NF-κB) activation to play an active role [46, 47], suggesting a positive feedback mechanism between expression levels of NF-κB and COX.

The LOX Pathway

In human cells, generally, four types of LOXs have been identified, namely 5-, 12- and 15-LOX-1 and -2 [48 , 49]. Collectively, they catalyze the dioxygenation of AA into hydroperoxyeicosatetraenoic acids (HpETEs). Ultimately, this is followed by their conversion to their corresponding hydroeicosatetraenoic acids (HETEs), leading to the formation of the leukotrienes (LKs), lipoxins (LOs) and hepoxilins (HOs).

5-LOX has received the greatest attention as drug target, in particular due to its role in the synthesis of the pro-inflammatory mediators, the LKs. The initial enzymatic step in the 5-LOX metabolic pathway (Figure 3) requires presentation of AA to LOX by 5-LOX activating protein (FLAP) in a calcium- and ATP-dependent manner [50]. Subsequently, AA is oxygenated to give 6E,8Z,11Z,14Z-5S-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5S-HpETE). 5S-HpETE acts as precursor for the formation of 5S-HETE and 6E,8Z,11Z,14Z-5-

oxoicosa-6,8,11,14-tetraenoic acid (5-oxo-6,8,11,14-ETE) by peroxidase and dehydrogenase activity respectively or is metabolized by 5-LOX to form the unstable epoxide leukotriene A_4 (LTA_4) [51, 52]. Overall, the LOX pathway is relatively complex in that several eicosanoid production pathways are interlinked and synthesis of the LOs, for example, is 5-, 12- and 15-LOX dependent [53, 54]. This is evident in the 5-LOX pathway, where synthesis of the lipoxins A_4 (LXA_4) and B_4 (LXB_4) requires 12-LOX activity [55, 56]. Further metabolites for which LTA_4 serves as precursor are leukotriene B_4 (LTB_4), catalysed by LTA_4 hydrolase [57] and the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), LTC_4 being synthesised with the aid of a specific glutathione-S-transferase [58]. Formation of 5-oxo-7E,9E,11Z,14Z-eicosatetraenoic acid (5-oxo-7,9,11,14-ETE) is the result of non-enzymatic metabolism [59]. Within the 15-LOX pathway, two isoforms have been identified [60], where 15-LOX-1 preferentially metabolizes linoleic acid into 13S-hydroxyoctadeca-9Z,11E-dienoic acid (13S-HODE), whilst 15-LOX-2 is mainly responsible for the production of 15S-HETE from 15S-HpETE [61, 62].

Figure 3

In a more generic approach, a number of investigations have found a correlation between mRNA expression levels of 5- and/or 12-LOX and cancer pathobiology, whereby increased LOX expression levels were noted in a broad range of cancers including breast, pancreatic, prostate, lung, urinary bladder, leukaemia and colon cancer [63, 64, 65, 66, 67, 68, 69]. With regards to 15-LOX, there is evidence for opposing theories on correlation of expression levels with carcinogenesis where both over- and under-expression has been observed in cancerous cells [70, 71]. However, an increasing number of investigations seem to indicate that 15-LOX-1 is positively, whilst 15-LOX-2 is negatively correlated with cell proliferation and carcinogenesis. In particular, 15-LOX-1 overexpression was found associated with decreased peroxisome-proliferator activated receptor γ (PPARγ) activity and subsequent increase in

MMP-9 signalling, whilst 15-LOX-2 expression was found associated with increased PPAR γ activity and a subsequent reduction in MMP-9 signalling [72, 73, 74, 75], These findings suggest that there is potential for both 15-LOX-1 and 15-LOX-2 inhibitors and metabolites, respectively, to act in an anti-inflammatory and tumour suppressive manner by decreasing cell proliferation and differentiation and inducing apoptosis [76, 77, 78]. In general, the 5-LOX pathway leads to proliferative and pro-apoptotic effects in various forms of cancer, with exogenous 5-HETE and cysteinyl leukotrienes having up to a fourfold proliferative effect on four different types of breast cancer cell lines [79]. Stimulation of 5-LOX activity was found to arise due to tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and histamine signalling, ultimately resulting in ROS-mediated NF-κB activation [80, 81]. Furthermore, cancer cell growth was demonstrated in human testicular cancer tissue, where both 5- and 12-LOX were found to promote induction of cell proliferation, an effect which was suppressed upon inhibition of 5-LOX [82]. In addition to its role in neoplastic transformation, 5-LOX and its AA metabolite 5-HETE have been shown to be involved in angiogenesis and mesothelial cell carcinogenesis through increased VEGF release and mRNA expression levels [83]. Contrary to the majority of LOX products, LXA_4 and LXB_4 have shown to generate effective anti-inflammatory responses, which may antagonize pro-inflammatory signals mediated by other LOX catalyzed AA derivatives [84].

CYP450 Pathway

The CYP450 metabolic pathway is the least well-characterized pathway in connection with lipid metabolism in the AA cascade. Several isoforms of CYP450 catalyze the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent conversion of AA (Figure 4). The corresponding metabolites include a family of lipoxygenase-like HETEs, epoxyeicosatrienoic acids (EETs) and ω-HETEs which are formed by bis-allylic

monoxygenation, olefin epoxydation and ω-hydroxylation respectively [85]. In addition, the CYP450 pathway gives rise to ROS termed HpETEs, although the EETs and ω-HETEs are the major products of the CYP450 pathway [86].

Figure 4

A vast number of recent studies suggest the involvement of CYP450 metabolites in carcinogenesis. In particular, this has been noted in renal carcinoma, where CYP450 is believed to be the main catalytic pathway since COX and LOX are basically undetectable [87]. Aberrant CYP450 epoxygenase activity and EET synthesis was found to promote tumour metastasis, independent of tumour growth, in several human cancer cell lines [88]. In addition, it was shown to affect mitogen-activated protein kinase phosphatase-1 (MKP-1) mediated inactivation of c-Jun N-terminal kinase (JNK), which ultimately leads to the expression of cyclin D1 and cell proliferation [89]. In addition, there is evidence that EETs not only elicit cell proliferation but also promote neo-angiogenesis under hypoxia-induced enhanced activity of CYP 450 epoxygenase [90]. In addition, 14,15-EET was found to inhibit apoptosis by a PI3/Akt signalling pathway [91]. Furthermore, overexpression of CYP450 ω-hydroxylase, and in particular its catalytic product 20-HETE, is believed to be implicated in renal carcinoma [92] as well as tumour-angiogenesis mediated by VEGF, angiotensin II, fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) signalling [93, 94, 95, 96, 97, 98]. The downstream angiogenic signals triggered by the various angiogenic factors acting on CYP450-derived metabolites are believed to be mediated by Akt-dependent phosphorylation and activation of eNOS as well as phosphorylation of growth factor receptors and mitogen activated protein kinase (MAPK) [97, 99].

INHIBITION OF AA METABOLISM

Molecular Mechanisms of Inhibition

The physiological functions of AA metabolites have been mainly identified due to pharmacological inhibition studies. Without the use of inhibitors with known enzyme affinity, the abundant evidence for a correlation between overexpression and aberrant signalling of COX, LOX and CYP with pathogenesis of human carcinomas would not have been possible. The identified capabilities of these inhibitors to date have lead to the development and/or further investigation of a series of novel or already existing selective and non-selective inhibitors, some of which are currently in phase II and III clinical trials for cancer chemoprevention and treatment. All three enzymes share a trait for iron dependency, whereby COX and CYP carry their iron in a haeme-bound moiety, whilst LOX binds its metal cofactor as a single ion atom bound directly to the protein itself.

COX Inhibition

COX inhibitors include the classical NSAIDs such as aspirin, ibuprofen, naproxen and sulindac and are generally classified according to their chemical structure. The majority of NSAIDs are considered to be competitive inhibitors of COX, since they require the same set of binding site interactions as the natural substrate AA, whereas aspirin is a covalent modifier of COX [100].

The crystallographic structure of COX-2 (Figure 5) reveals a homodimer with each monodimer containing three structural domains, the EGF-like, the membrane-binding and the catalytic domain (CD). The CD contains the active sites of both the cyclooxygenase and peroxidase activity. The cyclooxygenase active site is located at the end of a long hydrophobic channel, formed by residues Tyr385, Phe381, Phe518, Leu384 and Trp387. Substrate-binding requires hydrophobic interactions and hydrogen bonds to Arg120 and

Tyr335 as well as a salt-bridge formation between residues Arg120 and Glu524 [101]. Catalytic activity is exerted by residue Tyr385, which, upon binding of AA, removes its 13-pro-S hydrogen to initiate PGG_2 formation [102]. The binding of inhibitors does not seem to greatly influence either the conformation of the residues directly in contact with the inhibitor or the overall resting structure of the enzyme. No peroxidase-specific therapeutics have yet been developed, however, the peroxidase active site is believed to comprise substrate interactions between residues Gln203, His207, Val291 and Leu294 [103].

Figure 5

Although COX-1 and -2 have the same three-dimensional protein folds and share over 60% amino acid sequence identity, COX-2 displays a branched substrate binding site, whereas COX-1 has a non-branched, conformationally less flexible structure [104, 105]. Therapeutic inhibitors tend to exploit this difference in substrate binding sites to ensure selective COX inhibition [106]. Indeed, aspirin and sulindac inhibit both COX-1 and -2, whilst the more recently developed drugs such as celecoxib and rofecoxib (coxibs) target COX-2 selectively, which gives them a better gastrointestinal profile [107, 108]. Unfortunately, recent findings suggest negative cardiovascular associations with long-term use of selective COX-2 inhibitors [109, 110]. These results have prompted the need for further investigations, such as the APPROVE study and the Adenoma Prevention with Celecoxib (APC) trial respectively [111, 112], which have resulted in the recent withdrawal of rofecoxib from the global market. However, not all studies have found selective COX-2 inhibitors to be associated with greater cardiovascular risk [113, 114], which has resulted in the current controversy around the safety profile and application of COX-targeting drugs for the treatment of inflammatory conditions. This further suggests that a careful evaluation of a patient's individual attributable risks for cardiovascular and gastrointestinal events is required in order to determine the most

appropriate anti-inflammatory strategy for each subject. In particular, the recently raised COX-2-dependent cardiovascular effects seem to depend on a number of variables such as dosing, half-life and dosing intervals. It seems obvious, that cardiovascular safety and gastrointestinal risks are undoubtedly connected by the interplay between PGI_2 and TXA_2 biosynthesis [115] as a result of the varying mechanisms of action of different COX-inhibiting drugs (Table 1).

It has been reported that, in some cell lines, non-selective COX inhibitors as well as NSAID-derivatives with no affinity for COX are equally effective in tumour prevention [116, 117]. In addition, sulindac was shown to exert its growth inhibitory and anti-inflammatory action by inhibiting the activity of IκB kinase β (IKK^{β}) required to activate NF-κB [118]. Furthermore, NSAID treatment of COX-2 null cells were reported to induce arrest of cell proliferation, suggesting that NSAIDs also act through mechanisms not directly related to COX expression levels [119, 120]. Naturally, the above findings raise the question of the underlying mode of action responsible for these observations.

Table 1

Both *in vitro* and *in vivo* animal studies provide convincing evidence that a novel class of drugs that are currently in development may provide both reduced toxicity and increased therapeutic activity. Due to the previously mentioned gastrointestinal side-effects, nitric oxide-releasing NSAIDs (NO-NSAIDs) have been developed, which are meant to compensate for reduction in PG synthesis mediated by COX inhibition. By coupling NSAIDs with NO, it is hypothesized that once released, NO can exert its cytoprotective properties on the gastric mucosa. Investigations report significant results with chemopreventive measures being even greater than with traditional NSAIDs [121, 122].

Aspirin

Aspirin (Figure 6) is probably the best studied NSAID and a connection with long-term low dose aspirin treatment and reduction of cancer incidence in humans has been demonstrated [123].

Figure 6

As previously mentioned, aspirin induces a covalent modification to COX by acetylating residue Ser530 of COX-1 and Ser516 of COX-2 located just below Tyr385 (Figure 7), thereby inhibiting its usual enzyme activity [124, 100]. Furthermore, it has been noted that aspirin-acetylated COX-2 is able to synthesize an additional metabolite from AA, namely 15R-HETE, the enantiomer of 15S-HETE formed from AA by 15-LOX. As a result, aspirin-acetylated COX-2 leads to a decrease in PGG_2/PGH_2 production since 15R-HETE is instead converted by 5-LOX to give 15-epimeric lipoxin A_4 [125, 126]. Evidence suggests that 15-epi-lipoxin acts similarly to natural LXA_4, in that it has potent anti-inflammatory activity and exerts its activity by inhibiting NF-κB activation by attenuating peroxynitrite formation [127]. In addition, when exposed to aspirin, COX-2 expressing cells are capable of converting omega-3 docosahexaenoic acid (DHA) to a novel series of 17R-hydroxy docosanoids (17R-DHAs), termed resolvins and 17R-docosatrienes (17R-DTs) [128, 129].

Figure 7

LOX Inhibition

Although the LOXs share the same type of protein folding, their molecular interactions vary from enzyme to enzyme. These mechanistic differences are mainly due to size, shape and mode of interaction of the catalytic entity of their substrate binding channels.

5-LOX inhibitors generally exert their effect via three modes of action: redox mechanisms, iron-chelating effects or non-redox-related actions. Zileuton (Figure 8), is currently the only approved 5-LOX inhibitor on the market and is prescribed for the treatment of asthma. However, a growing body of investigations support its chemopreventive effects in cancer [130]. A drug with proven selectivity for 12-LOX, Baicalein, has its origin in Chinese herbal medicine and has been found to directly inhibit proliferation and induce apoptosis in human myeloma cells [131]. Another compound recognized for its pan-LOX inhibitory activity is nordihydroguaiaretic acid (NDGA), which has found frequent application in intervention studies but is not licensed for application in humans.

Figure 8

The crystallised protein structure of 5-LOX (Figure 9) reveals its three-dimensional protein folds and relative positions of the iron and substrate binding sites within the enzyme. The amino acid residues crucial for iron binding and enzyme activity were determined to include His367, His550, His372 and Glu376 [132]. Site-directed mutagenesis studies have identified the critical residue for enzyme activity and control of stereochemistry of oxygenation to be Ala404, which is located between the iron binding site and the likely entrance to the substrate binding channel [133]. LOX substrate is believed to bind to the protein through π-electron, charged and hydrophobic interactions. In particular, it is suggested that the C_{11} double bond contained in AA participates in π-π interactions in the substrate binding channel [134].

Figure 9

Another family of inhibitors such as MK-886 and AA-861 do not target 5-LOX directly but are rather aimed at competing for or altering the active site of FLAP, thereby interfering with

AA presentation to 5-LOX [135, 136]. However, *in vitro* studies suggest that FLAP inhibitors such as MK-886 may in fact exhibit their therapeutic effects by non-FLAP associated metabolic interactions [137]. Since the majority of 5-LOX inhibitors are known to act via a redox mechanism, it has been hypothesized that they may be responsible for the production of ROS which could be responsible for drug toxicity [138].

CYP450 Inhibition

Human CYP450 has several isoenzymes, all of which participate in the metabolism of AA and whose sequential identity may differ by up to 20%. CYP450s apply a certain flexibility to substrate choice, meaning they accept a broader range of ligands. Therefore, different ligands can induce a range of conformational changes to the overall protein structure.

The catalytic enzyme activity of CYP450 differs from the usual peroxidase, in that cleavage of the oxygen double bond is mediated by the Cys haeme ligand via electron donation. This is believed to be due to the fact that unlike other enzymes, CYP450 contains no acid–base catalytic groups near the oxygen binding site. Therefore, the oxygen binding cavity is lined with aliphatic and aromatic residues [139].

The three-dimensional structure of CYP450 (Figure 10) depicts the residues believed to exert most of the substrate-binding interactions, such as Leu208 and Gly296. In addition, the ligands are found to be stabilized in the binding groove by hydrogen-bonding interaction with residues Asp293 and Arg108 [139].

Figure 10

Recent findings suggest that, in addition to significantly reducing cell proliferation, CYP450 ω-hydroxylase inhibition has an affect on COX activity and can reduce PGE_2 synthesis by up to 50% [140]. This would indicate that there must be some sort of feedback mechanisms in

place between the metabolites of CYP450 and COX pathways and their enzymes and/or synthases.

Influence of Dietary Fats on AA Cascade

Epidemiological studies suggest an association between dietary fat intake and risk of carcinogenesis for various forms of malignant tumours [141, 142]. Most prominently, this association has been noted in cultures such as Greenland, Alaska and Japan, where a natural high dietary intake of fish oils is maintained. As a result, a number of publications have been able to demonstrate that an increased consumption of omega-3 fatty acids such as EPA and DHA lead to a reduction in colorectal [143, 144, 145] and breast [146] cancer risk respectively. Clearly, the main role of omega-3 in tumourigenesis lies in the reduction of cancer risk and inhibition of cancer cell growth [147].

Of the catalytic enzymes discussed, omega-3 has been found to bind to LOX and COX to produce a series of bioactive mediators (Figure 11). Metabolism of EPA notably produces 18R- hydroxy-eicosapentaenoic acid (18-R-EPA) termed resolvin E1 whilst DHA-derivatives include resolvins D1-D4, the 17S-hydroxy-docosahexaenoic acids (17S-DHAs), as well as the 17S-docasatrienes (17S-DTs) [148, 149]. The beneficial effects of resolvin E1 were shown to originate from blocking stimulation of LTB_4 and inhibiting LTB_4-induced NF-κB activation by binding to the LTB_4 receptor BLT1 [150].

Figure 11

A recent study has put forward supporting evidence that omega-3 fatty acids and their bioactive products significantly reduce pathological angiogenesis, both through reduction of hypoxic stimulus as well as through resolvin-mediated activity [151].

Furthermore, omega-3 has shown to reduce COX-2 expression in comparison to omega-6, which was found to increase COX-2 expression levels and induce *in vitro* invasion in brain-metastatic melanoma cells [152]. Other investigations were able to demonstrate COX-2 independent suppression of tumour cell growth both in an animal model and cultured cells [153]. The method of omega-3 mediated chemoprevention is believed to be partially due to the competition with AA for enzyme substrate. In addition, recent studies were able to assign its therapeutic properties to a marked increase in production of 13S-HODE as well as inhibition of protein kinase C (PKC)- and NADPH-mediated activation of NF-κB and ROS production respectively [154]. Intriguingly, a number of publications can be found suggesting both increased and decreased production of free radicals and ROS to be the reason for modification of carcinogenic processes by omega-3 [155]. Furthermore, omega-3 supplementation was reported to significantly reduce synthesis of pro-inflammatory 5-LOX metabolites LTA_4 and lipid peroxides, thereby inhibiting IL-1β and TNF-α release [156].

The above summarized findings support the importance of considering dietary fats and their ratios in tumour prevention and as therapeutic supplements for inflammatory related diseases and cancer.

DISCUSSION

A number of investigations document a correlation between aberrant expression of AA metabolites and disease prevalence and progression. The two regulatory factors influencing AA metabolism are substrate availability and expression levels and activity of the catalytic enzymes. Within the AA cascade, COX and LOX probably produce the most potent inflammatory signalling molecules and combined blocking of their metabolic pathways were shown to have additive effects in colon cancer cells [157].

Furthermore, a high incidence in expression levels of the G-protein coupled receptors of the LOX pathway, such as cysteinyl LT receptors CysLT1 and CyxLT2, as well as LTB_4 receptors BLT1 and BLT2, has been shown to correlate negatively with patient survival and cancer inhibition [158, 159, 160, 161, 162]. Along similar lines, evidence supports the involvement of the prostanoid receptors of the COX pathway. In particular, the four PGE receptors EP1-4 have been found to be positively correlated with COX-1 and -2 expression and tumour development, by affecting major signalling pathways such as the MAP kinase pathway as well as PPARγ-mediated transcription factor activation [163, 164]. Therefore, it is questionable whether pursuing further upstream inhibitors of the AA cascade is the right way forward. Evidence for an array of feedback loops is available, whereby coupling of PGE_2 levels and FLAP activation [165] as well as interaction between COX-2, 5-LOX and 15-LOX [166] are most likely only a subset of a much greater scale. Since inhibition of one pathway is likely to upregulate the other available metabolic routes of AA [167], it seems worthwhile to further investigate inhibition of the AA catalytic enzymes. As such, combined target inhibition such as COX/LOX, LOX/CYP450 or COX/CYP450, as well as inhibition of AA metabolites and/or their receptors, such as PGE_2 and TXB_4, may prove useful.

Although COX-inhibition studies have demonstrated a correlation between downregulation of COX-derived AA metabolites and inhibition of cell proliferation and apoptosis, other publications suggest that induction of apoptosis is in fact not a direct result of inhibition of COX-2. These contradictory propositions find their origin in the controversy around the 15-LOX pathway. Evidently, further investigations are required in order to conclusively confirm or deny a beneficial effect of 15-LOX metabolites. In addition, it seems necessary to gain further knowledge on cross-talk between phospholipases A2, C and D and the level and extent of their individual contribution towards AA metabolism.

Another demanding area of research is the constant quest to develop inhibitors with greater affinity and selectivity, both of which is critical for providing inhibition of the correct signalling pathways as well as in avoiding detrimental side-effects in long-term treatment.

Finally, contrary to some findings that suggest production of ROS to mediate the COX-independent therapeutic effects of NSAIDs [168], it is suggested that NSAIDs in fact act as antioxidants and inhibit ROS formation. By inhibiting superoxide-mediated peroxynitrite formation and NF-κB activation, NSAIDs maintain inhibitory nitric oxide levels to block further ROS formation. Although inhibitory action of NSAIDs on NF-κB, in particular through sulindac, has been suggested in the past [169], no underlying molecular mechanism for sulindac-mediated inhibition of IKK^{β} activity has been put forward. This novel mechanism of action of NSAIDs is supported by studies on the antioxidant properties of NSAIDs in the brain [170] and by investigations on the scavenging activity of sulindac and its metabolites [171]. In a similar fashion, it can be hypothesized that the protective effects of omega-3 find their origin in inhibiting NF-κB and ROS activity rather than in direct inhibition of COX-2.

In an attempt to combine summarised findings of the current understanding of the AA cascade and its molecular interactions in existing literature, an overview (Figure 12) based on a systems-integrated approach is proposed. Based on the assumption that the primary mode of action of catalytic AA enzyme inhibition is mediated by NO, ROS and NF-κB activity, a novel hypothesis is put forward. This idea is further supported by the observation made with the NO-NSAIDs, where an increase in NO has shown to have a positive therapeutic effect and has even significantly increased inhibitory action in comparison to that of the common NSAIDs. Further supportive data can be derived from the reports on COX- and LOX-targeted NDGA treatment. As an antioxidant and free radical scavenger, NDGA has been associated with profound inhibitory action, especially on the LOX pathway [172]. These findings are of

particular relevance, since the LOX pathway seems to generate a greater number of lipid peroxides and/or ROS than COX. Consequently, treatment with an antioxidant would be expected to give satisfactory results.

Interactions with radical nitrogen species affect COX, LOX as well as CYP450 pathways and may well account for the effects of dietary omega-3 in reducing overall cancer risk. The underlying basis of action is not through NO itself but through its interaction with superoxide and subsequent production of peroxynitrite, leading to increased NF-κB activity, a connection which has been previously noted [173]. Naturally, these interactions depend on a number of factors, among which the cell types and their preferred eicosanoid signalling pathways appear to be key determinants.

Figure 12

CONCLUSION

The examples discussed thus far illustrate that altered AA metabolism in the tumour microenvironment has profound impact on the pathogenesis of tumour development. Clearly, the complex and partially interconnected pathways as well as cross-talk and signal transduction mechanisms between the various players within the AA cascade have not yet been sufficiently considered or explored. However, investigations to date, in particular on the basis of inhibition studies, have identified NF-κB as one of the key signal transducers within the AA cascade. Collated evidence points towards its involvement in cell proliferation, survival, migration, inflammation, and neo-angiogenesis.

The summarized findings contained in this review support this novel hypothesis, providing both a mechanistic basis of action for omega-3 intervention and NSAID-mediated inhibition of pro-inflammatory and oncogenic signalling. However, it is expected that the suggested

interactions still represent an over simplistic schematic representation of the actual processes, with a lot of missing links to be filled. In particular, a better understanding of the reported feedback mechanisms between COX, LOX and CYP450 and components of their downstream signal transduction pathways is required. In order to evaluate and verify these mechanisms *in vivo*, further research is necessary. Among the investigations that may hold a promise in the future for resolving tumourigenesis due to AA metabolism are the study of the significance and interaction of the formation of reactive lipid oxygen species, the better understanding of the precise molecular mechanisms of endogenous AA metabolites and their physiological role and, seeing that their presence and activity determines eicosanoid production, investigations of inhibitors of downstream isomerases of the AA cascade.

It is evident that the NO/ROS/NF-κB pathway provides an interesting and challenging target and promising possibilities for inflammatory-mediated disease and cancer chemoprevention and treatment.

Acknowledgements This work was supported by The Open University and the MSc course S807 'Molecules in Medicine', in particular.

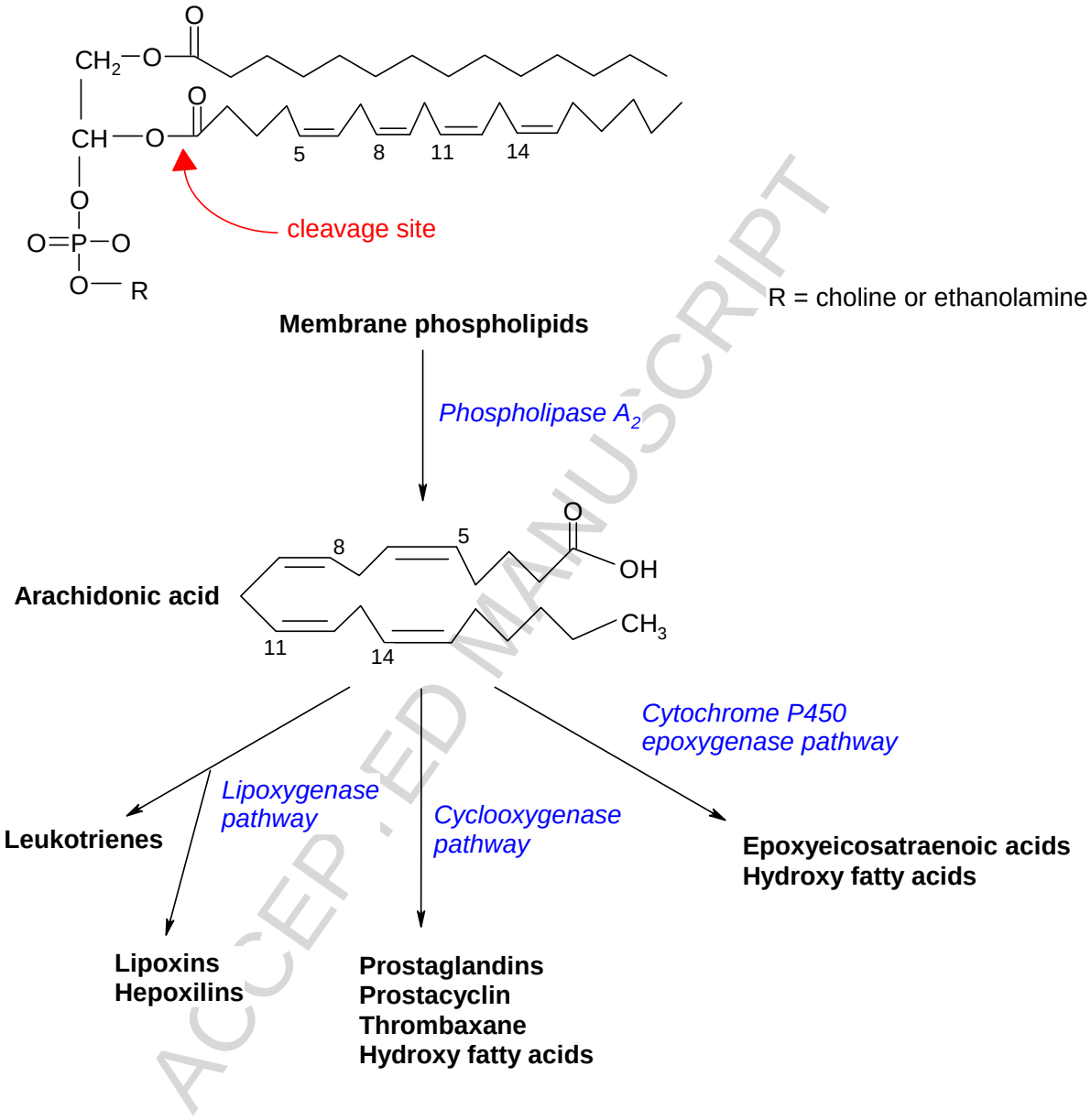


Figure 1. The Classical Arachidonic Acid Pathway. The three key enzymatic metabolic pathways of AA.

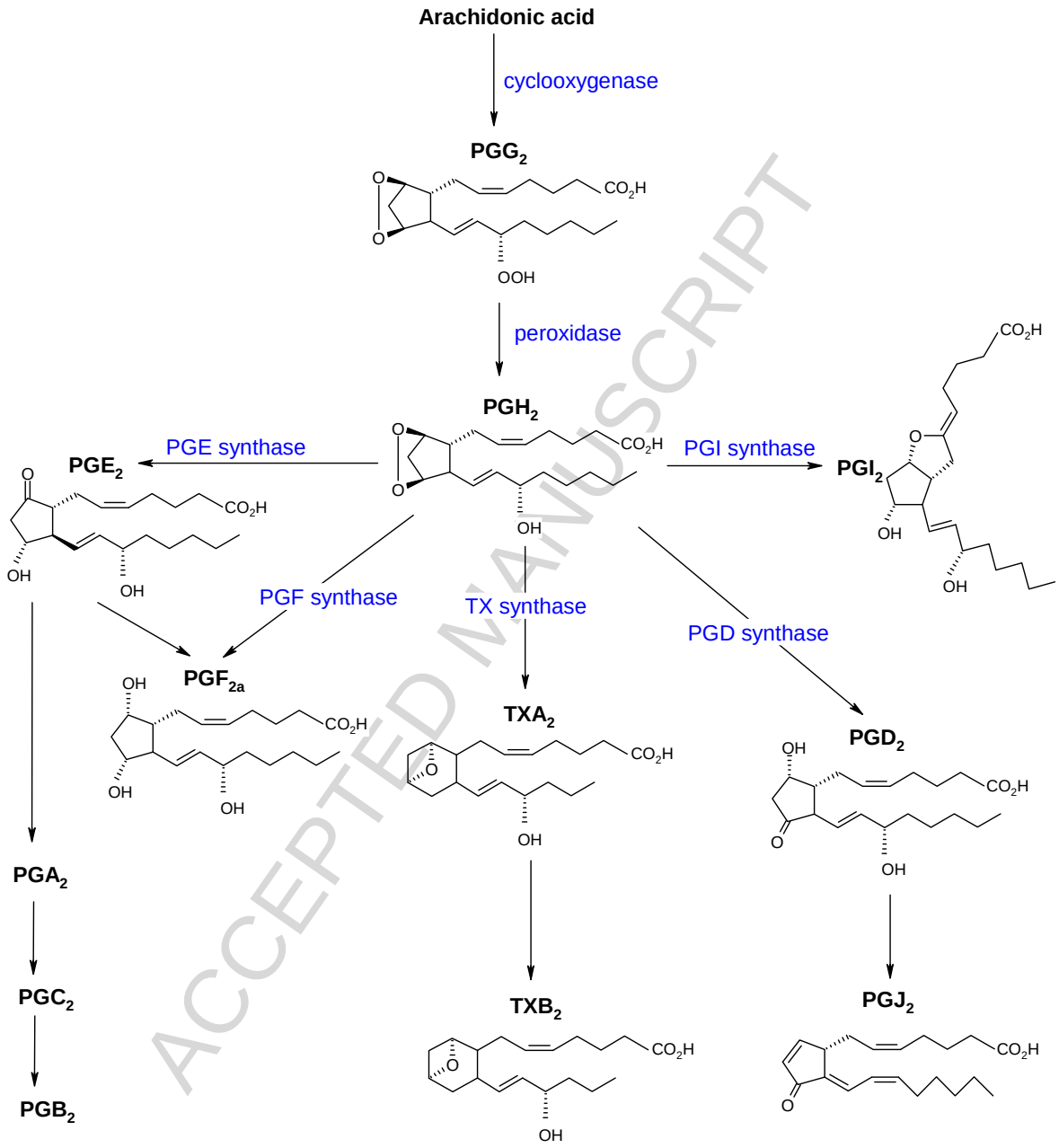


Figure 2. The COX pathway. The main AA derivatives as catalyzed by COX and its isomerases including chemical structures.

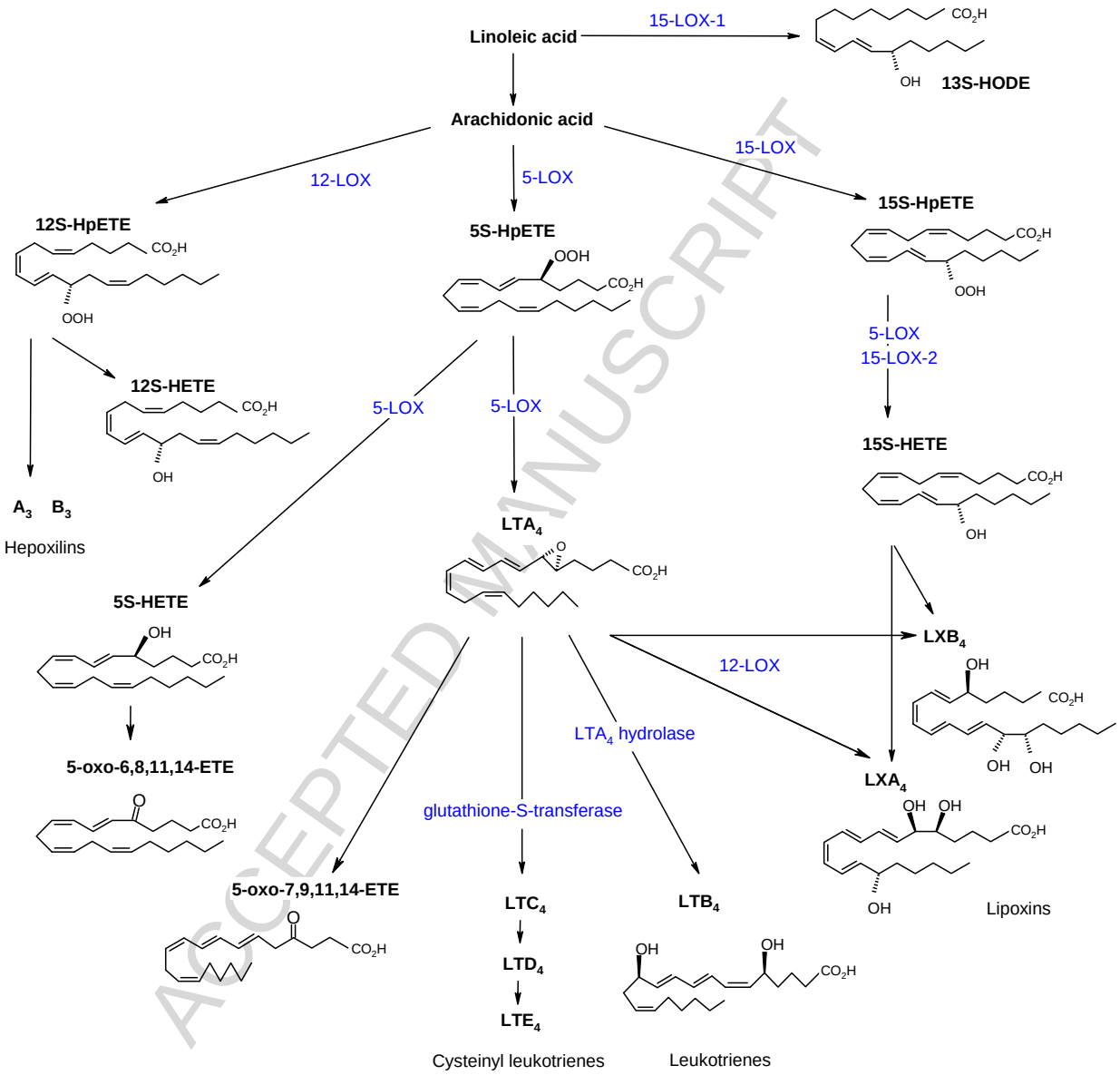


Figure 3. The LOX pathway. The main LOX-catalyzed AA derivatives including chemical structures.

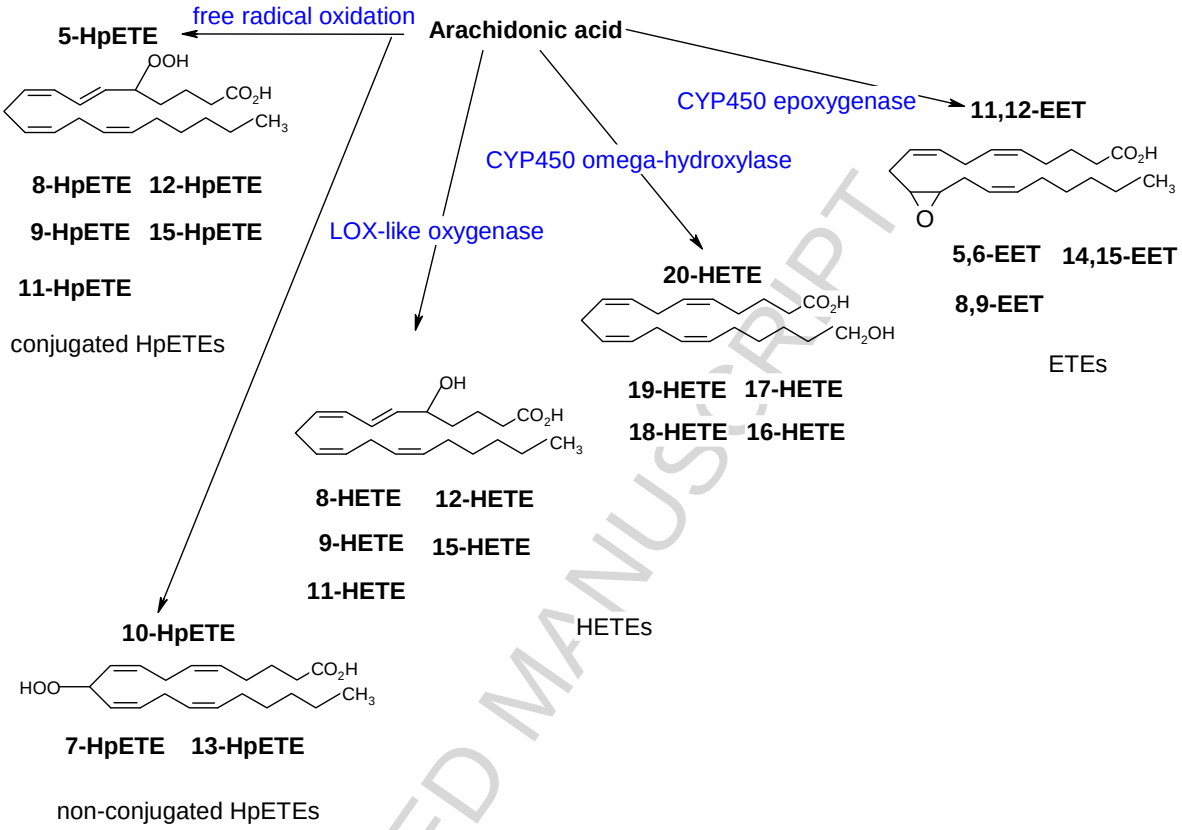


Figure 4. CYP450 catalytic pathway. The main CYP450 catalyzed AA derivatives including their chemical structure.

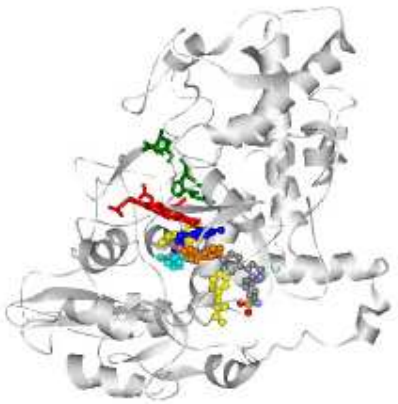


Figure 5. Mouse COX-2 coupled with selective inhibitor SC-558 (PDB: 6COX). The inhibitor is bound to the COX active site, displayed in ball and stick form. Selected amino acid residues are highlighted as follows: Tyr385 (blue) Phe381 and Phe518 (yellow), Leu384 (cyan) and Trp387 (orange). The peroxidase site is located in proximity to the bound haeme molecule (red) with the iron atom as red ball and residues Gln203, His207, Val291 and Leu294 in dark green stick form.

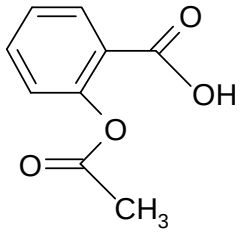
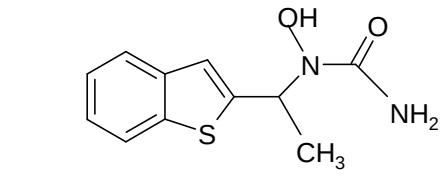


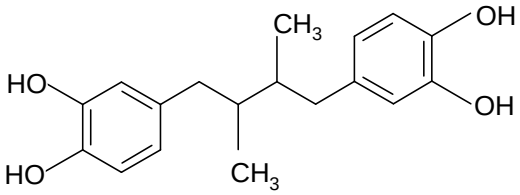
Figure 6. Chemical structure of aspirin.

A)  B) 

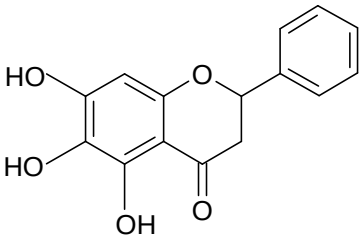
Figure 7. Close-up view of ovine COX-1 (PDB: 1EQG) and mouse COX-2 (PDB: 6COX) acetylation sites. Structural differences are visible by comparing residues Ser 530 in COX-1 and Ser516 in COX-2 (yellow). Tyr385(orange) and Arg120(blue). A) COX-1; B) COX-2.



Zileuton



NDGA



Baicalein

Figure 8. Chemical structures. Structural comparison between zileuton, NDGA and baicalein, a selective 12-LOX inhibitor for comparison.

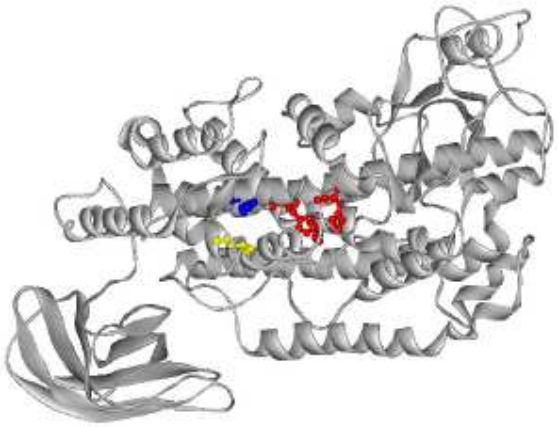


Figure 9. Human 5-LOX (Swissprot: P09917). Relative position of enzyme active site: protein as gray ribbon; residues His367, His550, His 372 are highlighted in red, Glu376 in blue and Ala404 in yellow.

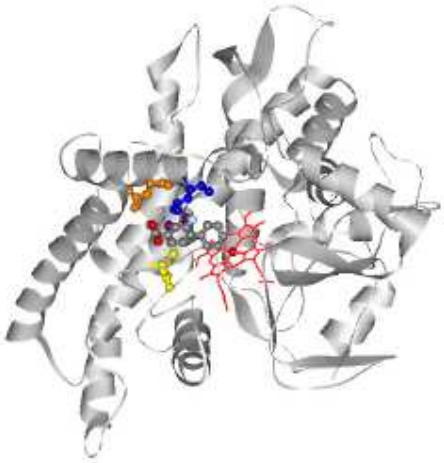


Figure 10. Human CYP450 2C9 bound to flurbiprofen (PDB: 1R9O). CYP450 protein (gray), flurbiprofen in ball and stick conformation coloured according to atoms, haeme group with iron atom as ball (red). Residues Leu208 (blue), Gly296 (purple) and hydrogen-bonding residues Arg108 (green) and Asp293 (yellow) are highlighted.

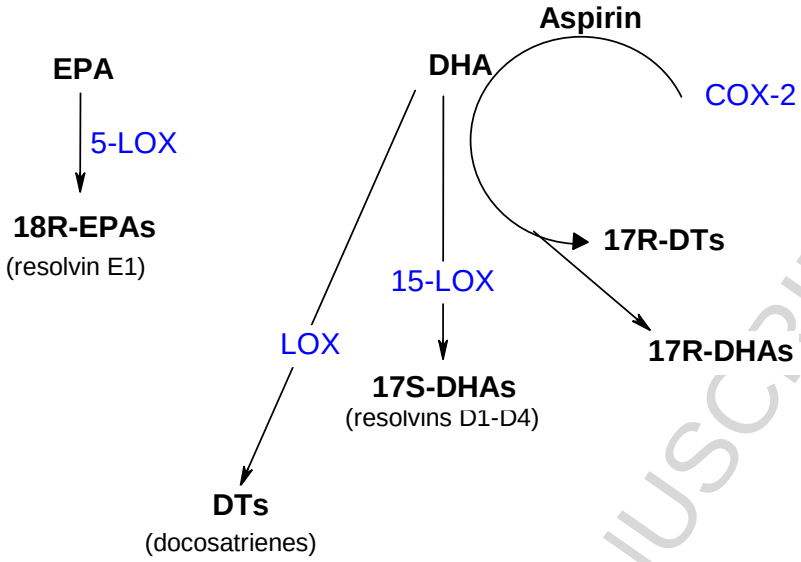


Figure 11. Overview of COX and LOX-catalyzed omega-3 derivatives. Both aspirin-triggered and non-intervened metabolism of omega-3.

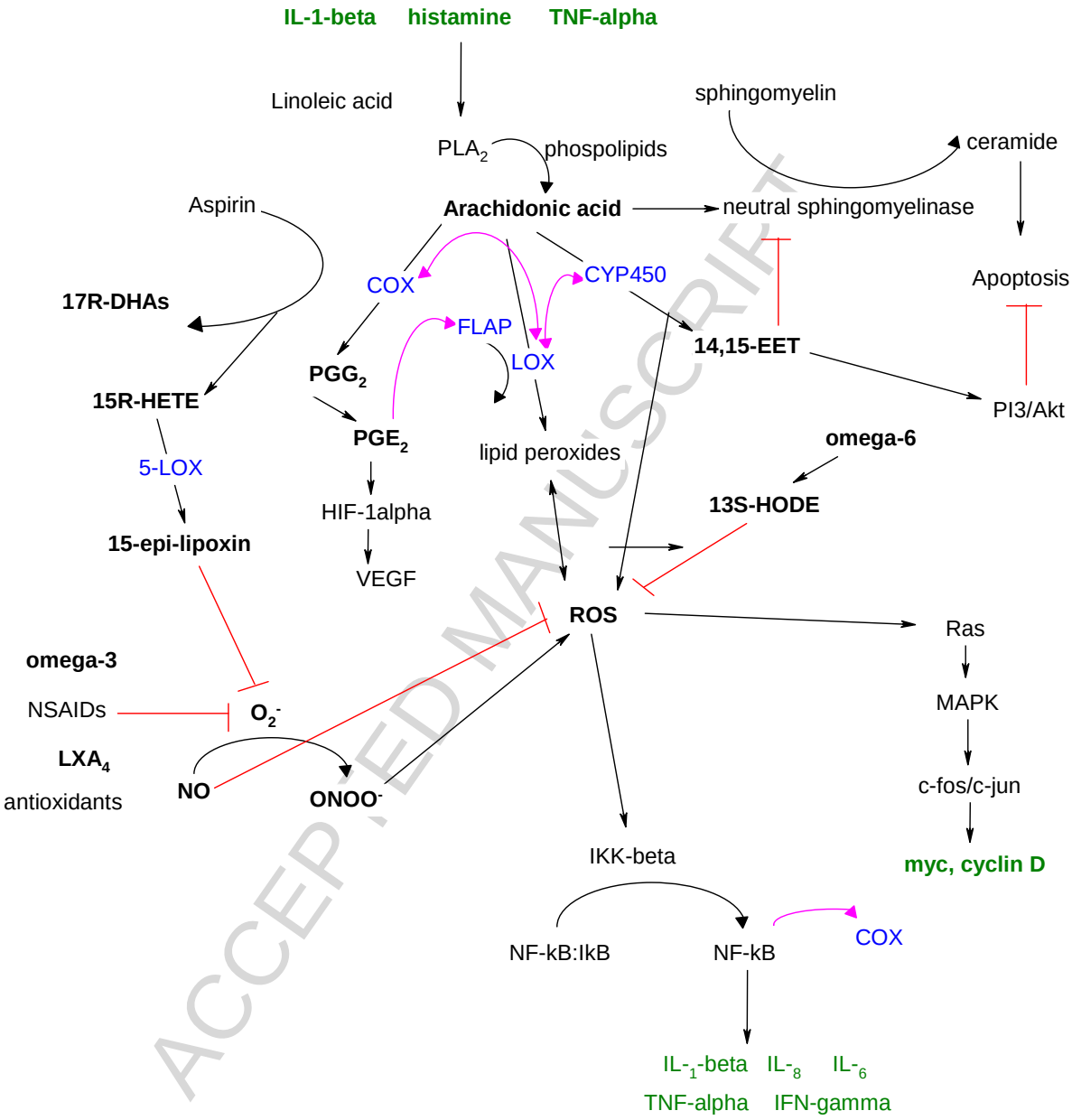


Figure 12. Schematic representation of signalling interactions in the AA cascade. indicates cross-talk and signalling circuitry leading to carcinogenesis including a hypothesis for the mode of actions responsible for success of NSAIDs and omega-3 fatty acids in tumour prevention.

Chemotherapeutic compound:	Mechanism of action:	Reference:
analgesics & anti-pyretics:		
Paracetamol (Acetominophen)	unestablished; selective COX-3 inhibitor?	Chandrasekharan NV et al. (2002) [174]; Schwab JM et al. (2003) [175]; Anderson BJ et al. (2008) [176]
Phenacetin	dual COX-1/COX-2 activity	Kankuri E et al. (2003) [177]
traditional NSAIDs:		
Aspirin	dual COX-1/COX-2 activity; preference for COX-1	Huls G et al. (2003) [178]; Chan AT et al. (2007) [179]
Diclofenac	dual COX-1/COX-2 activity; LOX activity?	Falkowsk M et al. (2003) [180]; Cannon CP et al. (2006) [181]; Kearney PM et al. (2006) [182]
Etodolac	dual COX-1/COX-2 activity; preference for COX-2	Sugimoto T et al. (2007) [183]; Okamoto A et al. (2008) [184]
Ibuprofen	dual COX-1/COX-2 activity	Yao M et al. (2005) [185]; Li W et al. (2008) [186]
Indomethacin	dual COX-1/COX-2 activity; preference for COX-1	Tavares AI (2000) [187]; Touhey S et al. (2002) [188]
Ketoprofen	dual COX-1/COX-2 activity	Marjanović M et al. (2007) [189]
Meloxicam	dual COX-1/COX-2 activity; preference for COX-2	Tavares AI (2000) [187]; Del Tacca M et al. (2002) [190]; Naruse et al. (2006) [191]
Nabumetone	dual COX-1/COX-2 activity; preference for COX-2	Nakanishi A et al. (2001) [192]; Vural F et al. (2005) [193]
Naproxen	dual COX-1/COX-2 activity	Farkouh ME et al. (2004) [194]; Kearney PM et al. (2006) [182]
Nimesulide	dual COX-1/COX-2 activity; preference for COX-2	Genç S et al. (2007) [195]; Inoue T et al. (2008) [196]
Piroxicam	dual COX-1/COX-2 activity; preference for COX-1	Palmerini E et al. (2005) [197]
Sulindac	dual COX-1/COX-2 activity; preference for COX-1	Dvory-Sobol H et al. (2006) [198]
selective COX inhibitors:		
DFU	selective COX-2 inhibitor	Riendeau D et al. (1997) [199]; Matsumoto G et al. (2004) [200]; Hétu PO et al. (2005) [201]
FPA-306	selective COX-2 inhibitor	Ahmed F et al. (2007) [202]
JTE-522	selective COX-2 inhibitor	Hashimoto H et al. (2002) [203]; Kobayashi H et al. (2004) [204]
MF tricyclic	selective COX-2 inhibitor	Hétu PO et al. (2005) [201]; Dvory-Sobol H et al. (2006) [198]
NS-398	selective COX-2 inhibitor	Futaki N et al. (1994) [205]; Tavares AI (2000) [187]; Minter HA et al. (2003) [206]
SC-560	selective COX-1 inhibitor	Hétu PO et al. (2005) [201]; Brenneis C et al. (2006) [207]; Li W et al. (2008) [186]
SC-58125	selective COX-2 inhibitor	Sheng GG et al. (1997) [208]; Ding J et al. (2005) [209]
Coxibs:		
Celecoxib	selective COX-2 inhibitor	Silverstein FE et al. (2000) [210]; Salomon SD et al. (2005) [211]; Bertagnolli MM et al. (2006) [112]; Arber N et al. (2006) [114]
Rofecoxib	selective COX-2 inhibitor	Bombardier C et al. (2000) [212]; Bresalier RS et al. (2005) [111]
Etoricoxib	selective COX-2 inhibitor	Cannon CP et al. (2006) [181]
Valdecoxib	selective COX-2 inhibitor	Ott E et al. (2003) [213]; White WB et al. (2004) [214]; Nussmeier NA et al. (2005) [215]

Lumiracoxib	selective COX-2 inhibitor	Farkouh ME et al. (2004) [194]
Parecoxib	selective COX-2 inhibitor	Ott E et al. (2003) [213]; Nussmeier NA et al. (2005) [215]
dual COX/LOX inhibitors:		
BW-755C	dual COX-1/COX-2 acitivty; 5-LOX inhibitor	Leval X et al. (2002) [216]
S-2474	selective COX-2 inhibitor; 5-LOX inhibitor	Inagaki M et al. (2000) [217]
Licofelone (ML-3000)	dual COX-1/COX-2 acitivty; 5-LOX inhibitor	Reginster JY et al. (2002) [218]; Skelly MM et al. (2003) [219]; Tries S et al. (2002) [220]
Phenidone	dual COX-1/COX-2 acitivty; 5-LOX inhibitor	Moon C et al. (2005) [221]
RWJ-63556	dual COX-1/COX-2 acitivty; 5-LOX inhibitor	Filliatre G et al. (2001) [222]
S-2474	selective COX-2 inhibitor; 5-LOX inhibitor	Inagaki M et al. (2000) [217]

Table 1. Overview of most clinically applied and studied prostaglandin inhibitors

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