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Phospholipase A_2 expression in tumours: a target for therapeutic intervention?

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Phospholipase A_2 (PLA_2) enzymes are involved in lipid metabolism and, as such, are central to several cellular processes. The different PLA_2s identified to date can be classified into three groups: secreted PLA_2 ($sPLA_2$), calcium-independent PLA_2 ($iPLA_2$) and calcium-dependent cytosolic PLA_2 ($cPLA_2$). In addition to their role in cellular signalling, PLA_2s have been implicated in diverse pathological conditions, including inflammation, tissue repair and cancer. Elevated levels of $sPLA_2$ and $cPLA_2$ have been reported in several tumour types. Here, we summarize the current views on the PLA_2s, and look at their expression, role in human malignancy and potential as targets for anticancer drug development.

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▼ A major drawback of anticancer agents used in the clinic today, is their lack of tumour specificity and their consequent activity against non-tumour cells. Therefore, a great deal of research is focused upon either increasing the tumour specificity of current therapeutics or developing new tumour-specific anticancer agents. Many of these strategies involve targeting of tumour vasculature, tumour hypoxia regions or tumour physiology. One area of tumour physiology under investigation for drug development is tumour-specific enzyme expression, either as a direct therapeutic target or as an activator of prodrugs to their cytotoxic derivative. This review highlights one potential group of enzymes, the phospholipase A_2 family (PLA_2), and examines their potential as targets for anticancer therapeutic agents.

The case for PLA_2s

Membrane phospholipids consist of a glycerol backbone, to which 2 long-chain fatty acids are bound at the sn-1 and sn-2 positions and a phosphate-containing head group at the sn-3 position (Fig. 1). The fatty acids located at position sn-2 are generally highly saturated, often possessing 1–5 double bonds, e.g. arachidonic acid (AA). The PLA_2 family consists of a wide variety of enzymes that hydrolyze the sn-2 ester bond of phospholipids, creating a lysophospholipid and releasing a fatty acid – predominantly AA [1]. Once released, AA is further processed by cyclooxygenases (COX-1 and COX-2) or lipoxygenase (LOX) to form eicosanoids, including prostaglandins and leukotrienes. Eicosanoids are central mediators of inflammation [2] and several, such as prostaglandin E_2 (PGE_2), are mitogenic [3]. The observation that many tumours have elevated eicosanoid levels [4,5] highlights their central role in tumour development and confirms their validity as potential targets for therapeutic intervention. Consequently, PLA_2s can be seen as fulfilling a significant role in cancer development and treatment.

The mammalian PLA_2 enzymes have been broadly classified into three groups: (1) low-molecular-weight Ca^{2+}-dependent secretory phospholipase A_2 ($sPLA_2$); (2) high-molecular-weight Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$); and (3) Ca^{2+}-independent phospholipase A_2 ($iPLA_2$). These will each be discussed in turn.

Secreted PLA_2s ($sPLA_2$)

Characteristics of $sPLA_2$

The secretory phospholipases are the largest branch of the mammalian phospholipase A_2 family, with ten structurally different members identified so far [1,6]. They share several characteristics: all are between 14–19 kDa, require millimolar concentrations of Ca^{2+} for enzymatic activation, are secreted from cells

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and are consequently found extracellularly. Despite these shared features, the $sPLA_2$s demonstrate a diverse range of physiological functions and signalling properties, many of which appear to be tissue-specific [1,7].

The first $sPLA_2$ was discovered in pancreatic juice and is now termed 'group IB' $sPLA_2$ [1]. Following this, a second $sPLA_2$ enzyme was isolated from human arthritic synovial fluid and named $sPLA_2$ or group IIA $sPLA_2$ [1]. This family member has subsequently been shown to be induced during inflammation in a wide selection of tissues. A further eight $sPLA_2$ family members have been identified: these are groups IIC, V, X, IID, IIE, IIF, III and XII in historical order of identification (reviewed in [1]).

By definition, all $sPLA_2$s hydrolyze the ester bond at the sn-2 position of membrane phospholipids (Fig. 1) in the presence of millimolar concentrations of Ca^{2+}. However, unlike other PLA_2 family members, $sPLA_2$s do not demonstrate any specific fatty acid selectivity. Differences also exist within the $sPLA_2$ subfamily with respect to the type of phospholipids cleaved. For example, group II $sPLA_2$s (IIA, IIC, IID, IIE, IIF) preferentially act upon anionic rather than neutral phospholipids, whereas groups V and X demonstrate no such preference. These differences in substrate specificity suggest diverse cellular functions for the various $sPLA_2$ enzyme subtypes [1]. With the exception of $sPLA_2$-IIA, little is known about the other group II $sPLA_2$ members (IID, IIE, IIF, V and X) with regards to their biological function, relationships to other cellular systems or roles in disease processes [8].

One of the main functions of $sPLA_2$-IIA and, potentially, other group II members, is in inflammation mediation (Fig. 2). The expression of $sPLA_2$-IIA is inducible by pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) (Fig. 2) [9,10], and is downregulated by anti-inflammatory cytokines [11] and glucocorticoids [12] in several diverse cell and tissue types [8]. In addition to a pro-inflammatory role, $sPLA_2$-IIA also has anti-bacterial and atherosclerotic properties [13,14].

$sPLA_2$ expression in tumours

More recently, $sPLA_2$-IIA has been suggested to have a central role in both tumour development and progression [15–17]. The elevated gene and protein expression of $sPLA_2$-IIA observed in human colorectal adenomas from familial adenomatous polyposis (FAP) patients, supported the hypothesis that $sPLA_2$-IIA dysregulation is an early step in colorectal tumourigenesis [17]. Significantly higher levels of $sPLA_2$-IIA have also been demonstrated in neoplastic prostatic tissue, compared with benign prostatic glands [15]. However, in this study, no correlation was observed between $sPLA_2$-IIA expression and either

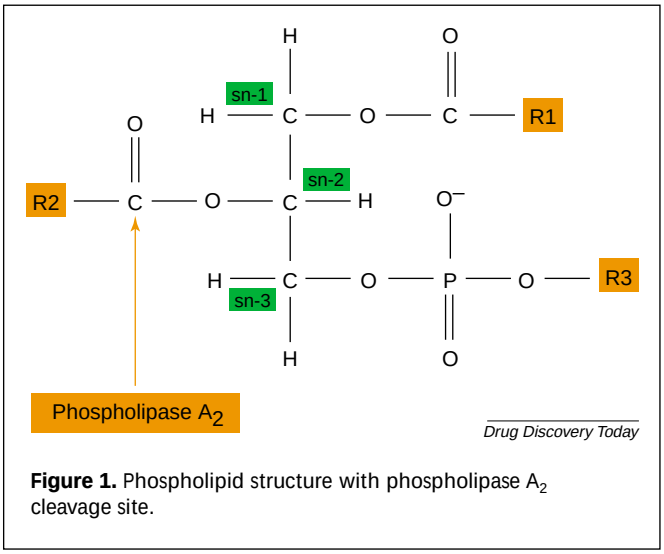

Figure 1. Phospholipid structure with phospholipase A_2 cleavage site.

Gleason grade (indicating severity of prostate cancer), patient age, tumour stage or presence of metastasis [15]. Conversely, Graff *et al.* demonstrated that $sPLA_2$-IIA expression in the prostate increased with progression to androgen independence, and was inversely related to patient survival [16]. Taken together, these data also suggest $sPLA_2$-IIA upregulation as an early event in prostate tumourigenesis. Comparative expression of $sPLA_2$-IIA protein expression was also observed in other organs and their malignant counterparts, such as the gastric mucosa [18,19], pancreas [20], small bowel and colon [21,22]: this further supports a central role for $sPLA_2$ in human tumourigenesis.

$sPLA_2$ as a tumour suppressor gene?

Mutations of the Adenomatous Polyposis Coli (APC) gene have been associated with the development of both sporadic and familial human colorectal tumours [23]. Similarly, mice carrying a dominant mutation in their APC homologue are also predisposed to intestinal adenoma formation, a condition termed Min1 (multiple intestinal neoplasia). In these mice, a second locus was identified, termed Mom1 (modifier of Min1), which modifies both the number and size of polyps produced [23,24]. Interestingly, Mom1 has now been identified as the $sPLA_2$-IIA gene [23–25], highlighting it as a negative modifier of tumour development. Further to this, the addition of a cosmid transgene overexpressing $sPLA_2$-IIA has the effect of reducing tumour multiplicity in Min1 mice [7]. These data strongly suggest a tumour-suppressive role for $sPLA_2$-IIA, albeit in murine colorectal carcinogenesis.

At present, information regarding $sPLA_2$-IIA as a human tumour suppressor remains inconclusive. Hemizygous deletion of the $sPLA_2$-IIA gene, observed in

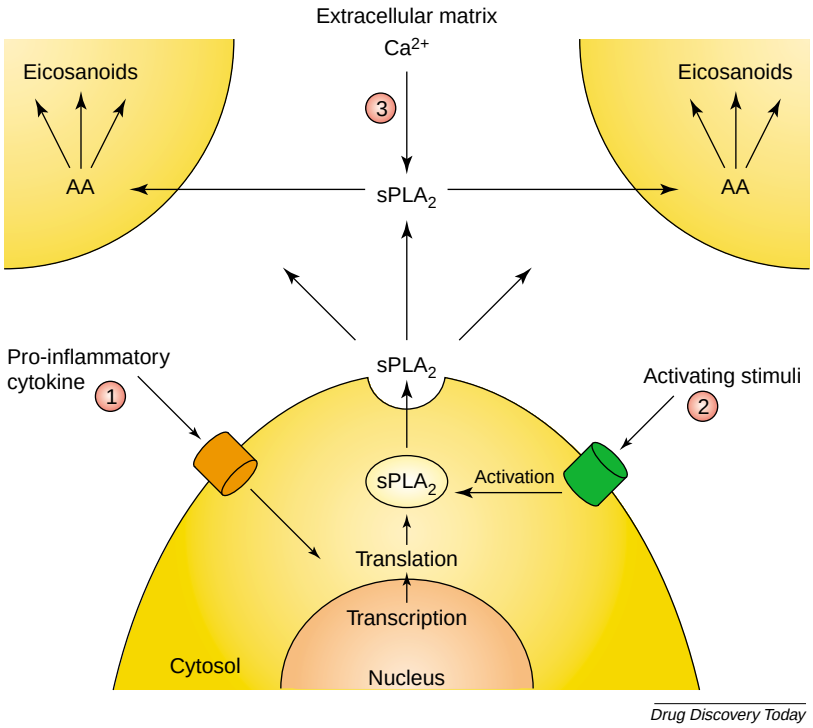

Figure 2. $sPLA_2$ participating in an inflammatory response. (1) Pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) or interleukin 1β (IL-1β) induce cellular expression of $sPLA_2$. (2) Activating factors cause release of $sPLA_2$ from secretory granules into the extracellular matrix. (3) In the presence of millimolar concentrations of Ca^{2+}, $sPLA_2$ hydrolyzes membrane-bound phospholipids of neighbouring cells. Released fatty acids, such as arachidonic acid, are further metabolized into eicosanoids, generating an inflammatory response in neighbouring cells.

human neuroblastoma and colorectal carcinomas [26,27], supports this theory, as do positive correlations between $sPLA_2$-IIA expression, prolonged patient survival and decreased metastatic frequency in human gastric adenocarcinoma [19]. By contrast, the lack of genetic mutations of $sPLA_2$-IIA in human colorectal carcinomas [23,27–29], neuroblastoma [26,29] or melanoma [29] tumours and cell lines would suggest the converse. Similarly, the elevated $sPLA_2$-IIA levels observed in small bowel adenocarcinomas [22], and positive correlations observed between $sPLA_2$-IIA expression, proliferative index and increased tumour grade in the prostate [15,16], offer no support for $sPLA_2$-IIA as a tumour suppressor. Inhibition of $sPLA_2$ activity has been shown to inhibit proliferation, induce apoptosis and suppress angiogenesis [30–32], suggesting that $sPLA_2$-IIA expression is pro-tumourigenic rather than tumour-suppressive. Furthermore, the recent identification of a second Min1 modifier locus, termed Mom2 [33,34], demonstrates a stronger effect on polyp multiplicity in mice than Mom1. This, therefore, questions the putative tumour suppressor role of $sPLA_2$ (Mom1). Further studies are required to clarify whether the contradictory roles of $sPLA_2$-IIA are due to species, tissue- or cell-specific responses. Despite this, it is clear that $sPLA_2$-IIA has a potentially important role in tumour development and, thus, also in therapy.

$sPLA_2$ as a target for cancer therapy

As a result of its central role in eicosanoid formation and the inflammatory response (Fig. 2), inhibition of $sPLA_2$-IIA expression and activity is currently under investigation as an anti-inflammatory therapeutic approach [35,36]. Several PLA_2 inhibitors have been proposed as potential anti-inflammatory drugs [37]. Of these inhibitors, those which do not enter the cell are proving most effective [35]: cell impermeable or extracellular PLA_2 inhibitors have the potential to limit inflammation by selectively inhibiting extracellular PLA_2s, at the same time enabling the continued 'housekeeping' activities of intracellular PLA_2s [35].

Recently, the increased expression and activity of $sPLA_2$-IIA observed in human tumours has been suggested as a putative target for anticancer therapeutics [35,38], with $sPLA_2$ inhibitors being shown to significantly retard proliferation of intestinal tumour cells *in vitro* [30] and inhibit prostate tumour cell invasion [39]. Moreover, $sPLA_2$ inhibition has been shown to inhibit endothelial cell proliferation, retard endothelial cell migration and suppress blood vessel formation [32], suggesting the use of $sPLA_2$ inhibitors as anti-angiogenic therapeutics.

More recently, an $sPLA_2$-IIA-mediated anticancer therapeutic strategy was suggested by Davidsen *et al.* [38]. They proposed that the increased $sPLA_2$-IIA levels present in the tumour extracellular matrix could be used to enzymatically activate liposomal prodrugs to potent anticancer etherlipids [38]. The enzymatic cleavage of these inactive prodrugs by tumour-secreted $sPLA_2$ was shown to cause tumour growth inhibition, an effect that was significantly hindered by an $sPLA_2$ inhibitor [38]. This concept is also backed by Zerouga *et al.*, who synthesized a prodrug containing a fatty acid and the cytotoxic drug, methotrexate [40]: this was shown to cause dose-dependent inhibition of leukemia cell proliferation *in vitro* [40]. If this theory is valid, such liposomal prodrugs could be introduced systemically and should only be activated in

those regions of the body where high concentrations of $sPLA_2$ would exist, such as in the microenvironment surrounding a tumour [38]. It is clear, however, that further research is needed to substantiate these theories.

Ca^{2+} independent enzymes ($iPLA_2$)

Characteristics of $iPLA_2$

The Group VIA or $iPLA_2$s were first isolated from a murine macrophage-like cell line. At least three active forms of this 85–88 kDa enzyme have now been identified, termed VIA1, VIA2 and VIB (reviewed in [1]). Several other splice variants of these proteins have also been identified but these are believed to act as dominant-negative inhibitors for active $iPLA_2$s [1]. Both $iPLA_2$-VIA and -VIB are ubiquitously expressed in several cell and tissue types [1,41], show no requirement for Ca^{2+} to function, and show no clear phospholipid substrate specificity [1,37,41]. Unlike other phospholipases, $iPLA_2$s do not preferentially target phospholipids that will yield AA [42].

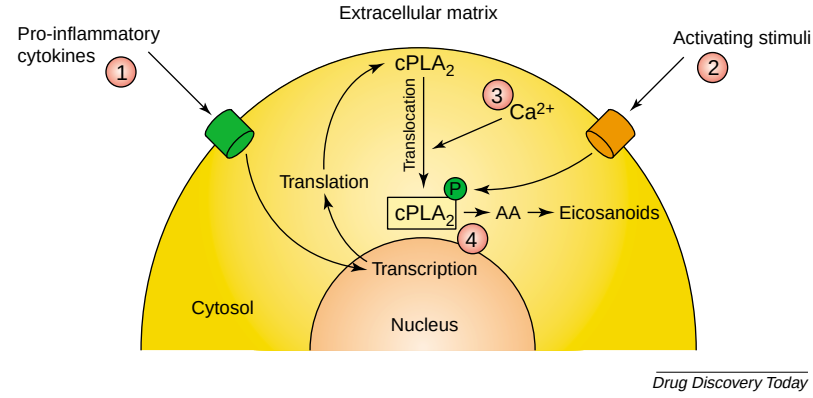

Figure 3. Activation of $cPLA_2$-α. (1) Pro-inflammatory cytokines including tumour necrosis factor α induce expression of $cPLA_2$-α. (2) Activation leads to mitogen-activated protein kinase-pathway-directed phosphorylation of $cPLA_2$-α. (3) Extracellular influx or mobilization of intracellular stores of Ca^{2+} bring about $cPLA_2$-α translocation from the cytosol to perinuclear membranes. This brings $cPLA_2$-α in close proximity to both its substrate and enzymes involved with eicosanoid synthesis. (4) Activated $cPLA_2$-α lyses membrane phospholipids providing arachidonic acid (AA) to a range of enzymes involved with eicosanoid synthesis, specifically COX and LOX.

$iPLA_2$ as a housekeeper enzyme

The main function of the $iPLA_2$s is believed to be as a 'housekeeper' within cells, controlling phospholipid levels and maintaining homeostasis through remodelling of membrane structures [37,43]. In contrast to $sPLA_2$, $iPLA_2$s do not appear to partake directly in the inflammatory response. Cells modified to express $iPLA_2$ and then stimulated with IL-1β (a promoter of inflammation), show the same production of AA as wild-type cells [44]. Other than its housekeeping role, studies have suggested that $iPLA_2$ might be involved in stimulus-coupled fatty acid release and apoptosis [1,45]. For example, agonist-induced PGE_2 production is attenuated by antisense oligonucleotides for $iPLA_2$, whereas overexpression of $iPLA_2$ leads to increased fatty acid release [1]. The link between $iPLA_2$ and apoptosis is demonstrated by the observation that $iPLA_2$ inhibition delays cell death induced by the Fas receptor, whereas Fas-induced apoptosis is accompanied by increased phospholipase activity [45]. Furthermore, in apoptotic cells, $iPLA_2$-VIA is cleaved by caspase proteases resulting in $iPLA_2$ activation and increased fatty acid release [45]. These findings would suggest that $iPLA_2$ is active during apoptosis and might be responsible for the associated remodelling of cell membranes. Although $iPLA_2$ has been implicated in apoptosis, no study has yet identified differential activity in human tumours, suggesting a lack of suitability as an exploitable anti-cancer therapeutic target.

Calcium dependent PLA_2 ($cPLA_2$)

Characteristics of $cPLA_2$

Three different $cPLA_2$s – $cPLA_2$α, β and γ – have been isolated and classified into groups IVA, IVB, IVC, respectively [6,46]. Of these isoforms, $cPLA_2$α has been studied most extensively. $cPLA_2$α is an 85 kDa serine esterase, which is found in a wide range of tissues except lymphocytes [6,47]. By contrast, $cPLA_2$β is a 114 kDa enzyme expressed predominantly in the cerebellum and pancreas, and $cPLA_2$γ is a 61 kDa enzyme expressed predominantly in skeletal muscle [46]. In its inactive state, $cPLA_2$α is located within the cytosol of the cell. Activation of $cPLA_2$α is regulated by cytoplasmic Ca^{2+} levels and by phosphorylation, which, in turn, causes it to translocate from the cytosol to perinuclear membranes (Fig. 3), such as the golgi, endoplasmic reticulum and nuclear envelope [1,47]. Sufficient increased intracellular levels of Ca^{2+} to cause $cPLA_2$α translocation are brought about by mobilization of Ca^{2+} from internal cellular stores or from extracellular sources [48]. The translocation of $cPLA_2$α is important for two reasons: first, it enables interaction between the enzyme and its substrate membrane phospholipids and, second, it brings the enzyme into close proximity to other downstream enzymes involved with eicosanoid synthesis, specifically COX and LOX [6].

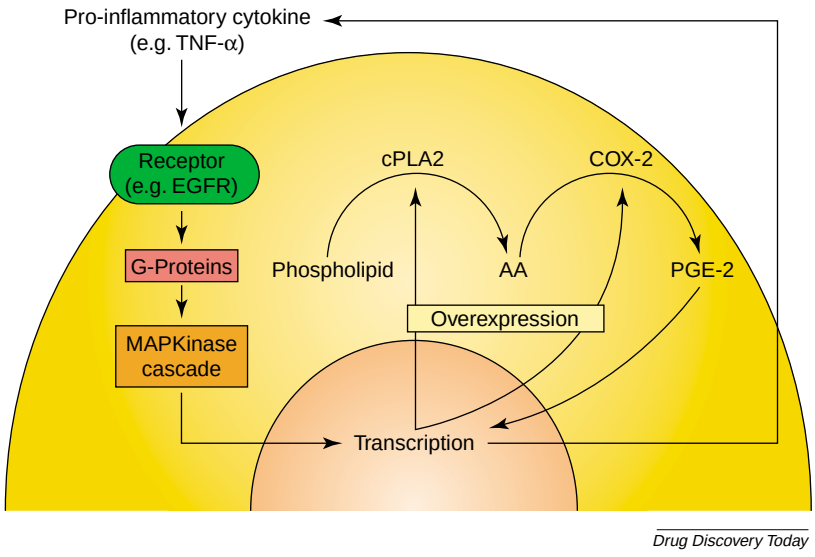

Figure 4. Model of constitutive overexpression of cPLA$_2$ and COX-2 in tumour cells (reviewed in [4]).

In addition to a requirement for Ca^{2+}, maximal activity of cPLA$_2\alpha$ requires sustained phosphorylation of defined amino acids within its structure. Examination of the molecular structure of cPLA$_2$ revealed four serine residues that might undergo phosphorylation (Ser^{437}, Ser^{454}, Ser^{505}, Ser^{727}) [49,50]. Further studies revealed that the phosphorylations of Ser^{505} and Ser^{727} were the most important for enzymatic activity: they are mediated by mitogen-activated protein kinases (MAPK) and by MAPK-activated protein kinases, respectively [49,50]. Phosphorylated cPLA$_2\alpha$ is generally unable to release arachidonic acid without an accompanying increase in Ca^{2+} levels, indicating that both phosphorylation and sub-micromolar Ca^{2+} concentrations are required for activity.

cPLA$_2\alpha$ is highly selective towards phospholipids that have AA at the sn-2 position (Fig. 1) [48,51]. Sequence analysis of cPLA$_2\beta$ revealed regions homologous to the Ca^{2+} and lipid-binding domains of cPLA$_2\alpha$, and Ca^{2+} dependent activity [6]. cPLA$_2\gamma$ appears to be membrane-bound and, due to a lack of both the lipid and Ca^{2+}, binding domains would seem to be Ca^{2+} independent [6]. Unlike cPLA$_2\alpha$, the substrate preferences and activity of cPLA$_2\beta$ and cPLA$_2\gamma$ are not as specific; both enzymes also demonstrate phospholipase A_1 activity [46]. Little is known about the cellular functions of cPLA$_2\beta$ and γ, therefore, the focus here will be specifically on cPLA$_2\alpha$.

The possible role for cPLA$_2$ in tumours

Although cPLA$_2\alpha$ is expressed in several tissue types [47], elevated expression of cPLA$_2\alpha$ has been shown in a range of human tumour types, such as colorectal [22,52,53], small bowel [22] and lung [54]. Within these, high levels of AA and eicosanoids are observed as a consequence of increased activity of cPLA$_2\alpha$ and the COX and LOX enzymes (see Fig. 4) [4]. Like cPLA$_2\alpha$, elevated levels of COX-2 have been associated with human tumourigenesis (Fig. 4) [22,52]. Accordingly, selective inhibition of COX-2 activity has gained considerable interest as an anticancer therapeutic strategy (reviewed in [55]). This serves to reinforce the importance of AA in human tumourigenesis and, thus, disturbance of AA metabolism as a potential anticancer therapeutic strategy.

The presence of mutated Ras genes is a common genetic characteristic of several human tumour types, including colon, prostate and lung [54,56]. Oncogenic Ras proteins in these tumours result in persistent activation of intracellular signalling pathways involved in proliferation and transformation [54,56]. Recently, in human non-small-cell lung cancers, expression of oncogenic forms of Ras were associated with increased expression and activity of cPLA$_2\alpha$ [54,56] – a relationship that was strengthened by the observation that Ras inhibition led to decreased cPLA$_2\alpha$ expression and prostaglandin synthesis [54]. The ability of Ras to influence cPLA$_2\alpha$ activity is thought to be due to the effect of Ras on MAPK activity and subsequently, on cPLA$_2\alpha$ phosphorylation [56]. These data demonstrate the key role cPLA$_2\alpha$ might have in human tumourigenesis, and outline the potential use of specific cPLA$_2\alpha$ inhibitors as therapeutics for cancer treatment.

Is cPLA$_2$ an oncogene?

In direct contrast to the potential tumour-suppressive role of sPLA$_2$-IIA, cPLA$_2\alpha$ expression in mice has been suggested to be pro-tumourigenic [57]. Homozygous deletion of the cPLA$_2\alpha$ gene in Min1 mice resulted in an 83% decrease in small intestinal polyp number and an accompanying decrease in polyp size [57,58]. The intestinal epithelium in cPLA$_2\alpha$ null mice contained numerous small ulcerative lesions, indicating that cPLA$_2\alpha$ has a role in tumour promotion, rather than tumour initiation [58]. Furthermore, these studies support the notion that cPLA$_2\alpha$ is the predominant source of AA for the COX-2 pathway (Fig. 4), and argue against a protective role for AA in tumour formation [57].

At present, there is little evidence for extrapolating this oncogenic role for cPLA$_2\alpha$ to humans. Although cPLA$_2\alpha$

has been demonstrated to be elevated in human small bowel and colorectal adenocarcinomas [22,53], the expression profile of other human tumour types or the presence of activating genetic mutations have yet to be reported.

Recently, studies have suggested that the effects of $cPLA_2\alpha$ on tumour formation might be tissue-specific [57–59]. Whereas homozygous deletion of $cPLA_2\alpha$ produced a significant reduction in tumour number in the murine small intestine, no significant effect was observed in the large intestine [57,58]. Overall, these findings suggest that the role of $cPLA_2\alpha$ in murine colon tumourigenesis might be fundamentally different from that observed in the small intestine and could be under the control of distinct physiological mechanisms [59]. Furthermore, a preliminary study showed diminished expression of $cPLA_2\alpha$ in human colonic tumours, compared with adjacent histologically normal tissue [59]. These findings suggest a tissue-specific variation in tumourigenesis, raising questions as to whether or not $cPLA_2\alpha$ is an oncogene that can be comprehensively addressed in all tumours.

Disruption of $cPLA_2$ activity as a potential therapeutic tool

The blocking of eicosanoid production through the deactivation of enzymes downstream to $cPLA_2\alpha$, has been exploited for some time. Indeed, the inhibition of the COX enzymes using non-steroidal anti-inflammatory drugs (NSAIDs) has therapeutic effects on several human tumour types [55]. However, as well as inhibiting the required target, COX-2, many of these drugs also inhibited the housekeeping enzyme COX-1, resulting in several serious side effects (discussed in [4,35]). Although the use of COX-2-specific inhibitors avoids the deleterious side effect of COX-1 inactivation, many of these selective drugs have complications [35]. These could be the consequence of shifting AA metabolism from the inhibited COX-2 enzyme to alternative pathways, such as COX-1 and LOX [35], which itself causes problems.

Based on this, an alternative therapeutic approach would be to avoid complications of COX-2 inhibition by limiting AA availability and subsequent eicosanoid production (see Fig. 4). In addition to being rate-limiting for eicosanoid biosynthesis, $cPLA_2\alpha$ has been suggested as a modulator of $sPLA_2$-IIA activity [60]. These data support inhibition of $cPLA_2\alpha$ activity as an attractive anticancer therapeutic strategy. Several $cPLA_2\alpha$-specific inhibitors, both chemical and genetic, are currently in development (reviewed in [37]) and their effectiveness as anticancer agents is yet to be addressed.

Towards tumour-specific drug delivery

The role that each of the phospholipase A_2 subtypes have in tumourigenesis is diverse, complex and, as yet, unresolved. One feature that is common to all phospholipase A_2 family members is their ability to hydrolyze the sn-2 ester bond of phospholipids, either intracellulary ($cPLA_2$ and $iPLA_2$), or extracellularly ($sPLA_2$). Roles for phospholipase A_2 members have been demonstrated on both sides of the tumourigenic equation, as tumour suppressors and oncogenes. A better understanding of the expression, activity and function of each family member in human tumourigenesis might lead to the design of drugs to either treat, inhibit or prevent human cancers. Such enzyme inhibitors or enzyme-activated prodrugs could enable tumour-specific drug delivery and activity, and as such a favourable therapeutic strategy.

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