COX-2 inhibitors: a novel strategy in the management of breast cancer

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Cyclooxygenase-2 (COX-2) inhibitors are common anti-inflammatory drugs with pleiotropic, endogenous actions that could be useful in the management of breast cancer. Here, we provide a complete understanding of the biochemistry of COX-2 and discuss the various molecular mechanisms behind its increased expression in breast cancer. We also analyze the possible mechanisms responsible for the anticancer effect of COX-2 inhibitors and provide an overview of the available preclinical and clinical data on the use of COX-2 inhibitors in breast cancer. Finally, we describe a mathematical model of the relation between the structure and biological potency of promising new COX-2 inhibitors (*trans*-stilbenes) using a 2D quantitative structure–activity relationship (QSAR) technique.

Introduction

Breast cancer is the most common malignancy and the most common cause of cancer mortality for females in industrialized, Western-lifestyle countries. In the USA, for example, there are approximately 182,000 new cases diagnosed annually, while each year around 40,000 women die from this disease [1]. Unfortunately, the incidence of breast cancer is still on the increase, although the mortality index has decreased over past few years. This decrease is likely to be the result of the widespread implementation of improved screening techniques, early detection and novel therapies [2]. In fact, intense scientific research in the field of breast cancer pharmacotherapy has provided several advanced strategies, such as monoclonal antibodies and small-molecule kinase inhibitors, which have considerably extended the opportunities for treatment. However, their costs are relatively high. Furthermore, recent studies on the pathologic nature of this disease have revealed the mechanisms by which breast cancer develops. For instance, it has been found that the concentration of prostaglandins (PGs) in breast cancer cells is greater than that in the corresponding normal tissue, indicating that it is aberrant COX expression, especially COX-2,

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that could be associated with the development and progression of the malignancy. Thus, various procarcinogenic actions have been attributed to COX-2, including inhibition of apoptosis, increase in proliferation, and stimulation of angiogenesis, making its abolishment an encouraging strategy for the management and prevention of breast cancer [3–8]. This approach is additionally favored by the fact that appropriate inhibitory agents are already widely available, inexpensive, and relatively well tolerated [9]. In this article, we review the most important issues concerning the use of COX-2 inhibitors in breast cancer and provide a full understanding of the impact of COX-2 on tumorigenesis.

COX-2: structure and function

Human COX family (also named prostaglandin-endoperoxide synthase or prostaglandin-G/H synthase; EC 1.14.99.1) comprises three isoenzymes: constitutive (COX-1), inducible (COX-2), and the recently discovered COX-3. They are involved in the first two steps of prostanoid biosynthesis (i.e., PGs, thromboxanes, and prostacyclins). They catalyze the metabolic transformations of unsaturated fatty acids, such as arachidonic acid (AA), dihomo-γ-linolenic acid, and eicosapentaenoic acid, which are released by phospholipases A_2, C, or D from cell membrane phospholipids [10,11]. COX enzymes exhibit a dualistic nature by displaying both COX activity, converting AA into short-lived 15-hydroperoxide–PGG_2, and PG hydroperoxidase activity, which is crucial in the process of reduction of PGG_2 into PGH_2. PGH_2 is then spontaneously rearranged or enzymatically converted into the biologically active PGs D, E, and F, prostacyclin PGI_2, or thromboxane (TXA_2) [11]. The process of prostanoid biosynthesis by COX is detailed in Fig. 1.

In its constitutive form, COX-1 is expressed as a housekeeping enzyme in most human organs and tissues. It is responsible for the maintenance of internal homeostasis by participating in general body processes, such as cytoprotection of the gastric mucosa, platelet aggregation, vascular smooth muscle functioning, and regulation of glomerular filtration and renal blood flow. By contrast, COX-2 usually remains undetected in healthy tissues and organs. In adults, it is found only in the central nervous system, kidneys, vesicles, and placenta, whereas in the fetus, it occurs in heart, kidneys, lungs, and skin [10,11]. COX-2 is a highly inducible isoform and can be rapidly upregulated in response to various proinflammatory agents, including cytokines, tumor promoters, and mitogens, especially in cells involved in inflammation, pain, fever, tumor, Alzheimer's disease, or osteoarthritis [12,13]. The recently discovered COX-3 isoform is still not fully understood. However, in humans, it exists as a splice variant of COX-1, primarily in cerebral cortex and heart [13].

COX-1 is encoded by ubiquitously expressed housekeeping gene located on chromosome 9. Consistent with its structure, *COX1* is almost devoid of transcription factor-binding sites and, hence, is open to only slight regulation. The gene encoding COX-2 is located on chromosome 1 and comprises the following regulatory elements: TATA box, NF-IL-6 motif, two AP-2 regions, three SP1 regions, CRE motif, and E-box. Their presence makes the transcription of *COX2* susceptible to stimulation by bacterial lipopolysaccharides, the cytokines, such as interleukin (IL)-1β, IL-2 and tumor necrosis factor (TNF)-α, and the growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. By contrast, anti-inflammatory agents, such as corticosteroids, IL-13, IL-10 or IL-4, can inhibit the expression of COX-2 *in vivo* [10,11,14].

Both COX-1 and COX-2 exist as integral, membrane-bound proteins, located primarily on the lumenal side of the endoplasmatic reticulum (ER) and the nuclear envelope. Importantly, COX-2 is more concentrated in the latter position. Both enzymes share common mechanistic features as well as product-substrate profiles. They catalyze AA transformations with similar kinetics, although dihomo-γ-linolenic acid and eicosapentaenoic acid show a higher affinity to COX-2 [13]. Structurally, human COX-1 and COX-2 occur as homodimers stabilized by hydrophobic interactions, and hydrogen and electrolytic bridges. They also exhibit as much as 63% amino acid identity. They vary in terms of their chain length and glycosylation patterns. Human COX-1 comprises 599 amino acid residues (molecular weight 70 kDa) with three *N*-glycosylation sites at Asn67, Asn103, and Asn147. In addition, there are five disulfide bonds between Cys35-Cys46, Cys36-Cys158, Cys40-Cys56, Cys58-Cys68, and Cys568-Cys574 that stabilize its tertiary structure. By contrast, COX-2 comprises 604 amino acid residues (molecular weight 72 kDa) with four *N*-glycosylation sites at Asn53, Asn130, Asn395, and Asn580, and five disulfide bonds between Cys21-Cys32, Cys22-Cys145, Cys26-Cys42, Cys44-Cys54 and Cys555-Cys561 [10,11,14,15] (http://www.uniprot.org/uniprot/P23219; http://www.uniprot.org/uniprot/P35354) (Fig. 2).

The primary structure of COX-2 is functionally divided into four regions, starting from the N terminus: (i) signal peptide (between Met1 and Thr17) usually removed by post-translational modifications; (ii) EGF-like domain (between Ala18 and Ser55), which contains three conserved disulfide bonds; this area is implicated in the process of homodimerization; (iii) a membrane domain, which takes the form of four amphipathic alpha-helices and serves as an anchor sequence; and (iv) acatalytic domain containing two independent peroxidase and cyclooxygenase active sites, and a heme-binding sequence [10,14,15] (http://www.uniprot.org/uniprot/P23219; http://www.uniprot.org/uniprot/P35354).

The COX active site of COX-2 is described as a narrow, hydrophobic channel, approximately 8 Å wide and 25 Å long. It is restricted by the membrane domain and its outlet is located close to the cell membrane. Such construction favors the penetration of lipophilic AA deep inside the active site tunnel to the specific cyclization spot. The peroxidase catalytic site is on the surface of the enzyme and is easily accessible to solvent molecules. It contains a heme cofactor bound with His374 [14].

The catalytic mechanism of COX-2-dependent conversion of AA into PGH_2 can be defined as a series of radical reactions occurring in the following sequence: (i) two-electron reduction of a peroxide substrate and oxidation of the ferric heme of peroxidase active site with the production of ferryl-oxo-porphyrin radical; (ii) formation of a tyrosyl radical at Tyr385 of the COX active site, resulting in the enzyme activation; (iii) abstraction of the pro-S hydrogen atom from C_{13} of AA by the tyrosyl radical; the AA in the COX active site adopts an extended L-shape conformation and its carboxyl is bound with Arg120 and Tyr341 by hydrogen bonds; as a result, C_{13} remains perfectly positioned to interact with the tyrosyl radical; (iv) formation of the arachidonyl radical localized at C_{11} and C_9, and the interaction with oxygen to produce

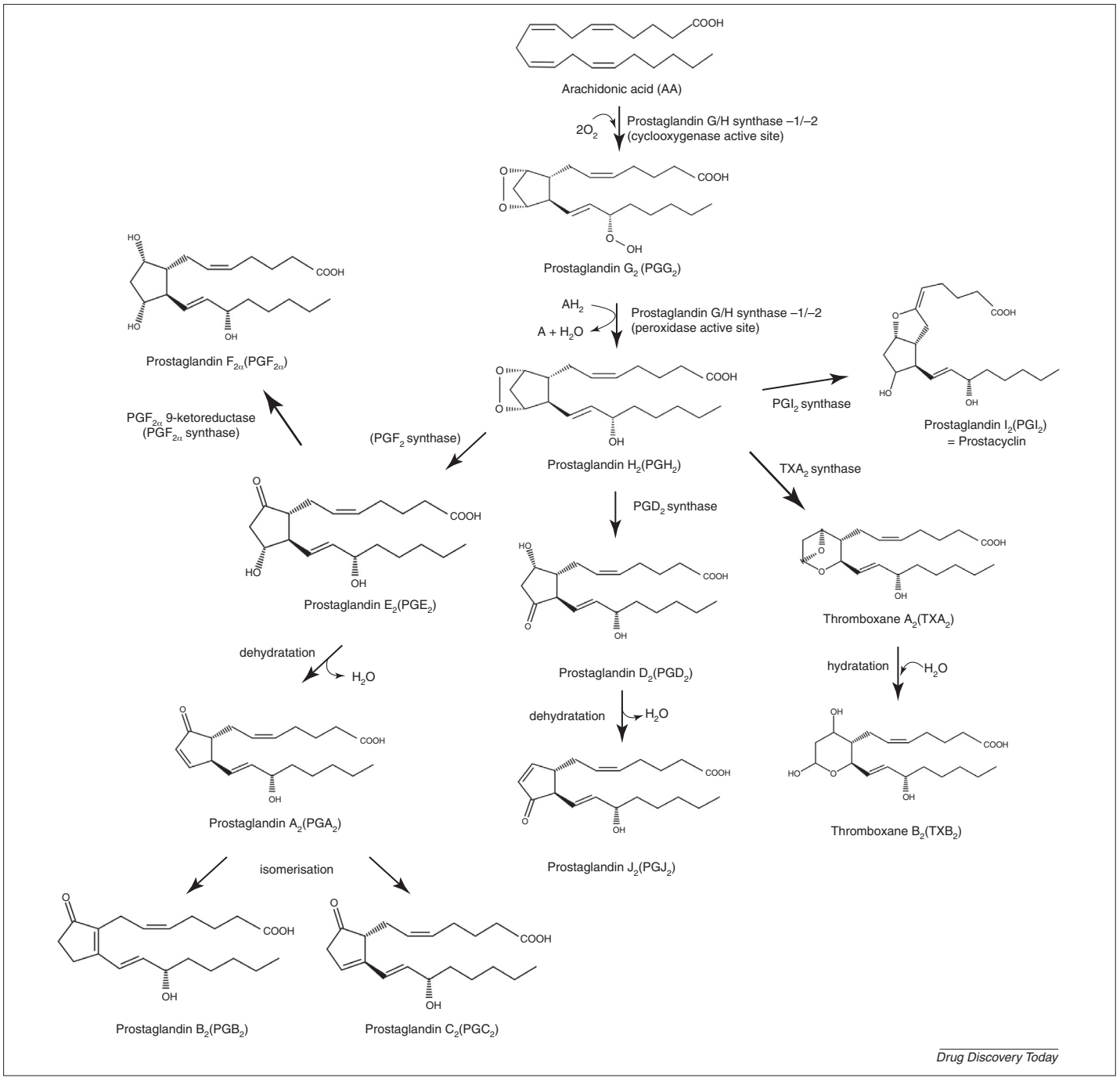


FIGURE 1

The biosynthesis of prostanoids by cyclooxygenase (COX) [10,11]. COX converts arachidonic acid (AA) into short-lived 15-hydroperoxide-PGG_2 and then reduces it to PGH_2. PGH_2 is subsequently spontaneously rearranged or enzymatically converted into the biologically active prostaglandins D, E, and F, prostacyclin (PGI_2), or thromboxane (TXA_2).

endoperoxide; (v) interaction of the radical localized at C_{15} with the second molecule of oxygen, facilitated by Ser530 and Val349; (vi) reabstraction of the proton from Tyr385 and formation of hydroperoxyl–PGG_2 with the concomitant regeneration of the tyrosyl radical; (vii) and conversion of PGG_2 into PGH_2 at the peroxidase active site [11,14].

There are three fundamental elements of the COX active site in COX-2 that distinguish it from COX-1: Val523, Arg513, and Val343. In COX-1, these are substituted by the long side-chain amino acids Ile523, His513, and Ile434. These subtle differences contribute to the 17% enlargement of the substrate-binding pocket in COX-2 and the increase in its hydrophilicity, enabling COX-2 to recognize bulkier substrates and increasing its substrate spectrum. This relation can also be used for the construction of selective COX-2 inhibitors with large hydrophilic functional groups, which fit easily into the wider catalytic site of COX-2.

Structural differences between COX-1 and COX-2 also determine their behavior in the presence of the irreversible inhibitor acetylsalicylic acid. COX-1 loses its capability for cyclooxygenation almost entirely when acetylated at Ser530, whereas COX-2

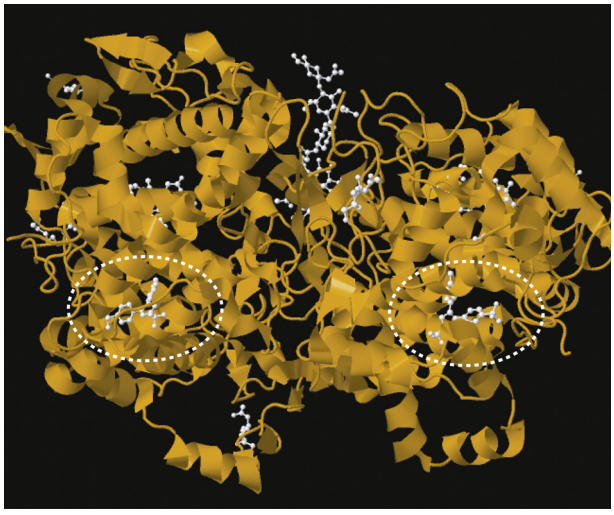


FIGURE 2

The secondary structure of the cyclooxygenase 2 (COX-2) homodimer with the inhibitor (celecoxib) bound to the active site (white-dashed oval). The COX active site is a narrow, hydrophobic channel restricted by the membrane domain with the outlet located close to the cell membrane. PDB ID: 3LN1, reproduced from [15].

preserves its catalytic function, although its product profile changes and, instead of PGG_2, 15-hydroxyeicosatetraenoic acid (HETE) is produced [11,14,16].

Prostaglandins and thromboxanes

PGs and thromboxanes (eicosanoids), the primary products of COX, constitute a large family of endogenous, regulatory agents derived from AA. Chemically, they are part of the prostanoid subclass of eicosanoids, and contain two side chains attached to the adjacent carbons of the cyclopentane ring (PGs) or the tetrahydropyran ring (thromboxanes). Their biosynthesis occurs in almost every human tissue, although each tissue can produce various eicosanoids depending on its physiological state. In general, prostaglandin D is synthesized primarily in mast cells and brain, prostaglandin F in uterus, prostaglandin I in endothelial cells, prostaglandin E in the whole body, and thromboxanes in thrombocytes and macrophages. Given that PGs and thromboxanes have a very short half-life, they act locally as autocrine or paracrine factors via the interaction with specific PG and thromboxane receptors [13,17–19]. Their main biological effects are listed in Table 1.

PGE_2 is the most physiologically abundant product of COX-2 because it exists at some level in nearly all cell types. Apart from its above-mentioned biological effects, such as induction of pain and inflammation, PGE_2 also participates in the mechanisms of cell proliferation, apoptosis, and metastasis, thereby contributing to the progression of several human cancers, including colon cancer, breast cancer, and lung cancer [18,20]. This hormone-like lipid compound acts on specific G-protein-coupled membrane receptors, termed EP_1, EP_2, EP_3, and EP_4 [21]. Each of these induces different signaling pathways. EP_1 is coupled with G_q and its second messengers are inositol trisphosphate (IP_3) and diacylglycerol (DAG), which are responsible for calcium mobilization. EP_2 and EP_4 are coupled with G_s, which stimulates adenyl cyclase to increase the production of cAMP [an activator of protein kinase A (PKA), phosphoinositide 3-kinase (PI3K) and glycogen synthase kinase 3 (GSK3)]. Importantly, GSK3 is implicated in cellular proliferation by promoting the phosphorylation of β-catenin and its subsequent degradation by the 26S proteasome. In addition to PGE_2-dependent activation, EP_4 is also associated with the induction of early growth response protein 1 (EGR-1). Finally,

TABLE 1

Main biological effects of PGs[a]

Location	Regulatory factor	Biological response
Female reproductive system	PGE_2, PGF_2	Uterine contraction; rupture of the follicle, ovulation; pregnancy accommodation
Male reproductive system	PGE_1, PGE_2, PGE_3, $PGF_{2\alpha}$	Stimulation of spermatozoa, ejaculation, stimulation of female reproductive tract after ejaculation
Cardiovascular system	TXA_2, PGI_2	Regulation of hemostasis
	TXA_2	Vascular permeability, thrombosis
	PGE_2, PGI_2	Vasodilation
	PGE_2	Angiogenesis
	TXA_2, $PGF_{2\alpha}$	Vasoconstriction of veins
	PGE_2, PGI_2	Fetal ductus arteriosus patency
Respiratory system	PGE_2, PGI_2	Bronchodilation
	$PGF_{2\alpha}$, TXA_2, PGD_2	Bronchospasm
	$PGF_{2\alpha}$, TXA_2	Constriction of pulmonary vessels
Excretory system	PGE_2, PGI_2,	Glomerular filtration rate and renal flow regulation, renin release
Gastrointestinal tract	PGE_2, PGI_2	Cytoprotection, reduction of gastric acid secretion, vasodilation in gastric mucosa, release of viscous mucus
Immune system	PGE_2, PGI_2, TXA_2, PGD_2	Inhibition of lymphocyte T and B proliferation, stimulation of immature thymocytes and B lymphocytes
Central nervous system	PGE_2	Pyrexia, wakefulness
	PGD_2	Sleep regulation, hypothermia
	PGE_2, PGI_2	Hyperalgesia

[a] Based on [9,17,25–27].

EP_3 is coupled with G_i and its interaction with PGE_2 causes down-regulation of adenyl cyclase and a decrease in cAMP [18]. Interestingly, the disturbed expression of EP receptors has been found in breast cancer cells, in the form of the upregulation of EP_1, EP_2 and EP_4, and the repression of EP_3 signaling. For this reason, the pharmacological modulation of EP activity by COX-2 inhibitors emerges as an attractive option in the management of patients with breast cancer [22–24].

COX-2 inhibitors

Inhibitors of COX, also termed nonsteroidal anti-inflammatory drugs (NSAIDs), exert anti-inflammatory, antipyretic and pain-relieving actions. They are the most frequently used group of pharmaceuticals, mainly in the treatment of degenerative joint disease, rheumatoid diseases, metabolic disorders, cardiovascular disturbances, infections as well as other diseases that are associated with pain and inflammation. First-generation NSAIDs were non-selective COX inhibitors, which demonstrate a similar affinity for both COX-1 and COX-2. The primary drug of this type, which has been in production since 1898, is aspirin (acetylsalicylic acid), which irreversibly blocks the target enzyme by its acetylation at Ser530 (COX-1 nomenclature). Other currently known nonselective NSAIDs act as competitive COX inhibitors that displace AA from the active site of the enzyme [11]. These include: diclofenac, indomethacin, ketoprofen, naproxen, ibuprofen, phenylbutazone, and meclofenamate. Within the group of nonselective COX inhibitors, there is also a subgroup of preferential COX-2 inhibitors that includes etodolac, meloxicam, nabumetone, and nimesulide. These compounds are more potent in inhibiting COX-2 than inhibiting COX-1. The pharmacological advantages of nonselective COX inhibitors are often counterbalanced by their relatively high toxicity, especially in the gastrointestinal tract and kidneys. In fact, their abolishment of COX-1 results in the decreased biosynthesis of homeostatic, cytoprotective, and hemostatic PGs, which further leads to gastric ulcers and bleeding. Furthermore, the inhibition of COX-1 in kidneys causes excessive water and sodium retention as well as reduced blood flow. These events could complicate the use, or increase the cost, of such inhibitors in the treatment of various diseases [10–13,16].

Coxibs

The detailed description of the pathophysiological nature of inflammation resulted in the development of second-generation NSAIDs that selectively (almost 50 times more) block the inducible form of COX, which is the form responsible for the biosynthesis of proinflammatory PGs. The key to their successful discovery was the identification of the structural differences between both COX isoforms; namely, the expanded hydrophobic substrate-binding channel and the presence of a hydrophilic side pocket in COX-2 [16]. Thus, the primary concept of drug design focused on the construction of molecules with steric hindrance that would prevent the inhibitor from forming unwanted interactions with COX-1. The group of compounds identified was diaryl derivatives with sulfonamide ($–SO_2NH_2$) or methylsulfonyl ($–SO_2CH_3$) functional groups located in a *para*-position in one of the pendant phenyl rings. Interestingly, the feature that determined their high selectivity was the oxidation state of sulfur, because the reduction of sulfonamide to sulfoxide or sulfide turned out to abolish or reverse

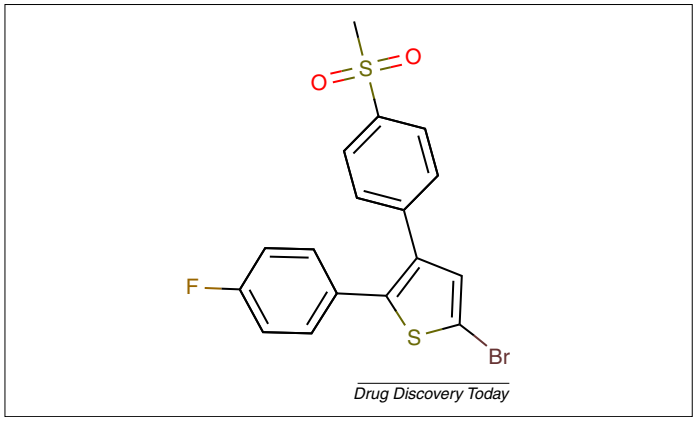


FIGURE 3

The chemical structure of 2,3-diaryl-thiophene with a *cis*-stilbene-like moiety (DuP-697), which is the prototype compound for cyclooxygenase inhibitors [25].

their selective inhibition of COX-2. The prototype compound for future COX-2 inhibitors was DuP-697 (Fig. 3), which is a 2,3-diaryl-thiophene with a *cis*-stilbene-like moiety. In structure–activity relationship (SAR) analyses, this group was found to have high COX-2 selectivity [25–27].

The evolution of the above structures led to the development of the first selective COX-2 inhibitors: celecoxib and rofecoxib. These were introduced into clinics in 1998 and 1999 (http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20998.cfm; http://www.accessdata.fda.gov/drugsatfda_docs/nda/99/021042_52_Vioxx.cfm). Currently, the family of selective inhibitors of COX (Coxibs) includes four more members: valdecoxib, parecoxib, lumiracoxib, and etoricoxib [28]. Structurally, they can be divided into two subcategories: (i) heterocyclic diaryl derivatives (celecoxib, rofecoxib, valdecoxib, parecoxib, and etoricoxib); and (ii) phenylacetic acid derivatives (lumiracoxib; chemically resembling diclofenac) [28].

These drugs, being very weak COX-1 inhibitors and very strong inhibitors of COX-2, do not influence the metabolism of cytoprotective PGs and, hence, exhibit only a limited toxicity profile, especially with respect to the gastrointestinal tract and kidneys. However, their use is associated with an increased risk of cardiovascular events, including heart attack or stroke, which are thought to result from disturbed hemostatic homeostasis. In fact, COX-2 inhibitors are theoretically responsible for the decreased biosynthesis of antithrombotic PGI_2 in blood vessels, which is not compensated for by the concomitant reduction in the prothrombotic thromboxanes produced by COX-1 [13,28]. Therefore, increased cardiotoxicity was the direct reason for the withdrawal of rofecoxib in 2004 and valdecoxib in 2005. Lumiracoxib, in turn, was withdrawn in 2007 because of its excessive hepatotoxicity (http://www.pbm.va.gov/vacenterformedicationsafety/vioxx/DearHealthcareProfessional.pdf) [29,30].

The detailed X-ray crystallographic description of the COX-2–celecoxib crystal structure confirmed the validity of this adopted concept of drug design. In its bound form, celecoxib adopts a *cis*-stilbene-like conformation that enables it to interact with three fundamental regions of COX-2 active site in the following manner (Fig. 4): (i) the oxygens of the sulfonamide moiety are

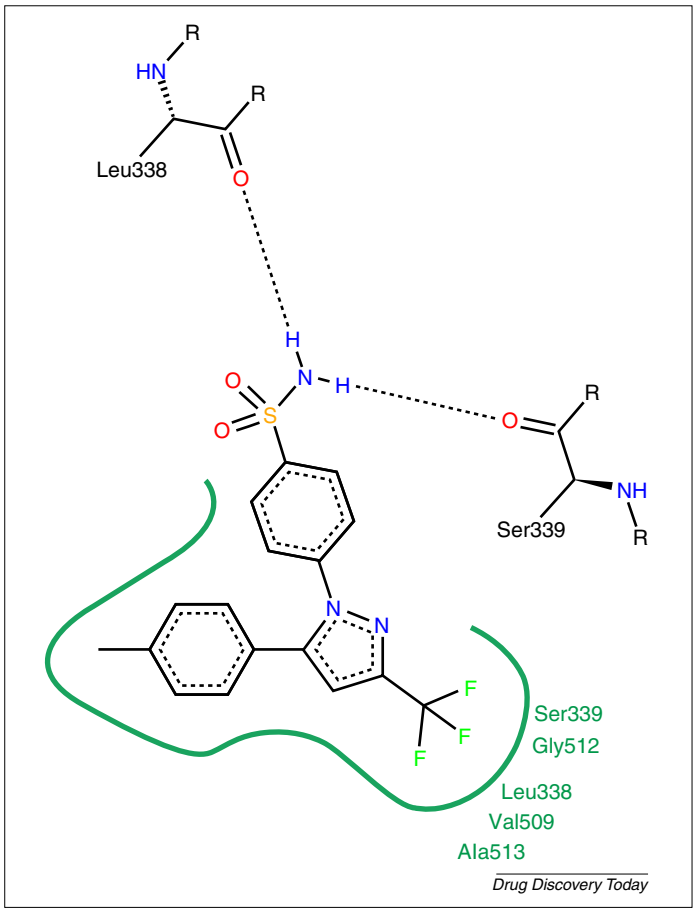


FIGURE 4

The selective cyclooxygenase 2 (COX-2) inhibitor celecoxib bound to the active site of murine COX-2 (PDB ID: 3LN1). The hydrogens of its sulfonamide moiety are H-bound with Leu338, and Ser339. The pyrazole ring and the trifluoromethyl functional group interact through Van der Waals bonds with Ser339, Gly512, Leu338, Val509, and Ala513. Black-dashed lines show hydrogen bonds, whereas green lines, show hydrophobic interactions. Image generated using the PoseView software.

H-bound with the amino acids located in the hydrophilic side pocket (His75, Arg499 and Gln178); the pyrazole ring and the trifluoromethyl functional group interact through Van der Waals bonds with Ser339, Gly512, Leu338, Val509, Tyr341, and Arg106, which line the side hydrophobic channel of the enzyme; and (iii) the aryl ring substituted with the methyl functional group in a *para* position forms Van der Waals interactions with the amino acids from the main hydrophobic channel (Tyr371 and Ser516) [15,31–33].

trans-Stilbene derivatives

Although coxibs are the only clinically available selective COX-2 inhibitors, there are also other agents that display a promising COX-2 selectivity confirmed in *in vitro* and *ex vivo* tests. In fact, numerous compounds without *cis*-stilbene moiety have been discussed in the literature and one such group deserving special attention are the *trans*-stilbene derivatives. The main stimulus for their research was the discovery of the biological properties of resveratrol. This natural compound shows nonselective affinity to COX-1 ($IC_{50} \sim 0.5$ μmol/l) and COX-2 ($IC_{50} \sim 1$ μmol/l) *in vitro*

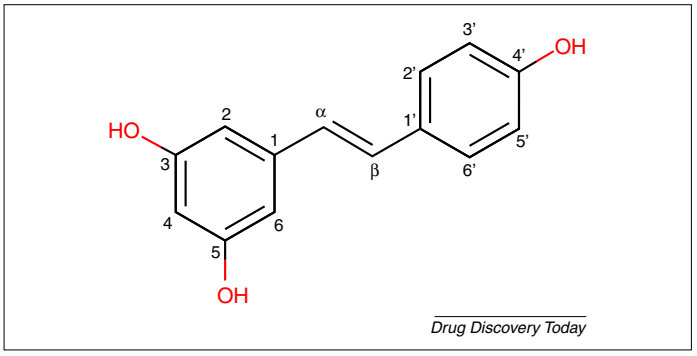


FIGURE 5

The structure of *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), which is a naturally occurring nonselective cyclooxygenase inhibitor that exhibits anti-inflammatory, fungicidal, cytotoxic, chemopreventive, and cardioprotective actions [34].

[34,35] and also demonstrates anti-inflammatory, fungicidal, cytotoxic, chemopreventive, and cardioprotective actions [34]. Importantly, resveratrol is a stilbene analog that occurs as a *cis* or *trans* isomer, yet only *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is biologically active (Fig. 5) [34,35].

Based on these findings, various *trans*-resveratrol and *trans*-stilbene derivatives have become appealing targets for drug design as potential selective COX-2 inhibitors. The most interesting structures are presented in Table 2.

One of the most promising groups of selective COX-2 blockers are hydroxy *trans*-resveratrol analogs, with 3,3′,4,4′,5,5′-hexahydroxy-*trans*-stilbene (3,3′,4,4′,5,5′-HHS) and piceatannol (3,3′,4′,5-tetrahydroxy-*trans*-stilbene) being the most potent members. In an immunoenzymatic *in vitro* assay, the former compound had 700 times higher affinity to COX-2 than to COX-1, whereas the latter had almost 400 times higher affinity. Their significant increase in COX-2 selectivity relative to *trans*-resveratrol was attributed to the presence of numerous hydroxyl groups (–OH) in the aromatic rings, especially in the 3′ position. However, appropriate docking experiments showed that both compounds, unlike coxibs, do not accommodate the hydrophilic side pocket of COX-2, indicating that their hydroxylation pattern enables them to create additional hydrogen bonds with the enzyme [35].

Further investigations with *trans*-stilbenes provided evidence that the substitution of hydroxyls in resveratrol with methoxy functionals substantially potentiated their cytotoxic activity, confirmed in SAR studies. However, *in vitro* assays revealed only low selectivity and weak affinity to COX-2 in this group, with the exception of 3,3′,4,4′,5,5′-hexamethoxy-*trans*-stilbene, which exhibited moderate potency and selectivity (approximately 45 times higher than to COX-1). These results clearly suggest that the cytotoxic effects of the studied compounds cannot be attributed to their COX-2-related actions (Table 2) [35].

Another interesting population of *trans*-stilbene derivatives comprises the analogs substituted with the following groups in *meta* or *para* positions: –OH, $–OCH_3$, $–N(CH_3)_2$, –F, $–CF_3$, and $–NO_2$, with one phenyl group substituted with naphthyl, or with an ethylene bridge modified with $–CH_3$ or $–C_6H_5$ (Table 2). Interestingly, in *in vitro* assays, the compounds with –OH and $–CF_3$ at position 4 displayed a high affinity to COX-2 ($IC_{50} < 2$ μmol/l). Of these, almost all were substituted with –OH or $–OCH_3$ at position 3

TABLE 2

***trans*-Stilbene derivatives that selectively (>50×) and/or potently (IC_{50} < 2 μmol/l) inhibit COX-2**

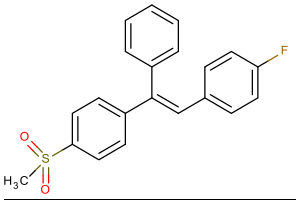
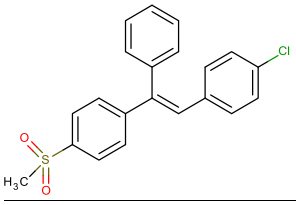
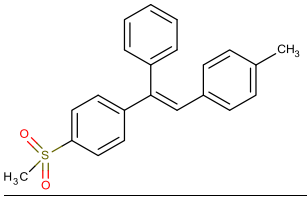
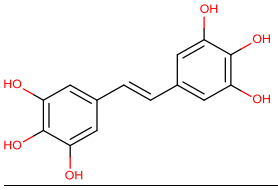
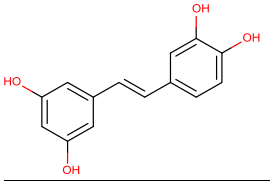
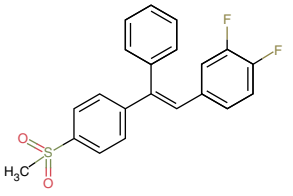
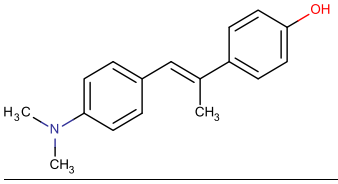
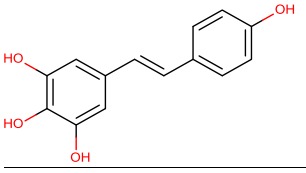
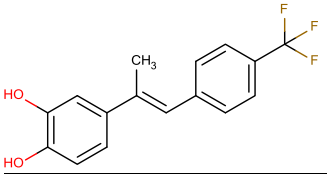
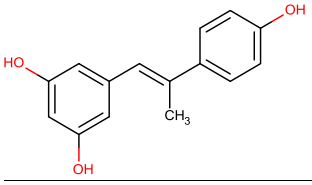
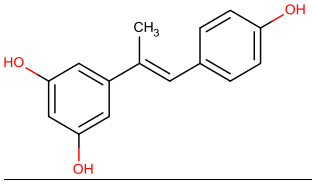
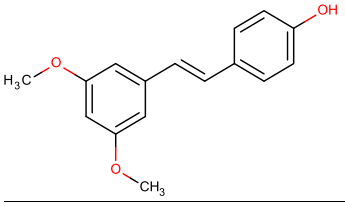
Chemical formula and name of *trans*-stilbene derivative (name of compound in the reference)		COX-1 IC_{50} (μmol/l)	COX-2 IC_{50} (μmol/l)	Selectivity index (COX-1 IC_{50}/COX-2 IC_{50})	Refs
	4-methylsulfonyl-4′-fluor-α-phenyl-*trans*-stilbene (10c)	>100	0.0316	>3164	[37]
	4-methylsulfonyl-4′-chlor-α-phenyl-*trans*-stilbene (10e)	>100	0.1138	>878	[37]
	4-methylsulfonyl-4′-methyl-α-phenyl-*trans*-stilbene (10b)	>100	0.12	>833	[37]
	3,3′,4,4′,5,5′-heksahydroxy-*trans*-stilbene (3,3′,4,4′,5,5′-HHS) (12)	0.748	0.00104	719.23	[35]
	3,3′,4′,5-tetrahydroxy-*trans*-stilbene (3′-hydroxy-*trans*-resveratrol, piceatannol) (10)	4.713	0.0113	417.08	[35]
	4-methylsulfonyl-3′,4′-difluor-α-phenyl-*trans*-stilbene (10d)	>100	0.97	>103	[37]
	4-(*N*,*N*-dimethyl)amine-4′-hydroxy-β-methyl-*trans*-stilbene (1M13)	36.3	0.47	77.23	[36]
	4-hydroxy-*trans*-resveratrol (8)	2.072	0.04537	45.67	[35]

TABLE 2 (*Continued*)

Chemical formula and name of *trans*-stilbene derivative (name of compound in the reference)	COX-1 IC_{50} (μmol/l)	COX-2 IC_{50} (μmol/l)	Selectivity index (COX-1 IC_{50}/COX-2 IC_{50})	Refs
 3,4-dihydroxy-4′-trifluormethyl-α-methyl-*trans*-stilbene (2M16)	10.7	1.74	6.15	[36]
 β-methyl-*trans*-resveratrol (1M2)	1.9	1.57	1.21	[36]
 α-methyl-*trans*-resveratrol (2M1)	1.9	1.78	1.07	[36]
 4′-hydroxy-3,5-dimethoxy-*trans*-stilbene = pterostilbene (DL2; 1H6)	4.84 [35]; 0.70 [36]	1.19 [35]; 0.82 [36]	4.07 [35]; 0.85 [36]	[35,36]

and 5 as well as by $–CH_3$ or $–C_2H_5$ in the ethylene bridge. Furthermore, the compound 1M13 (Table 2) with no substituents at positions 3 and 5, and $–N(CH_3)_2$ at position 4 also demonstrated strong inhibitory activity and moderate selectivity to COX-2 (almost 80 times higher than to COX-1). These findings, in turn, provided several structural clues determining the selectivity and potency of *trans*-stilbene derivatives: (i) the presence of functionals with high electron density at positions 3, 4, and 5 in the aromatic ring; and (ii) the presence of alkyl functionals in the ethylene bridge to provide steric hindrance for the flat conformation of *trans*-stilbene and to change the special orientation of the rings relative to the flat structure of the bridge, thereby conferring a better docking opportunity in the COX-2 active site [36].

A final group of *trans*-stilbenes includes the derivatives of *trans*-1,1,2-triarylethene, four of which exhibit high potency ($IC_{50} < 1$ μmol/l) and high selectivity (>100 times) to COX-2. Their representative [4-methylsulfonyl-4′-fluor-α-phenyl-*trans*-stilbene (10c)] is over 3000 times more selective to COX-2 and over six times more potent than celecoxib. Interestingly, the general structure of these compounds resembles coxibs, with three aromatic rings and the methyl sulfonyl functional group in a *para* position. The discussed agents also interact similarly to coxibs with the COX-2 active site: (i) the ring substituted with $–SO_2CH_3$ accommodates the hydrophilic side pocket (His90, Arg513, and Gln192); (ii) the benzene ring binds with the side hydrophobic channel (Arg120, Leu359, and Tyr 355); and (iii) the benzene ring substituted with fluorine, chlorine, or methyl in the *para* position enters the main hydrophobic channel (Ser530, Ala527, and Val349). Importantly, the lack of the above substituents completely abolishes the selectivity to COX-2. This interaction pattern also indicates that the derivatives of *trans*-1,1,2-triarylethene preferably adopt a *cis* conformation when bound to COX-2 [37].

Unfortunately, the published data on *trans*-stilbene derivatives and their ability to inhibit COX-2 are scarce and, hence, more studies are required (only 100 compounds have been analyzed to date). The need to extend the scope of this research is primarily substantiated by the fact that several analogs exhibit pronounced anticancer activity that could be both COX-2 dependent and independent. Therefore, we report here some *in silico* analyses to support the process of *trans*-stilbene development.

QSAR 2D

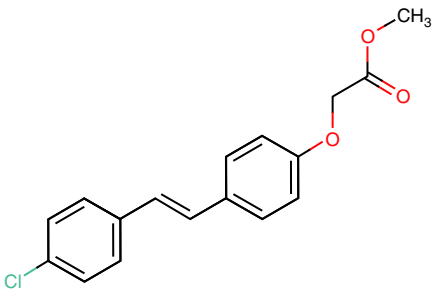
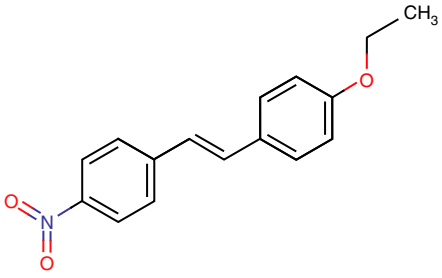
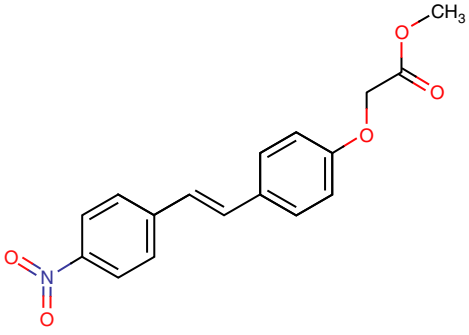
A crucial technique in modern drug discovery is computer-aided drug design (CADD), which involves the optimization of a drug candidate structure using computer analysis before *in vitro* and *in vivo* experiments. This scientific approach can result in a decrease in the time and costs necessary for drug elaboration [38,39]. A common method of CADD is QSAR, which determines the relation between biological activity and physiochemical properties of a compound. Such analyses provide essential data on which features of a compound are most responsible for its biological actions and then depict these relationships as a mathematical model in the form of an equation with one, two or more variables [40].

Experimental analysis

We qualified 40 *trans*-stilbene derivatives in our study, 37 of which were obtained from the literature [35–37]. The remaining three new compounds (Table 3) were synthesized and identified according to [41]. Out of this group, a training set of compounds was selected to determine the best 2D QSAR equations. The remaining

TABLE 3

New *trans*-stilbene derivatives as potential COX-1 and COX-2 inhibitors[a]

The chemical formula and name of *trans*-stilbene derivative		COX-1 IC_{50} (μmol/l)[b]	COX-2 IC_{50} (μmol/l)[b]	Selectivity index (COX-1 IC_{50}/COX-2 IC_{50})
	4′-(2-methoxy-2-oxo)-ethoxy-4-chlor-*trans*-stilbene (Z6)	40.980	7.522	5.448
	4′-ethoxy-4-nitro-*trans*-stilbene (Z9)	84.980	4.017	21.155
	4′-(2-methoxy-2-oxo)-ethoxy-4-nitro-*trans*-stilbene (Z10)	30.830	7.461	4.132

[a] Based on [41].

[b] The biological activity (IC_{50}) was determined using a commercial ELISA test: COX (ovine/human) Inhibitor Screening Assay Kit – item no. 560131 (Cayman Chemical, USA).

compounds were used as a test set for the verification of the parameters of the training set.

For calculation purposes, the biological activity of the compounds (IC_{50}) was expressed as COX-1 pIC_{50} (= −log COX-1 IC_{50}) and COX-2 pIC_{50} (= −log COX-2 IC_{50}). The appropriate equations were then established basing on three different mathematical approaches: single linear regression (SLR); multiple linear regression (MLR); and principal component analysis (PCA). SLR and MLR were performed in Microsoft Excel 2007, whereas PCA was calculated using PQStat Software v. 1.6.0 (http://pqstat.pl/pl).

Validation of each training set was performed by using Eqns 1 and 2 [42]:

$$R^2_{training} = \frac{SSE}{SST} = 1 - \frac{SSR}{SST} = 1 - \frac{\sum_{i=1}^{n}\left(y^{\exp}_{i,training} - y^{pred}_{i,training}\right)^2}{\sum_{i=1}^{n}\left(y^{\exp}_{i,training} - \gamma^{\exp}_{training}\right)^2} \quad (1)$$

$$Q^2_{training} = 1 - \frac{\sum_{i=1}^{n}\left(y^{\exp}_{i,training} - y^{pred,LOO\,cv}_{i,training}\right)^2}{\sum_{i=1}^{n}\left(y^{\exp}_{i,training} - \gamma^{\exp}_{training}\right)^2} \quad (2)$$

$Q^2_{training} = R^2$ obtained from leave-one-out cross-validation (LOO cv), where *SSE* was the sum of squared errors of prediction; *SSR* was the sum of squared residuals; *SST* was the total sum of squares (SSE + SSR); $y^{\exp}_{i,training}$ was the experimental value of the biological activity of inhibitor *i* in the training set; $\gamma^{\exp}_{training}$ was the mean experimental value of the biological activity of the training set; $y^{pred}_{i,training}$ was the predicted value of the biological activity of inhibitor *i* in the training set; and $y^{pred,LOO\,cv}_{i,training}$ was the predicted value of the biological activity of inhibitor *i* left out from the training set.

Each test set was verified with by the calculation of Eqns 3 and 4 [42]:

$$R^2_{test} = \frac{SSE}{SST} = 1 - \frac{SSR}{SST} = 1 - \frac{\sum_{i=1}^{n}\left(y^{\exp}_{i,test} - y^{pred}_{i,test}\right)^2}{\sum_{i=1}^{n}\left(y^{\exp}_{i,test} - \gamma^{\exp}_{test}\right)^2} \quad (3)$$

$$Q^2_{test} = 1 - \frac{\sum_{i=1}^{n}\left(y^{\exp}_{i,test} - y^{pred}_{i,test}\right)^2}{\sum_{i=1}^{n}\left(y^{\exp}_{i,test} - \gamma^{\exp}_{training}\right)^2}, \quad (4)$$

where $y^{\exp}_{i,test}$ was the experimental value of the biological activity of inhibitor *i* in the test set; $y^{pred}_{i,test}$ was the predicted value of the biological activity of inhibitor *i* in the test set; $\gamma^{\exp}_{test}$ was the mean experimental value of the biological activity of the test set; and

$\gamma^{exp}_{training}$ was the mean experimental value of the biological activity of the training set.

A model was qualified as good and acceptable only if all the calculated parameters were ≥0.5 and the differences between corresponding R^2 or Q^2 were ≤0.3 [42]. The results of the best 2D QSAR models are presented in Tables S1 and S2 in the supplementary material online [43].

Our observations led to the following conclusions: (i) the molecular weight, number of H-bond acceptors, dipole moment, number of rotatable bonds, and sum of atomic polarizabilities are weak 2D descriptors because of their lack of correlation with the biological activity of the tested COX-1/-2 inhibitors; (ii) pIC_{50} against COX-1 correlated with the greater number of descriptors compared with the pIC_{50} obtained for COX-2 (e.g., in the PCA, the prediction of COX-1 affinity was based on six descriptors, whereas the prediction of COX-2 affinity was based on only 4); (iii) more H-bond donors (e.g., –OH, –SH, and $–NH_2$ moieties) and a greater polar surface (TPSA) in the molecule increased the potency of inhibitors against both COX isoforms in the same manner (similar coefficients in the corresponding equations), but only the TPSA model for COX-2 inhibitors had good parameters; (iv) lipophilic compounds (higher ClogP values) with high mass, surface and volume (high MR and VdWSA) were weaker COX-1 inhibitors compared with small hydrophilic molecules with polar groups and a low-branching or linear structure; (v) hydrophobic compounds with a linear structure and smaller polar surface (i.e., a reduced number of polar atoms, such as O, S, N, Cl, and F) are weaker COX-2 inhibitors compared with hydrophilic, branched compounds that are rich in polar moieties (i.e., lower ClogP, higher TPSA, and/or VdWSA), such as *trans*-stilbenes with sulfonamide, nitro, trifluoromethyl, hydroxyl, or hydroxyl-alkoxy groups in one or both aromatic rings.

Similar relations have been suggested by Kang *et al.* [36], who observed a higher inhibitory activity against COX-2 for compounds with one aromatic ring substituted with the resveratrol-like, resorcinol group (H-bond donor and lower ClogP), and with small hydrophilic or polar moieties (e.g., –OH or $–CF_3$) in the second ring. However, in terms of COX-1 inhibitors, and contrary to our findings, the potency was suggested to increase for compounds with the resorcinol group in one ring (H-bond donor and lower ClogP) and hydrophobic substituents (higher ClogP and VdWSA, but lower TPSA), such as the naphthalene group, or electron-rich hydrophobic moieties (lower TPSA and higher MR), such as $–N(Me)_2$ or –OMe in the other ring.

Our results are consistent with the observations of Murias *et al.* [35], who tested 12 resveratrol analogs (methoxy- and hydroxyl-derivatives) and determined the following relations: (i) a moderately negative correlation between COX-1 pIC_{50} and MR; and (ii) strongly positive dependence between COX-2 pIC_{50} and TPSA (more –OH than –OMe substituents favor COX-2 selectivity). Interestingly, no correlation was estimated for logP, suggesting that lipophilicity makes no significant contribution to the biological activity of the tested molecules. However, unlike this observation, we found a moderately negative relation between ClogP and pIC_{50} values for COX-1/-2.

Finally, the study performed by Uddin *et al.* [37] within the group of 1,1,2-triarylethenes leads to some ambiguous conclusions on the relation between their biological activity (inhibitory potency and selectivity against COX) and physicochemical properties. In fact, the researchers have been unable to establish any clear correlation between pIC_{50} for COX-1 or COX-2 and 2D descriptors (mainly ClogP, MR, and TPSA). However, 1,1,2-triarylethenes have an additional third aromatic ring at the ethylene bridge, which changes their geometric structure, unlike classic *trans*-stilbenes, which comprise only two aromatic rings. This feature might influence their activity inconsistently with the classic *trans*-stilbenes. Therefore, the discussed compounds should not be used for *in silico* analysis (e.g., QSAR 3D) with *trans*-stilbenes because they might cause difficulty in obtaining a reliable model for the prediction of activity against COX.

Based on the above-discussed experiments, the clear differentiation of the descriptors responsible only for COX-1 or COX-2 selectivity is difficult. Nevertheless, we can hypothesize that small, but more branched and more hydrophilic compounds (richer in polar groups at both aromatic rings) might be stronger COX-2 than COX-1 inhibitors. To confirm this, further QSAR studies, such as 3D analysis, are required. Such data would facilitate the development of selective *trans*-stilbenes that might serve as drug candidates for use in preclinical and clinical trials.

Inflammation and breast cancer

Inflammation is a complex biochemical and immune process that occurs in vascular tissue in response to various harmful stimuli, including pathogens, aberrant cells, or irritants. Physiologically, its main aim is to stimulate host defense mechanisms against infections or induce tissue repair after damage. One of the primary mediators of inflammation is PGs biosynthesized by COX-2. They rapidly accumulate at the damaged site and induce a vascular response that is responsible for the initiation and maintenance of the inflammatory state [44]. PGs also participate in the mechanism of chronic inflammation, which, based on available epidemiologic studies, has been associated with the development of more than 15% of all human malignancies, including [45–47]: *Helicobacter pylori* infection and gastric cancer; chronic viral hepatitis and liver cancer; Human papilloma virus (HPV) infection and cervical carcinoma; parasitic infections, such as *Schistosoma haematobium*, and bladder cancer; chronic obstructive pulmonary disease associated with smoking and lung cancer; chronic inflammation of the intestines (e.g., ulcerative colitis and Crohn's disease) and gastrointestinal cancer; and ductal carcinoma *in situ* (a premalignant form of breast cancer), characterized by high levels of COX-2, and malignant breast cancer [48].

How does inflammation contribute to cancer? The current belief is that local inflammatory foci, which undergo a gradual generalization, are already formed at the stage of carcinogenesis initiation. They are thought to produce appropriate conditions for various genetic changes. For instance, one inflammatory mechanism that facilitates tumor promotion and progression is the local excessive generation of reactive oxygen species and reactive nitrogen intermediates. These factors cause oxidative damage and nitration of DNA leading to the accumulation of genetic mutations and genome destabilization. As a consequence, tumor cells can acquire two fundamental features of immortality: uncontrolled proliferation and resistance to apoptosis. Apart from DNA damage, inflammation also facilitates the release of growth factors, such as EGF and fibroblast growth factor (FGF),

into the tumor microenvironment. This additionally accelerates neoplastic proliferation and, hence, has a crucial role in cancer promotion. Furthermore, there are many hypoxic and acidic areas in tumor tissue in which massive cell death occurs, similarly to that in the areas of tissue injury or infection. This phenomenon constitutes a misleading signal to the host immune system which immediately activates signaling pathways responsible for tissue repair and regeneration, thereby enabling the tumor cells to survive. Immune cells that exist within the tumor microenvironment, including dendritic cells, lymphocytes, and macrophages, release various proinflammatory cytokines, such as TNFα, IL1β, IL6, and IL8, inducing secondary inflammation that further facilitates cancer development [10,18,49].

Breast cancer: epidemiology, etiology, and treatment

Breast cancer is the most common invasive cancer in women worldwide, with an incidence equaling 22.9% of all invasive cancers in the population as a whole (second place) and 16% of all female cancers (first place). Furthermore, current prognosis suggests that the worldwide incidence of breast cancer and related mortality are both on the rise. According to the WHO, in 2008, there were over 1,380,000 new cases diagnosed and over 458,000 deaths. However, in 2012, almost 1,700,000 women (25% of all female cases and 12% of the total) were diagnosed with breast cancer and 522,000 died, indicating an 18% increase on the 2008 figures. It has been also predicted that the worldwide incidence of female breast cancer will reach approximately 3.2 million new cases per year by 2050 [50,51].

The current international incidence of breast cancer varies significantly, being the lowest in less-developed regions, such as Eastern Africa and South Asia, and the highest in Northern Europe, Australia, and the USA (the American Cancer Society estimates that one in eight women in the USA will develop breast cancer in her lifetime). Furthermore, female breast cancer is strongly related to age, with almost 80% of cases diagnosed in the over 50s (Fig. 6) [50–52].

In most cases, the etiology of breast cancer remains unknown. However, there are several risk factors predisposing to the development of this disease, including: personal or family history of breast cancer, genetic predisposition (BRCA1 and BRCA2 mutation), female gender, advanced age, hormonal activity exceeding 30 years (early first menstruation and late menopause), long-term use of hormone replacement therapy, late first delivery or nulliparity, exposure to ionizing radiation therapy, alcohol consumption, and some noncancerous breast diseases [50].

As in other cancers, the primary determinant of successful therapy is early diagnosis. However, treatment is a multistep process involving surgery, radiotherapy, and systemic therapy (i.e., hormone therapy, chemotherapy, and targeted treatments). The drugs of choice include: antiestrogens (tamoxifen, idoxifene, toremifene, and droloxifene); aromatase inhibitors (letrozole, anastrozole, and exemestane); gonadotropin-releasing hormone agonists (goserelin and buserelin); progestogens (megestrol acetate and medroxyprogesterone acetate); pyrimidine analogs (5-fluorouracyl and capecitabine); folic acid analogs (methotrexate); alkylating antineoplastic agents (cyclophosphamide); topoisomerase inhibitors (anthracyclines); microtubule-affecting drugs (taxanes, such as paclitaxel and docetaxel); monoclonal antibodies (trastuzumab and bevacizumab); and small-molecule tyrosine kinase inhibitors (lapatinib) (http://www.cancer.gov/types/breast/hp/breast-treatment-pdq).

The selection of appropriate chemotherapy regimen for treatment of patients with breast cancer is determined by the individual phenotype of neoplastic cells and their corresponding receptor status, described by the level of expression of estrogen (ER), progesterone (P4), and HER2 receptors. In fact, the increased response ratio to hormone therapy is associated with the increased expression of ER and P4. By contrast, Her2-positive breast cancers (20–30% of all breast cancers) are more susceptible to molecular treatment with monoclonal antibodies and small-molecule kinase inhibitors (http://www.cancer.gov/types/breast/hp/breast-treatment-pdq) [53].

Expression of COX-2 in breast cancer

The increased expression of PGE_2 and TXA_2 in neoplastic cells of breast cancer, especially in patients with metastatic disease, has been known since the 1980s, while long-term treatment with NSAIDs was noted to correlate with a lower risk of breast cancer development [54–56]. Further analysis of *COX2* gene expression confirmed these observations, showing an elevated concentration of *COX2* mRNA in breast cancer as well as in other human solid cancers, including colorectal, lung, gastric, bladder, pancreas, endometrial, and prostate cancer [3]. Furthermore, it has been shown that, although COX-2 is not physiologically present in breast epithelium, *COX2* expression is increased in 63–85% of cases of premalignant-stage breast cancers, such as ductal carcinoma *in situ*. Similarly, in approximately 40% of human breast malignancies (although this varies from 5% to 100% depending on the approved research protocol), the concentration of COX-2 is increased [4]. For example, in one immunohistochemical study of 1576 invasive breast carcinomas, there was moderate to strong *COX2* expression in 37% of the samples analyzed [57]. Other researchers found the increased *COX2* expression in all 13 tumor samples analyzed using RT-PCR [58]. Finally, the overexpression of *COX2* was detected only in two out of 44 tumor samples in a study using Western blots [59]. Moreover, the excessive expression and activity of *COX2* has been confirmed in experimental rats with carcinogen-induced breast cancers [using dimethylbenz(a)anthracene, *N*-nitrosylmethyl urea, or 2-amino-1-methyl-6-phenylimidazol(4,5-b)pyridine], as well as in transgenic mice overexpressing *HER2* 53,60,61]. In addition, the most common malignant breast cancer cell lines (ER+ MCF-7 and ER− MDA-MB-231) have been shown to have fluctuating levels (from very low to high) of *COX2* mRNA and COX-2 protein, which influenced their epithelial–mesenchymal transition and invasiveness [62–66].

The overexpression of *COX2* occurs predominantly in HER2-positive breast cancers as well as in cells with Ras mutation. This effect probably stems from the activation of the HER2-coupled Raf/Mek/ERK pathway, Janus kinase (JNK), and p38 kinases, which stimulate COX-2 *transcription* [5,53]. The increased activity of COX-2, in turn, affects the signaling pathways that involve proproliferatory PGs and, hence, the upregulation of this enzyme correlates with worse patient prognosis and a more aggressive profile of the tumor. In summary, the features of tumors that overexpress COX-2 are: negative ER but positive HER2 status, increased microvessel density, and a tendency to form distant metastases [6,67].

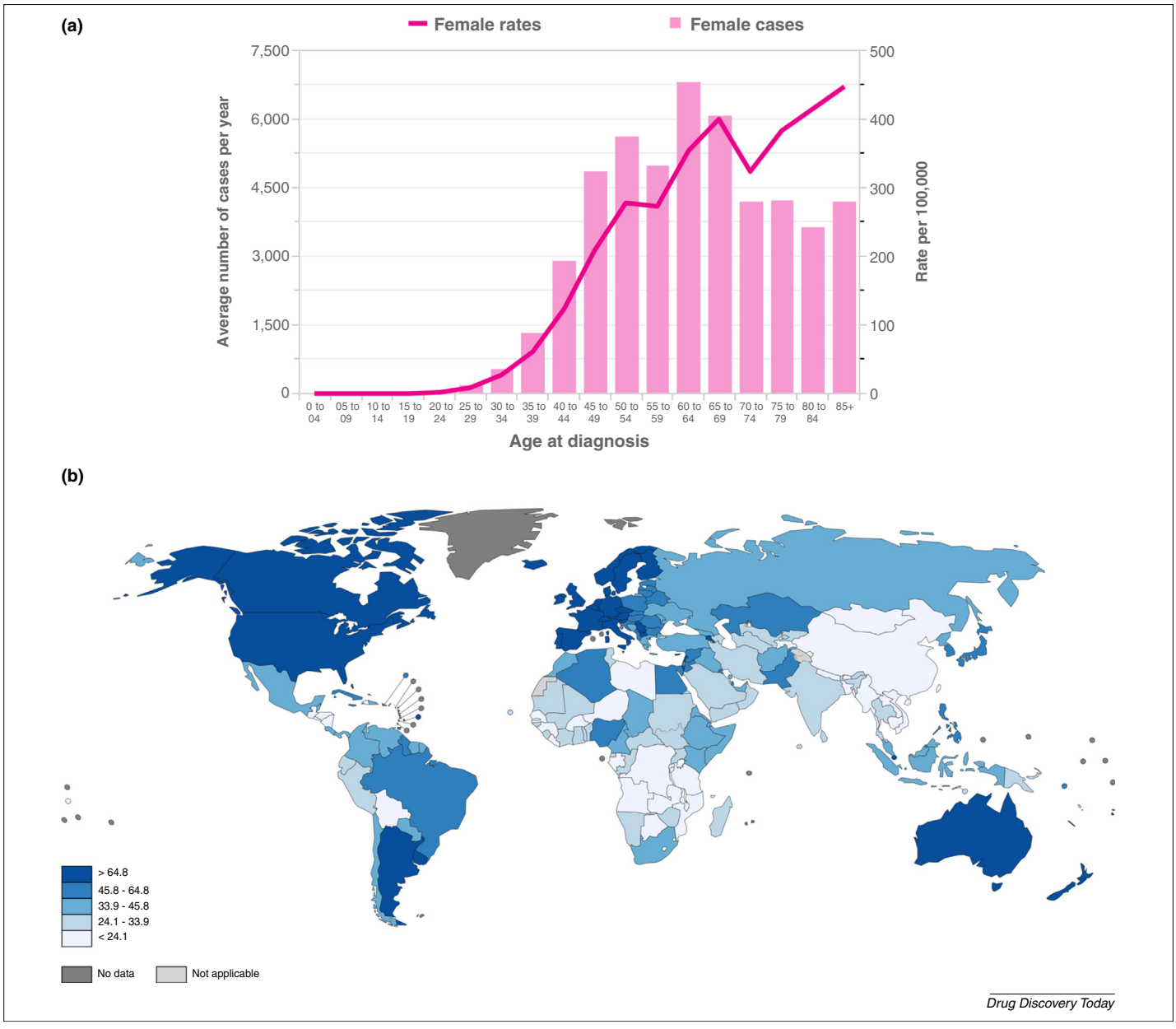


FIGURE 6

Incidence of breast cancer. **(a)** Breast cancer prevalence in the UK showing the average number of new cases per year (pink bars) and age-specific incidence rates (pink line) (Data from http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer). Female breast cancer is strongly related to age with most cases diagnosed in the over 50s. **(b)** Worldwide incidence of breast cancer showing the age-standardized rate. Breast cancer is less frequent in less-developed regions, such as Eastern Africa and South Asia, whereas the highest incidence occurs in Northern Europe, Australia, and the USA [52].

The association between COX-2 and breast cancer

Numerous *in vivo* experiments involving various mouse models of breast cancer clearly indicate that the inhibition of COX-2 restrains the development of breast tumors. For example, nonselective inhibitors, such as indomethacin, flurbiprofen, and aspirin, have been found to reduce the frequency of carcinogen-induced tumors [68–72]. In addition, preferable or selective COX-2 inhibitors, such as celecoxib and nimesulide, delayed the onset of tumorigenesis and decreased the disease incidence. Importantly, celecoxib was more effective than ibuprofen [60,73]. Furthermore, available genetic studies show that the suppressed expression of *COX2* in transgenic *HER2/neu* mice decreased the number of neoplastic lesions in breast when compared with wild-type animals [53]. Finally, the overexpression of *COX2* was sufficient for the initiation of carcinogenesis in *HER2/neu* mice after multiple gestations, which confirms that COX-2 has a crucial role in breast cancer development [74].

Further research on animal models proved that COX-2 influences both cancer initiation and/or growth and vascularization. Indeed, a decreased density of microvessels was observed in animals with the induced downregulation of *COX2*, which also correlated with the decreased expression of genes responsible for angiogenesis, including *VEGF*, Angiopoietin 1 and 2 (*Ang1* and *Ang2*), Fetal liver kinase 1 (*Flk1*), and Fms-related tyrosine

kinase 1 (*Flt1*). Interestingly, the excessive production of COX-2 in breast cancer, accompanied by the increased biosynthesis of PGE_2, also impacts the formation of bone metastases, an effect that is associated with the induction of IL-8, IL-11, RANK ligand (RANKL), and osteoclasts [3,7].

On the molecular level, several mechanisms of COX-2-dependent neoplastic initiation and progression in breast cancer have been identified. These include antiapoptotic and proangiogenic activity, induction of matrix metalloproteinases (MMPs), increased synthesis of antiadhesive proteins, immunosuppression, stimulation of aromatase P450, and formation of mutagens *in vivo*.

Apoptosis

Apoptosis, as one of the most vital elements associated with body functioning and physiological cell turnover, is recognized as programmed cell death. In physiology, it is defined as the process of elimination of redundant, damaged, mutated, or used cells that might be harmful to the host organism. The principal rule of apoptosis is based on the degradation of cellular components by cysteine proteases, named caspases, that can be activated through either extrinsic or intrinsic pathways. Both mechanisms are sophisticated and energy dependent. The extrinsic (or death receptor) pathway is initiated by an external apoptotic signal that activates death receptors on the plasma membrane (e.g., TNF receptor 1). This is followed by formation of the death-inducing signaling complex and stimulation of the caspase cascade, leading to lysis. Intrinsic (mitochondrial) apoptosis is associated with DNA damage and the consequent overexpression of cytoplasmic proapoptotic proteins, such as B-cell lymphoma-extra large (BCL-xL), BH3 interacting domain death agonist (BID) and BCL2-associated X protein (BAX) (normally counterbalanced by BCL-2 in nonapoptotic cells). Their accumulation disturbs the integrity of the mitochondrial membrane and causes the release of cytochrome c into the cytosol. Cytochrome c further interacts with ATP, caspase 9, and Apoptotic peptidase activating factor 1 (Apaf-1) forming an apoptosome that stimulates the caspase cascade. This event finally causes protein degradation and cell death [75,76]. The mechanism of apoptosis is tightly regulated and any abnormalities can lead to significant pathologies, such as cancer, autoimmune lymphoproliferative syndrome, AIDS, ischemia, Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis [77].

The dysfunction of normal cell turnover in breast cancer stems from the COX-2-dependent overexpression of antiapoptotic BCL-2 in malignant cells, accompanied by downregulation of proapoptotic BAX and BCL-xL. Breast cancer cells can also become resistant to apoptosis by the COX-2-related activation of serine–treonine kinase Akt, which is a vital component of the Akt/phosphatidylinositol 3,4,5-trisphosphate (PIP_3)/mammalian target of rapamycin (mTOR) signaling pathway, which promotes cell survival. This effect was confirmed in an *in vitro* study with ER–HER2+ cell lines [78]. In addition, other *in vitro* studies on breast cancer cells indicate that the selective COX-2 inhibitor celecoxib activates the intrinsic pathway of apoptosis by stimulation of caspase 3 and caspase 9, but not caspase 8 [3]. Finally, COX-2 could effect its antiapoptotic activity by the degradation of AA, which is a known promoter of programmed cell death (it participates in the conversion of sphingomyelin to ceramide). Therefore, the overexpression of *COX2* in breast cancer leads to its increased metabolism, thereby decreasing the apoptotic potential of malignant cells [53,79,80].

Proliferation

Cell proliferation occurs as a cyclic process of cell growth and division. It is associated with the biochemical and biophysical transformations that are regulated by a highly controlled series of events, named the cell cycle. Physiologically, this crucial molecular mechanism is divided into four phases: G_1, S, G_2 (collectively called interphase), and phase M (mitosis). During G_1, the intense synthesis of lipids and proteins occurs, leading to an in increase in cell mass and size. In S, DNA replication and duplication of chromatin in the cell nucleus occurs. In G_2, cells continue protein (mainly tubulin) synthesis and any damage of DNA is repaired. Cell division occurs during the M phase and involves karyokinesis (i.e., the division of the nucleus) and cytokinesis (i.e., the division of the cytosol). After division, each daughter cell enters the interphase of its own cycle [81]. The organization of the cell cycle is controlled in an ordered and directional manner by the regulatory cyclins and cyclin-dependent kinases (CDKs). Their main function is to supervise the coordinated progression of the cell cycle from one into the following phase by phosphorylation of the target cell cycle proteins [82]. Another key protein regulating the cell cycle is tumor protein p53, which ensures the integrity of DNA and functions as a tumor suppressor. Interestingly, COX-2 and its products, such as PGE_1, PGE_2 and PGI_2, also participate in the cell proliferation because they influence the increased synthesis of DNA during tissue regeneration, as confirmed in an *in vitro* study on rat hepatocytes [80] and in mammary epithelial cells in the presence of EGF [83].

The fundamental nature of cell proliferation means that any abnormalities in its progress result in the development of neoplastic tissue. Published data clearly indicate that disturbed *COX2* expression in breast cancer is one of the elements that determines this uncontrolled proliferation. The upregulation of COX-2 contributes to the increased intracellular concentration of cyclin D_1, which regulates the progression from G_1 to S phase [84]. Thus, the inhibition of COX-2 with celecoxib in breast cancer cells results in cell cycle G_1 arrest, which decreases the number of cells in S and G_2/M phase, thereby suppressing cell division [3,85]. This beneficial effect has been confirmed in several animal studies as well as in one randomized Phase II clinical trial conducted within a population of patients with primary breast cancer. Interestingly, this study showed that the preoperative administration of celecoxib causes G_2/M arrest associated with the activation of p53 and GADD45A, the protein that inhibits cyclin B_1 and B_2, both of which have a role in the G_2/M transition [86,87]. In addition, there is also the hypothesis that the increased proliferation of breast cancer cells is dependent on the PGE_2-related stimulation of EGFR and the consequent stimulation of MAPKs, which are critical mediators of mitogenic pathways in cells [18].

Angiogenesis

Angiogenesis is an important part of neoplastic development because it is the formation of new blood vessels on the basis of the pre-existing ones. This complex multistep process is required for tumor growth, spread, and metastasis. The function of the pathologic cancer vasculature is to provide an essential blood

supply for nutrients, oxygen, and metabolite exchange. It also serves as a way to invade other tissues. Physiologically, the regulation of angiogenesis depends on the balance between proangiogenic substances [e.g., VEGF, PDGF, TGFα, TGFβ, basic FGF (bFGF), IL-8 and hypoxia inducible factor alpha (HIFα)] and antiangiogenic factors, which include angiostatin, endostatin, and thrombospondin [88]. The increased concentration of the former stimulates the adjacent endothelial cells to proliferate and migrate towards tumor tissue in a process facilitated by the digestion of extracellular matrix by MMPs [88]. Importantly, the overexpression of *COX2* in tumor blood vessels also has a crucial role in tumor angiogenesis, especially in colon, breast, lung, prostate, pancreas, and head and neck cancers [8,89]. In fact, it is COX-2 that activates MMPs in a complex mechanism involving nuclear factor (NF)-kappa B. This enzyme also influences endothelial migration by TXA_2 [8,90,91]. Furthermore, it has been observed that the increased activity of COX-2 correlates with the secretion of proangiogenic substances by epithelial neoplastic cells, endothelial neoplastic cells, fibroblasts, and macrophages [90–92]. Hence, the mechanism of COX-2-dependent angiogenesis has been defined as the interaction between the above elements of the tumor microenvironment, and it proceeds as demonstrated in Fig. 7. In detail, the process begins with the production of proangiogenic prostaglandins (mainly PGE_2) by cancer cells, which increases the concentration of VEGF and bFGF. The former growth factor directly stimulates COX-2 in endothelial cells, whereas the latter one directly stimulates COX-2 in fibroblasts to synthesize PGs that activate the PKA pathway through the EP_2 receptor. In this event, VEGF is also induced in fibroblasts and it interacts with endothelial cells in paracrine manner. Consequently, vascular COX-2 is activated, causing the increased permeability, proliferation, and morphogenesis of blood vessels [92]. The extensive microvessel density, in turn, determines the higher metastatic potential of neoplastic cells, which is a predictor of poor patient prognosis [8,92,93]. In addition, there is also evidence that PGE_2 stimulates angiogenesis by the activation of EP_4 and its second messenger, PKA, in endothelial cells [74,94].

Apart from their direct proangiogenic effect, PGs can also stimulate angiogenesis indirectly, through the activation of

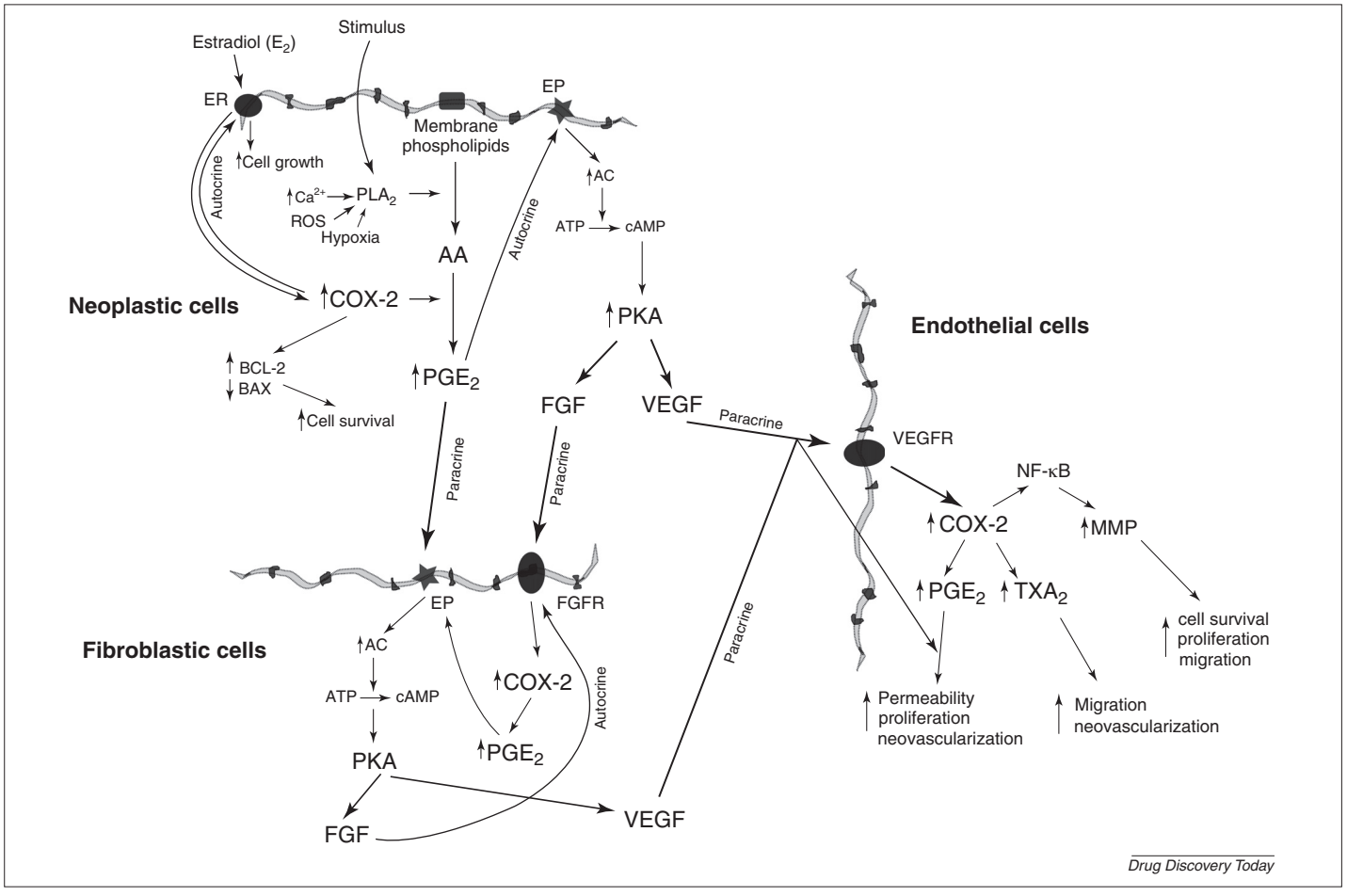


FIGURE 7

The mechanism of the cyclooxygenase 2 (COX-2)-dependent cancer angiogenesis. This process is based on the interaction between epithelial neoplastic cells, endothelial neoplastic cells, fibroblasts, and macrophages. It begins with the production of proangiogenic prostaglandins (mainly PGE_2) by cancer cells, which increases VEGF and bFGF. These growth factors stimulate directly COX-2 in fibroblasts and endothelial cells to synthesize prostaglandins, which bind with the EP_2 receptor and activate the PKA pathway. This also induces the secretion of VEGF by fibroblasts, which interacts with endothelial cells in a paracrine manner and causes the activation of COX-2 in blood vessels, leading to an increase in their permeability, proliferation, and morphogenesis [8,88–92]. *Abbreviations*: AA, arachidonic acid; AC, adenylyl cyclase; BAX, BCL-2-associated X protein; BCL-2, B cell lymphoma 2 protein; ER, estrogen receptor; EP, PGE_2 receptor; (b) FGF, (basic) fibroblast growth factor; FGFR, FGF receptor; NK-κB, nuclear factor kappa B; MMP, matrix metalloproteinase; PGE_2, prostaglandin E_2; PKA, protein kinase A; ROS, reactive oxygen species; TXA_2, thromboxane A_2; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

monocytes that infiltrate cancer tissue. In fact, the increased production of proinflammatory factors, such as IL-2, results in further recruitment of macrophages to the so-formed inflammatory foci, recognized by macrophages as the area of damaged tissue. Thus, the arriving macrophages induce a healing process, which involves the connection of the damaged region to blood vessels, formation of supportive connective tissue, and stimulation of regeneration [84,92].

Finally, in one Phase II clinical trial in patients with breast cancer, preoperative adjuvant therapy with celecoxib resulted in improved matrix stability associated with the downregulation of MMP-2 and MMP-9. Interestingly, this observation was explained by the celecoxib-dependent activation of TIMP metallopeptidase inhibitor 1–3 (TIMP1, TIMP2, and TIMP3), and Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), which are known antagonists of MMPs [87].

Immunosuppression

COX-2 has a significant role in breast tumor immune escape. PGs, especially PGE_2, negatively influence the activity of T and B lymphocytes, natural killer (NK) cells, and dendritic cells. They also reduce the synthesis of TNFα and augment the activity of immunosuppressive IL-10 [53]. These abnormalities, in turn, abolish the efficiency of host defenses in detecting and rejecting malignant cells, thereby promoting their uncontrolled proliferation [95]. The hypothesis of COX-2-related immunosuppression is supported by published studies in which the decreased expression of *COX2* in breast cancer cells resulted in increased tissue infiltration by cytotoxic T lymphocytes (CD8+). Also, the application of selective COX-2 inhibitors in human breast cancers induced the enhanced recruitment of immune cells to the tumor microenvironment [85,95].

Association between COX-2 and aromatase P450

Aromatase P450 belongs to the family of proteins associated with cytochrome P450. It is responsible for the conversion of androgenic steroids into estrone and estradiol, and has a crucial role in the local production of these hormones in estrogen-dependent breast cancer (approximately 60–70% of all breast tumors). The interaction between estrogens and their receptors, in turn, constitutes the critical stimulus for the development of these tumors because it directly induces the growth and proliferation of malignant cells. Hence, it is no surprise that the upregulation of aromatase P450 determines the progression of breast cancer with an ER+ status. Interestingly, the expression of *CYP19* encoding aromatase P450 is dependent on auto- and paracrine activation by PGE_2, which, by interacting with EP_2 and EP_4, causes the accumulation of cAMP, PKC, and PKA [96]. For this reason, in human breast cancer, there is a positive correlation between *CYP19* expression and the concentration of COX-2 within the tumor microenvironment [97]. Available experimental data support these hypotheses. In fact, in model transgenic mice with silenced *COX2*, the activity of aromatase appears to be significantly lower, whereas in mice overexpressing *COX2*, the enzyme is upregulated [47]. In addition, the impact of COX-2 on the local production of estrogens could explain the chemopreventive properties of NSAIDs in postmenopausal estrogen-dependent breast cancer [47].

Production of mutagens in vivo

A mutagen is any endo- or exogenic substance that damages DNA directly, thereby contributing to the development of neoplasia. One endogenic mutagen [malondialdehyde (MDA)] is formed consequently to the enzymatic or nonenzymatic degradation of PGH_2, which is a known product of COX-2. The detrimental effect of MDA is, in turn, associated with its tendency to form adducts with deoxynucleotides, leading to point and frameshift mutations [53]. In addition, COX-2 acting as a peroxidase could catalyze the oxidation of aromatic amines, heterocyclic amines, dihydrodiol derivatives, and multiring aromatic hydrocarbons, producing various carcinogenic compounds [98]. In fact, it has been experimentally shown that the preferential COX-2 inhibitor nimesulide reduces the production of a mutagen named 8-oxo-7,8-dihydro-2′-deoxyguanosine in mucosa during early colonic inflammation in rats [99].

COX-2 inhibitors in breast cancer: current and future directions

The use of COX-2 inhibitors in breast cancer could be primarily justified by their chemopreventive properties, which have been found to be beneficial in both primary and secondary prophylaxis. Appropriate studies with animal models have shown such strategies to be effective. For instance, the decreased incidence of carcinogen-induced breast malignancies after the application of flurbiprofen and indomethacin (nonselective COX inhibitors) has been shown. Similarly, some chemopreventive actions have been observed for selective and preferential COX-2 inhibitors, including celecoxib, rofecoxib, and nimesulide. What is more, these agents additionally resulted in growth inhibition in cancer cells with ER+, and ER− HER2+ phenotypes (http://www.cancer.gov/types/breast/hp/breast-treatment-pdq), whereas celecoxib has also been found to delay the onset of *HER2/neu*-induced tumors. Importantly, celecoxib also acts more effectively in retarding the development of DMBA-induced breast cancer compared with nonselective ibuprofen [100].

Moreover, the available epidemiological data indicate that the long-term use of NSAIDs significantly lowers the incidence of breast cancer. This effect was confirmed in 1980 within a group of 4876 patients treated with indomethacin (compared with age-matched controls) [101]. In 1996, it was estimated that the administration of NSAIDs three times a week for at least 1 year lowered the risk of breast cancer development by 66% (a study comparing 511 females with newly diagnosed breast cancer using NSAIDs versus 1534 controls) [102]. Consistent results were achieved in studies conducted in 1994 (the incidence ratio among aspirin users was 0.7) [103] and 2000 (retarded growth of breast cancer among NSAIDs users) [104]. In addition, a more recent meta-analysis of nine case–control studies and seven cohort studies from 2003 showed a slight but significant decrease in breast cancer incidence among users of acetylsalicylic acid and other nonaspirin NSAIDs []105. Also, in a case–control study from 2006, a reduction of breast cancer risk of 71% was observed in women using selective COX-2 inhibitors (celecoxib and rofecoxib) in standard doses for 2 years [106]. Finally, large meta-analyses from 2012 confirmed that aspirin is protective against cancer development and its regular intake significantly reduces the risk of breast cancer (odds ratio = 0.88) [107]. However, there

are also epidemiological studies indicating that there is no relation between NSAIDs use and breast cancer incidence [108–110]. This inconsistence could be explained by the nonuniform expression of COX-2 in different subtypes of this malignancy.

Apart from their prophylactic effects, COX-2 inhibitors could be also used in the treatment of breast cancer. This concept is substantiated by the results of experiments on rats that showed that 100-day therapy with ibuprofen decreased the size of DMBA-induced breast cancer by 40%, whereas in nontreated controls the neoplastic lesions increased by 180% [61]. Similarly, celecoxib used for 6 weeks contributed to the decrease of tumor mass by 32% [111].

Alternatively, COX-2 inhibitors could be introduced into adjuvant anticancer therapy. A promising strategy in this respect involves their co-administration with taxanes, because these cytostatic drugs have the ability to induce the transcription of *COX2*, which might result in the decreased efficiency of monotherapy [112]. Another possibility is the combination therapy of a COX-2 inhibitor and an aromatase inhibitor, such as letrozole or anastrozole, in the treatment of hormone-dependent breast cancers. Finally, a therapeutic regimen including a COX-2 inhibitor and trastuzumab in malignancies overexpressing HER2 should also be considered [113]. However, the results of available studies are not satisfactory. In trastuzumab-refractory metastatic breast cancer, COX-2 inhibition was shown to be inactive yet well tolerated in a group of 12 patients (a randomized Phase II clinical trial) [114]. In postmenopausal metastatic breast cancer, the improved efficacy and end-point benefits of celecoxib in combination with an aromatase inhibitor were reported, but only in tamoxifen-resistant women [115]. Finally, the results of a randomized Phase II study in 111 postmenopausal women with advanced breast cancer treated with exemestane and celecoxib indicated a longer time to progression with no additional adverse effects associated with the use of combination therapy [116].

There is also a study that analyzed the benefits of simultaneous use of celecoxib (400 mg) with FEC chemotherapy (5-FU 500 mg/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 500 mg/m^2) in neoadjuvant treatment of locally advanced breast tumors. Interestingly, the results showed that the clinical and pathological responses for the combined treatment arm (FEC + celecoxib) equaled 81.3 and 87.5%, respectively, versus 62.5 and 62.5% in the group treated with FEC only [117].

Despite these promising results, more clinical data are required to decide whether the described strategies provide any substantial benefits to patients with breast cancer.

Concluding remarks

Great progress has been made since COX-2 was first established as a potential therapeutic or preventive target in breast cancer, especially in our understanding of the link between the activity of the enzyme and the malignancy. The detailed description of the pathomechanism of COX-2-related cancer progression revealed that selective COX-2 inhibitors together with conventional therapy (radiotherapy or chemotherapy) could represent a modern and effective approach in the management of breast cancer. Indeed, this idea is supported by the results of several experimental studies, yet the clinical benefits of this novel therapeutic mode are currently too limited [61,86,88,101–120]. Appropriate, randomized clinical trials are needed for the final assessment of the clinical value associated with the strategies discussed here. Also, the identification of patients who could benefit from their implementation is required. Finally, more attention should be paid to the discovery and development of new safer and more effective inhibitors, such as *trans*-stilbenes, to provide patients with more convenient and less toxic therapeutic options. This can be done by the use of different analytical methods, including *in silico* QSAR techniques, which constitute an important research tool in contemporary pharmaceutical research.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.drudis.2015.12.003.

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