Review

The cyclooxygenase isoforms: structural insights into the conversion of arachidonic acid to prostaglandins

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Abstract

Despite the marked differences in their physiological roles, the structures and catalytic functions of the cyclooxygenase isozymes COX-1 and -2 are virtually identical. Nevertheless, a handful of amino acid substitutions give rise to subtle differences in ligand binding between the two isoforms. These ‘small’ alterations of isozyme structure are sufficient to allow the design of new, isoform-selective drugs. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cyclooxygenase (COX) catalyzes the first committed step in the biosynthesis of prostaglandins through the conversion of arachidonic acid to prostaglandin H$_2$, the common intermediate in all pros-
Prostaglandin synthesis [1]. COX is more formally referred to as prostaglandin endoperoxide H synthase (EC 1.14.99.1), a bifunctional, membrane-bound enzyme responsible for the COX reaction. Pharmacologists have long been interested in COX as it is the primary target of non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin, ibuprofen, and other NSAIDs have been readily available as ‘over-the-counter’ drugs for treatment of headache and fever, as well as to reduce soreness and inflammation resulting from work or exercise. For many individuals who suffer from rheumatoid arthritis and osteoarthritis, NSAIDs are also prescribed to reduce pain and to restore flexibility in inflamed joints. Unfortunately, the beneficial effects of NSAIDs are outweighed by serious side effects in some individuals: about 1% of chronic users per year develop ulcers or other serious gastrointestinal complications [2]. Because of the widespread use of NSAIDs, their toxicity is one of the most prevalent drug-associated health risks. Nonetheless, aspirin remains one of the widely used and most successful drugs on the world market.

The observation by Smith and Lands in 1971 that aspirin irreversibly inhibited ram seminal vesicle COX activity [3] demonstrated that the therapeutic properties of this drug were due to direct inhibition of prostaglandin synthesis. Over the next 20 years, the anti-inflammatory properties of NSAIDs were attributed entirely to the inhibition of prostaglandin synthesis by a single gene product, the COX-1 enzyme. This model for prostaglandin biosynthesis remained unchanged until two independent groups of researchers [4,5] discovered a second, highly regulated COX gene, which expressed a cyclooxygenase-2 (COX-2). Experiments comparing the patterns of expression and regulation COX-1 and COX-2 immediately suggested that these two isozymes have unique and separate physiological functions (see [1], for a review). COX-1 is constitutively expressed in most tissues, which allows this isozyme to produce prostaglandins that help maintain homeostasis, as happens in the cardiovascular system and the gastric mucosa. COX-2, on the other hand, is constitutively expressed in a very limited number of few tissues, notably brain and kidney, but is rapidly induced by inflammatory mediators such as IL-1 and LPS [6]. COX-2 expression is elevated at sites of inflammation, and in stimulated macrophages, fibroblasts and synoviocytes, while the anti-inflammatory cytokines

![Fig. 1. A ribbon drawing of the ovine COX-1 dimer structure [8] showing the relationship between the heme group (red), the binding site for flurbiprofen (yellow), and the putative position of the lumenal leaflet of the ER bilayer. The EGF, membrane binding, and catalytic domains are colored green, gold, and blue, respectively; some of the saccharide units at the sites of glycosylation are shown in the ball-and-stick form.](image-url)
IL-4 and IL-12, and glucocorticoids repress the expression of COX-2, but not COX-1 [6]. This pattern of expression suggested that COX-2 produced prostaglandins involved in inflammatory responses and that the anti-inflammatory activities of NSAIDs were due to inhibition of this inducible enzyme. Conversely, it also seemed likely that ulcers and gastrointestinal bleeding resulted from inhibition of COX-1. Although the exact role(s) of the prostaglandins produced by the two COX isozymes is still not clear, much is now known about the enzymatic characteristics, substrate specificities and the drug sensitivities of COX-1 and COX-2 and this has led to new advances in the design of isoform-selective drugs.

2. The general design of COX-1 and COX-2

The COX enzymes are glycosylated, integral membrane proteins found in the endoplasmic reticulum (ER) and the nuclear envelope [1]. After post-translational processing in the ER, the mature COX-1 and COX-2 proteins have apparent molecular masses of 67–72 kDa and exist as homodimers which bind 1 mole of high-spin ferric heme per mole monomer. Three crystal structures of COX have been published: ovine COX-1 [7,8], recombinant human COX-2 [9] and recombinant mouse COX-2 [10]. All the COX crystal structures reveal the complete dimeric form of these glycosylated, detergent-solubilized enzymes (Fig. 1). Due to the high degree of sequence homology between COX-1 and COX-2, it was not surprising that the tertiary and quaternary structures of the COX isozymes are virtually identical [9,10]. The sequence homology between COX-2 and -1 is high (60–65% sequence identity), within and between species [1]. The major isozyme differences in the primary structure are that COX-2 has a truncated signal peptide and an 18-amino acid C-terminal insertion. For the purposes of convenience, the numbering of the residues in the COX-2 sequence and structure is based on the sequence numbering in ovine COX-1.

The COX monomer reveals a unique structure comprised of three domains (Fig. 2): an N-terminal epidermal growth factor (EGF)-like module; a membrane-binding domain (MBD) consisting of a spiral of four amphipathic helices; and a globular catalytic peroxidase site.

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1 For clarity and ease of comparison, we have numbered the isoform amino acid sequences based on the ovine COX-1 enzyme [8]. The homologous amino acids in ovine COX-1 and human or mouse COX-2 that are specifically discussed in the text are listed here with their appropriate isoform numbering (COX-1:COX-2): Arg-120:Arg-104; Ile-434:Val-420; Phe-518:Phe-504; Ile-523:Val-509; His-523:Arg-519, and Tyr-385:Tyr-371.
domain containing the COX and peroxidase active sites. The tertiary structure of the catalytic domain bears striking homology to myeloperoxidase and other mammalian peroxidases [7,8,11]. The structural and sequence homologies between COX and myeloperoxidase suggest that the COX enzymes arose from an ancestral soluble peroxidase which had acquired a membrane binding capability [8,12], a hypothesis that was recently tested by DeWitt and coworkers [13]. Using a series of expression constructs containing the EGF and MBD domains, from either COX-1 or -2, fused to green fluorescent protein, they demonstrated that only chimeric proteins containing a MBD domain were localized to the membrane. The correlation between the EGF-like module and membrane anchoring [14] also suggests that the EGF-like module and the MBD may actually act as a single modular motif for creating membrane proteins from soluble precursors. Thus, the COX enzymes interact with the ER membrane via the penetration of the MBD into only one leaflet of the lipid bilayer (Fig. 1) and are therefore monotopic integral membrane proteins.

The COX enzyme structures have broader implications for membrane protein biosynthesis. The COX-1 and COX-2 are processed, folded and inserted into the bilayer of the ER. From this point, the mature enzymes are then localized throughout the ER and the outer and inner membranes of the nuclear envelope [1]. The structural features that regulate the maturation, post-translational modification, localization and targeting of the COX isozymes are not known, but the crystal structures of the two isozymes will provide the opportunity for the design of critical experiments probing the mechanisms of the membrane protein insertion and targeting in eukaryotes.

3. The interaction of fatty acids with COX-1 and COX-2

The first step of the conversion of arachidonate to PGH₂ takes place in the COX active site (Fig. 2) where two molecules of oxygen add to arachidonate to form the bicyclic peroxide intermediate, PGG₂. The monotopic nature of the COX enzyme has a functional significance for substrate binding: the MBD not only anchors the enzyme to the bilayer, but also forms the mouth of the COX active site. This structural feature allows AA to enter from the hydrophobic compartment of the bilayer directly into the COX active site, a long (25 Å), hydrophobic channel that extends from the MBD for Tyr-385 [7]. Within this channel, few polar residues are seen; two that are of interest are Arg-120, which is positioned about midway between the mouth and the apex of the active site, and Ser-530, the site of acetylation by aspirin [15].

How AA binds within the active sites of COX-1 and COX-2 is not known, but the known stereochemistry of the reaction (see [1] for a review) and the crystals structures of COX-1 and COX-2 complexed with NSAIDs [7,9,10,15,16] provide new and significant insights. Arg-120 is one of the few charged amino acids in the hydrophobic COX-1 active site and it interacts with the carboxylic moieties of the arylpropionic acid NSAIDs, like flurbiprofen and sulprofen [7,16]. Subsequent mutagenic studies [17,18] clearly established that Arg-120 is critical for COX activity and for most NSAID inhibition in COX-1. Most likely, Arg-120 is primarily responsible for liganding the carboxylate moiety of AA, other fatty acid substrates, and acidic NSAIDs.

From Arg-120, near the mouth of the hydrophobic pocket, arachidonate would snake its way up into the active site, eventually forming a hairpin turn between carbons 9 and 11. The bound arachidonate must then be positioned so that oxidized Tyr-385, in the

![Fig. 3. A stereo diagram of 'hairpin' binding conformation of arachidonic acid (AA) in the cyclooxygenase active site. Arg-120 (dark gray) is labeled and Tyr-385 (+) is shown interacting with the pro-(S) hydrogen of arachidonic acid.](image-url)
form of a tyrosyl radical, can remove the pro(S) hydrogen from carbon 13 of the fatty acid (Figs. 3 and 4). What happens beyond carbon 13 is not completely clear as there are two possible binding modes for the remaining portion of the substrate: carbons 14-20 could turn back down the hydrophobic channel (Fig. 3) or, alternatively, could kink off into a small ‘cul-de-sac’ in the active site (Fig. 4). Both conformations of bound AA are stereochemically feasible and detailed X-ray diffraction experiments will be needed to distinguish between the two possibilities. Once proper binding of AA occurs, Tyr-385 abstracts the hydrogen from carbon 13 and molecular oxygen forms the endoperoxide bridge connecting carbons 9 and 11. Ring closure then occurs between carbons 8 and 12, resulting the bicyclic cyclopentyl-dioxygen prostaglandin structure. The addition of a second molecule of oxygen at carbon 15 forms the product PGG2. PGG2 must then migrate to the peroxidase (POX) active site, located on the luminal side of the enzyme (Fig. 1) to be reduced to PGH2.

The mechanisms for COX and POX reactions are the same for both the COX isozymes [1], although differences in substrate binding [19] and in COX activation [20] have been observed between the two isozymes. COX-1 activity is crucially dependent on the proper orientation of fatty acids substrates within the cyclooxygenase active site, a process that relies on ionic bonding to Arg-120. When Arg-120 is replaced with positively charged lysine, the mutant is fully active [18]. However, the substitution with neutral glutamine produces an enzyme with less than 50% activity and a 1000-fold increased $K_m$ [17,18]. Substitution of Arg-120 with the negatively charged glutamate produces an inactive ovine COX-1, or a human COX-1 with only 5% activity. The situation is markedly different in COX-2: the R120Q mutant is fully active with AA [21], and the R120E mutant has near full activity albeit with a 30-fold increased $K_m$ [22]. The conclusion is that AA binding in COX-1 is critically dependent on an ionic interaction with Arg-120, while, in COX-2, hydrophobic interactions between AA and the residues in the COX active site dominate fatty acid binding.

Little is known about how other residues in the active site participate in the binding AA and affect product formation during this rather non-specific, free radical-based reaction [1]. COX-1 and COX-2 can also oxygenate a variety of $C_{18}$ and $C_{20}$ $n$-3 and $n$-6 fatty acids [19]. The native isozymes also are not 100% efficient in converting AA to PGG2 as other oxygenated derivatives of AA, such as 15- and 11-HETEs, have been detected [21]. Smith and co-workers (W.L. Smith, personal communication) have recently mutated several residues that line the ovine COX-1 and human COX-2 active sites and found that even subtle changes, such as a valine to alanine substitution, altered markedly the product distribution between PGH2, 15-HETE and 11-HETE. These mutations also have clear effects on the enzymes’ efficiency to oxygenate other fatty acid substrates. Interpreting these results in terms of structural models of AA bound to the enzymes (Figs. 3 and 4) does not yet yield clear conclusions. Obviously, detailed X-ray diffraction experiments will be needed to evaluate the results from these mutagenic studies.

4. Inhibition by non-selective COX-1 and COX-2 NSAIDs

The physical nature of ligand binding to the COX enzymes has been more extensively studied with regards to NSAID binding. The largest group of non-selective NSAIDs, the acidic cyclooxygenase inhibi-
tors, depend on Arg-120 for efficient binding and inhibition. Crystal structures of COX-1 complexed with flurbiprofen, aspirin, and iodo-suprofen clearly reveal that the carboxylates of these NSAIDs are always within hydrogen bonding distance of Arg-120 [7,15,16]. Mutational analysis also confirms the importance of this interaction for inhibition [17,18]. Substitution of Arg-120 in COX-1 with glutamine or glutamate abolishes or reduces inhibition by the acidic NSAIDs indomethacin, flurbiprofen and ketoprofen, diclofenate and meclofenamate. Flurbiprofen, which inhibits native COX-1 by a time-dependent mechanism, now inhibits the R120Q mutant only competitively, implying that interaction of the carboxylic acid moiety of the NSAID is also required for the conformational changes that accompany time-dependent inhibition by these acidic, non-selective NSAIDs [18]. Interestingly, the COX-2 selective NSAIDs which lack a carboxylic acid moiety (e.g. DuP-697 and L-746-483) inhibit the R120E mutant about 10 times more potently than the native COX-1, suggesting that the presence of Arg-120 may actually hinder the binding of non-acidic inhibitors in COX-1 [17].

In contrast to its function in COX-1, Arg-120 may play only an accessory role in the binding of many NSAIDs to COX-2. Crystal structures of COX-2 complexed with flurbiprofen and indomethacin suggest that these inhibitors bind to COX-2 similarly to that seen in COX-1 (i.e. via electrostatic interaction between a carboxylate moiety and Arg-120) [10] and the behavior of a R120Q COX-2 mutant [21] is consistent with this hypothesis. However, the inhibitory efficacy of some NSAIDs, like meclofenamate and diclofenate, is only minimally affected by the mutation of Arg-120 to a glutamate [22]. Thus, in contrast to the situation in COX-1, the presence of arginine at residue 120 provides, at most, a very small contribution to the binding of acidic NSAIDs to COX-2. As alluded to above, the positively charged arginine may actually begin to discriminate against binding of some acidic inhibitors to COX-2: the methyl ester of indomethacin displays an increased potency against COX-2 over that of the free acid [22]. For non-acidic, COX-2 selective NSAIDs, the charged guanido group of Arg-120 may also have an inhibitory effect on their binding to COX-2, as the IC₅₀ values for NS398, DuP697, and SC58125, are decreased by as much as 1000-fold when neutral glutamine is substituted for arginine in the R120Q COX-2 mutant [21].

No satisfactory and comprehensive physical explanation exists for the diminished role of Arg-120 in the binding of NSAID and fatty acids to COX-2. However, this effect may arise from the fact that the active site in COX-2 is larger and has a slightly different shape than in COX-1. By comparing crystal structures of ovine COX-1 and human COX-2, Luong et al. [9] estimated that the central channel of the NSAID binding pocket of COX-2 is about 17% larger than COX-1. The increased size may simply allow NSAIDs, and perhaps fatty acids, to bind more tightly or to find more optimal binding configurations in the COX-2 active site than in COX-1; this might then reduce the relative importance of ionic interactions with Arg-120. The larger COX-2 active site may also reduce unfavorable steric interactions involving Arg-120 at the mouth of this pocket, and thereby increase access, or permit more avid binding of non-acidic inhibitors to COX-2. These hypotheses raise the issue that some of the isozymic differences in drug binding may arise from a combination of static (e.g. steric clashes and active site size) and dynamic (e.g. ease of access to the active site) features of the isozymes’ structure. Nonetheless, the differing role of Arg-120 between such homologous isozymes is quite remarkable.

5. COX-2 selective inhibitors

The observation that ligand interactions with Arg-120 may indirectly contribute to drug selectivity by discriminating against non-acidic NSAIDs more effectively in COX-1 than in COX-2 is an encouraging starting point for the design of isozyme selective drugs. The physical basis for the altered behavior of Arg-120 and how can it be taken advantage of are issues that must still be addressed. The former is, as discussed above, unresolved, but the latter may be answered indirectly. The catalytic center of the COX isozymes is identical in terms of the residues which line the active site surface (the ‘first shell’ residues) save for one difference: Ile-523 in COX-1 is a valine in COX-2. However, a few more amino acid substitutions have occurred in residues that are ad-
jacent to the active site, but not part of it (i.e. in the ‘second shell’). As it turns out, only a small number of amino acid substitutions are responsible for the increase in the size, topography, and chemical environment of the COX-2 active site. Most important among these is the substitution of valine in COX-2 for Ile-523 in COX-1 (compare Figs. 5 and 6). The loss of a single methyl group in COX-2 permits access to a small pocket adjacent to the active site channel and thus markedly increases the volume of the COX-2 active site. The effect of this change is now compounded by the substitution of a valine in COX-2 for Ile-434 in COX-1 within the second shell of amino acids. The combination of these two substitutions in COX-2 allows a neighboring amino acid, Phe-518, to swing out of the way, which further increases access to the side pocket (Fig. 6). The larger main channel combined with this extra ‘nook’ increases the volume of the COX-2 NSAID binding site by about 25% larger over that in COX-1 [9].

This extra size are the structural features exploited by COX-2 selective NSAIDs: if access to this side pocket is restricted in COX-2 by switching Val-523 to an isoleucine, COX-2 is no longer differentially sensitive to these inhibitors [22,23].

Another essential amino acid difference between COX-1 and COX-2 does not alter the shape of the drug binding site, but rather changes its chemical environment. Within the now accessible side pocket in COX-2 (Fig. 6), lies residue 513, an arginine in COX-2 and a histidine in COX-1. This residue difference results in a stable positive charge being placed at the center of the pocket. This arginine appears to interact with the 4-methysulfonyl or 4-sulfonamoyl-phenyl substituents of diarylhetereocyclic inhibitors and is required for the time-dependent inhibition of COX-2 by these compounds [10]. In vitro mutagenesis experiments with COX-1 have revealed two interesting results. First, the I523V mutation in COX-1 increases its affinity for COX-2 selective NSAIDs. Second, the H513R mutation is needed for COX-2 selective drugs to exert time-dependent inhibition [24]. With these two mutations, COX-1 now behaves like native COX-2.
The preceding discussion illustrates one way in which ligands can discriminate the subtle structural differences found between COX isozymes. However, other mechanisms for drug binding and isozyme selectivity exist. For example, the zompirac class of inhibitors may require the presence of Arg-513 to exhibit tight binding and time-dependent inhibition although Arg-513 does not directly interact with these inhibitors [9]. In this case, Arg-513 forms hydrogen bonds with Glu-524 and Tyr-355 in the NSAID binding pocket which causes a change in the conformation of Arg-120. This structural perturbation is significant enough to alter the structure of the mouth of the active site [9]. Although the physical basis for the tighter binding is not completely clear, inhibition by these compounds does not involve binding to the side pocket in COX-2.

One intriguing conclusion arising from the work on COX-2 selective NSAIDs is that time-dependent inhibition plays critical roles in isozyme selectivity and drug efficacies. The structure of COX-2 co-crystallized with SC-588 [10] indicates that the phenylsulfonamide moiety of this diarylheteterocyclic compound fits into the side pocket made accessible by Val-523 and comes within bonding distance of the guanido group of Arg-513 (Fig. 6). However, there is increasing evidence that subtle conformational transitions in the enzyme–ligand complex enhance further a drug’s selectivity in vivo. Many non-selective NSAIDs like flurbiprofen and indomethacin inhibit both isozymes in a time-dependent, pseudo-irreversible manner [1]. Depending on the NSAID used and its concentration, it can take several seconds to minutes to achieve full inactivation. Upon the formation of the reversible enzyme–inhibitor EI complex, a relatively slow conformational transition converts EI to a EI* state, which is characterized by a very slow off-rate for the ligand. The formation of these non-covalent, tight-binding enzyme complexes slowly, but effectively, inactivate the COX enzyme. The new NSAIDs exhibit efficacious COX-2 selectivity because they inhibit this isoform by a time-dependent, pseudo-irreversible mechanism, while they inhibit COX-1 by a rapid, competitive, and reversible mechanism. The practical result of this mixed mode of inhibition is that if the blood concentration of a COX-2 selective NSAID is below the IC$_{50}$ for COX-1, the activity of COX-1 will be minimally affected by the inhibitor, while the inactivation of COX-2 effectively increases as more EI* state is formed.

The physical basis for time-dependent inhibition in the COX isozymes is not well understood, and indeed seems to vary among inhibitor classes, and even between drugs within a given class. For example, flurbiprofen inhibits COX-1 by a time-dependent, pseudo-irreversible mechanism, while ibuprofen, a close chemical relative, does not [1,18]. For most acidic time-dependent NSAIDs, interaction with Arg-120 appears to be required, as these drugs inhibit the R120Q and R120E mutants only competitively [17,18]. As mentioned earlier, time-dependent inhibition in COX-2 by selective NSAIDs containing sulfonamide or methylsulfoxide moieties apparently arises from their interaction with Arg-513 [24]. Curiously, inhibition by the methylsulfoxide inhibitor NS-398 appears to depend on interaction with Arg-120, but not with Arg-513. NS-398 binds in the COX-2 active site similarly to acidic NSAIDs and inhibits the R120E COX-2 mutant only competitively [22].

6. Implications for pharmacology

In summary, two amino acid differences, one in the cyclooxygenase active site and one outside of it are sufficient to account for the differential sensitivities of COX-1 and COX-2 to selective COX-2 inhibitors. The substitution of Ile to Val at position 523 provides access to a side pocket in COX-2, which increases the effective size of the COX-2 active site relative to COX-1. The Ile to Val substitution at position 434 outside the COX-2 catalytic center further increases the effective size of the active site channel by enhancing the local mobility of side chains within the side pocket. These subtle structural alterations of the COX-2 isozyme permits it to bind bulkier ligands than COX-1. The larger effective size of the central channel in the COX-2 may also preferentially reduce steric and ionic crowding by the charged Arg-120 in COX-2 and thus may enhance the binding of non-acidic NSAIDs by this isozyme. Finally, the substitution of an arginine in COX-2 for His-513 in COX-1 alters the chemical environment of the side pocket. This arginine can interact with polar moieties entering the pocket [10], which may give rise to the
time-dependent inhibition displayed by this class of inhibitors [24].

Detailed mechanisms for the binding and conversion of AA to PGG$_2$ or explanations for the observed differences in fatty acid substrate preferences between the isozymes do not yet exist. However it is clear that COX-1 and COX-2 catalyze the same reactions and that the differences in their enzymology do not really account for the physiologically significant differences in product formation. The difference in PGH$_2$ biosynthesis between the isozymes is seemingly regulated by a combination of differential isozyme expression in tissues and differential control of the substrate release. Thus, the physiological effects of inhibition by isozyme selective drugs will likely affect the local production of specific prostaglandins in ways that are clearly distinct from non-selective NSAIDs. The observations that COX-2 overexpression plays a role in the development of colon-rectal cancer [25] and that the prophylactic use of aspirin [26,27] can significantly reduce its incidence raise the possibility of using COX-2 selective inhibitors in cancer prevention.

The first COX-2 selective NSAID, Celebrex (celecoxib [28,29]), has recently been approved by the FDA for clinical use. These second-generation NSAIDs have the same anti-inflammatory, anti-pyretic and analgesic activities as current NSAIDs, but their COX-2 selectivity should reduce significantly the incidence of gastrointestinal side effects. In a market that amounted to US$ 1.9 billion in prescription NSAID sales in America in 1997, Wall Street estimates that worldwide sales of the first COX-2 inhibitors could eventually reach as high as US$ 1–3 billion annually [2]. COX-2 selective NSAIDs should not have any other adverse effects that have not previously been observed in over 20 years of experience with non-selective NSAIDs, an assumption supported by animal experiments and early clinical trials [29]. However, we cannot yet predict the outcome of long-term use of COX-2 selective drugs as the roles of the COX isozymes in the production of specific prostaglandins in different tissues have not been completely clarified. For example, selective COX-2 inhibitors reduce systemic prostacyclin synthesis [30], but unlike aspirin [31], these new compounds do not affect platelet COX-1 thromboxane synthesis. Thus, selective COX-2 inhibitors could bias vascular prostaglandin synthesis in favor of thromboxane production, and thus promote a more pro-thrombotic, vaso-constrictive state. While studies examining the consequences of COX-2 inhibition on vascular prostaglandin synthesis are still in their early stages, long-term monitoring stroke and heart attack rates in patient populations taking these drugs will be desirable.

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