Supporting Information

Protocatechualdehyde synergizes with aspirin at the platelet cyclooxygenase-1 level

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Materials and methods

Definition and calculation of platelet-associated aspirin clearance

The aspirin clearance in PRP (or PPP) was defined as the quantity of aspirin being cleared per minutes (pmol·min⁻¹) (Eq.1), and the platelet-associated aspirin clearance was defined as the difference of aspirin clearance in PRP and aspirin clearance in PPP.

In equation 1, $c$ is the concentration of aspirin added into PRP (or PPP), $V$ is the total reaction volume, which is 270μL in our study, and $t$ is the reaction time.

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\text{Aspirin clearance}_{PRP,PPP}(pmol\cdot min^{-1}) = \frac{SA(\mu M)}{SA(\mu M)+AS(\mu M)} \cdot c \cdot V / t
\]  (Equation 1)

Determination of plasma concentration of salicylic acid and aspirin by LC/MS
The concentrations of salicylic acid and aspirin were determined by LC/MS. An aliquot of 40 μL PRP (or PPP) was transferred to a 1.5 mL centrifuge tube, to which was added 10 μL of internal standard (dissolved in acetonitrile). An aliquot (0.25 mL) of acetonitrile containing 0.1% formic acid was added and vortexed for the sample extraction. The sample was centrifuged at 18,000 rpm for 10 min, and the resultant supernatant (0.2 mL) was transferred to another 1.5 mL centrifuge tube and further centrifuged at 20,000 rpm for 5 min. The supernatant (0.15 mL) was transferred to an analytical vial, and 5 μL was injected directly into the LC-MS system.

Analysis was carried out using a Shimadzu LCMS-2010A instrument (Shimadzu Technologies). The separation was performed on a Capcell-Pak stainless-steel column (C18, 5 μm, 100 mm × 2.0 mm I.D.; Shimadzu). The mobile phase consisted of water (containing 0.05% formic acid) (A) and acetonitrile (B), which was programmed with a linear gradient system, began with 15% B for 0.5 minutes, increased from 15% B to 50% B over a 5.5 min period, held at 50% B for 0.5 min, increased from 50% B to 90% B over a 0.5 min period, returned to 15% B over a 0.5 min period, and finally held at 15% B for another 4.5 min of equilibration. The flow rate was set at 0.2 mL/min. The mass spectrometer was operated in the negative ion mode in the m/z range of 136.95 (salicylic acid and aspirin) and 150.95 (3-methylsalicylic acid, internal standard).

In silico docking studies

The structures of ligands were built using Sybyl (Tripos), where energy minimization
was also performed. Three-dimensional coordinates of the X-ray crystal structure of the ovine PGHS-iodoindomethacin complex at a resolution of 4.5 Å were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB, code 1PGG). Docking was performed with the AutoDock program (Version 4.0; Scripps Research Institute). Five independent docking runs were carried out for each ligand. The active site space was defined with AutoGrid program (Version 4.0; Scripps Research Institute), which pre-calculated grids of van der Waals, hydrogen bonding, electrostatics, torsion, and solvation interactions between PGHS and ligands. A grid box with sufficient space to cover the whole arachidonic acid-binding channel of PGHS was defined with dimensions of 40×40×40 and a resolution of 0.375 Å in each dimension. Lamarckian genetic algorithm (LGA) in AutoDock was chosen to search the globally optimized conformations and orientations for ligands. The maximum number of energy evaluations and generations were set at 25,000,000 and 27,000, respectively. The population size was set at 300. During the docking simulation, all atoms of the ligands were allowed to move while the protein structure was fixed. For each ligand, 100 LGA runs were conducted and followed by a cluster analysis with an RMSD tolerance of 2.0 Å. The conformations with the lowest docking energy were selected for further binding mode analysis.

**Analysis of interacting data**

**Single drug inhibition**
Observed values were expressed as percent of changes in light transmission. The inhibitory concentration-effect relationship was modeled with the sigmoid $I_{max}$ model (Eq. 2):

$$1 = \frac{C}{IC_{50} \cdot \left( \frac{E - Back}{S_{max} - E} \right)^{-1/\gamma}}$$  
(Equation 2)

The $S_{max}$ was the maximum platelet aggregation of vehicle-treated PRP, while the back was defined as vehicle-treated PRP without AA challenge. The measured effects, $E$, was the percent of changes in light transmission at drug concentration. The maximal inhibitory effect ($I_{max}$) was equal to $(S_{max} - \text{back})$. IC$_{50}$ represented the concentration producing 50% of $I_{max}$ and the slope of the concentration-effect relationship was given by $\gamma$.

**Double drug interaction with the isobologram and URSA methods**

Combination data were analyzed with the isobologram and the URSA methods. The URSA method applied to the double interactions between Pro, CA, or aspirin was shown in equation 3.

$$1 = \frac{C_{Pro}}{IC_{50,Pro} \cdot \left( \frac{E - Back_{Pro}}{S_{max} - E} \right)^{-1/\gamma_{Pro}}} + \frac{C_{As}}{IC_{50,As} \cdot \left( \frac{E - Back_{As}}{S_{max} - E} \right)^{-1/\gamma_{As}}}$$

$$+ \alpha_{Pro,As} \cdot \frac{C_{Pro}}{IC_{50,Pro} \cdot \left( \frac{E - Back_{Pro}}{S_{max} - E} \right)^{-1/2/\gamma_{Pro}}} \cdot \frac{C_{As}}{IC_{50,As} \cdot \left( \frac{E - Back_{As}}{S_{max} - E} \right)^{-1/2/\gamma_{As}}}$$  
(Equation 3)

Where $\alpha$ was defined as an interaction parameter, and other parameters have been defined in the sigmoid $I_{max}$ model. If $\alpha$ was equal to zero, the interaction mode was
proposed as additive; when $\alpha$ was positive, synergism was indicated, while when $\alpha$ was negative, antagonism was suggested. The interaction intensity can be directly deduced from the $\alpha$ value.
**Fig. 1S** Crystal structure of COX-1. Close-up view of the binding mode of Pro (A) and CA (B) on COX-1. Atom type code of the ligands: carbon, yellow; oxygen, red; hydrogen, white. Atom type code of the amino residues: carbon, pink; oxygen, red; nitrogen, blue. Dotted lines represent hydrogen bonds.