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Magnetic ligand fishing combination with high-performance liquid chromatography–diode array detector–mass spectrometry to screen and characterize cyclooxygenase-2 inhibitors from green tea

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ABSTRACT

Cyclooxygenase-2 (COX-2) inhibitors may be used to efficiently treat inflammation or cancer diseases. In the present study, we established a new screening assay based on magnetic Fe_3O_4@SiO_2–COX-2 ligand fishing combination with high-performance liquid chromatography–diode array detector–mass spectrometry (HPLC–DAD–MS^n) to screen and identify COX-2 inhibitors from green tea. Optimized conditions (pH at 7.4, temperature at 30 °C, and incubation time for 30 min) for fishing out COX-2 inhibitors were achieved by testing positive control, celecoxib, with active and inactive COX-2. Notably, immobilized COX-2 showed high stability (remained 94.7% after ten consecutive cycles), reproducibility (RSD < 10% for batch-to-batch evaluation). Finally, eight catechins with COX-2 binding activity were screened in green tea, and their structures were characterized by ultraviolet (UV), accurate molecular weight, diagnostic fragment ions and nuclear magnetic resonance (NMR). Particularly, the COX-2 inhibitory activities of two rare catechins, [(−)-epigallocatechin-3-(3″-*O*-methyl)-gallate (3″-*O*-methyl-EGCG, IC_{50} = 0.17 ± 0.03 μM 0.16 ± 0.01), (−)-epicatechin-3-(3″-*O*-methyl)-gallate (3″-*O*-methyl-ECG, IC_{50} = 0.16 ± 0.02 μM)], were reported for the first time. The results indicated that the proposed method was a simple, robust and reproducible approach for the discovery of COX-2 inhibitors from complex matrix.

1. Introduction

Cyclooxygenase-2 (COX-2), an inducible COX isozyme rapidly in response to cytokines, growth factors and tumor promoters, is dramatically up-regulated during pathological conditions such as inflammation and cancers [1,2]. Its inhibition has been more directly implicated in reducing the extent of polyposis in patients with familial adenomatous polyposis [3], inducing apoptosis in colon, stomach and prostate cancer in human [2,4,5]. Therefore, COX-2 has become an important drug target for the discovery and development of anti-inflammatory or anti-tumor drugs. Up to now, great varieties of components with effective COX-2 inhibitory activity have been designed and synthesized [6–8]. Moreover, investigations on both animal models and human clinical trials have led to the hypothesis that synthesized selective COX-2 inhibitors might be endowed with better anti-inflammatory activity with fewer gastrointestinal side effects than nonselective classical nonsteroidal anti-inflammatory drugs [6]. Unfortunately, cardiovascular adverse effects with some selective COX-2 inhibitors (e.g. coxibs) have ultimately prompted them withdrawal from the market [9,10]. Therefore, the development of selective COX-2 inhibitors with improved safety profiles is the need of hour.

Newman and Cragg have reported that about 50% of all the marketed-new chemical entities were shown to be of natural origin during 1981–2010 [11]. Up to now, many natural products, such as *Krameria pauciflora*, *Radix aconiti*, *Curcuma longa*, *Terminalia bellerica* and *Camellia sinensis* (tea leaves), have been reported to exhibit significant COX-2 inhibition [12–15]. However, most reports focus on the COX-2 inhibitory activity of crude extracts or commercially isolated compounds [16]. For example, catechins, the main compounds in nonfermented green tea, have diverse pharmacological properties (i.e. antioxidative, antiatherosclerotic, anticarcinogenic, hypocholesterolaemic, antihypertensive, antiallergic, antimicrobial, and COX-2 inhibitory properties [17–19]), and

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the beneficial effects of catechins have increasingly stimulated the usage of green tea as food and cosmetic additives. However, no biochemical profile of catechins in green tea for COX-2 inhibition has been previously reported, and only commercial catechins have been investigated. Traditional bioassay-guided fractionation of natural products is a time-consuming, labor intensive and expensive strategy, which sometimes leads to the loss of activity because of the dilution or decomposition during the isolation and preparation process [20]. Therefore, the key step in natural product research is to develop a selective screening assay to reduce time, cost and the incidence of false positives/negatives.

Affinity based screening assay, depending on the macromolecular target–ligands binding, has been considered as the most convenient and efficient method to fish out potential ligands from complex matrix. Powerful approaches are elimination of non-binders from macromolecular target–ligands using centrifugation [21], ultrafiltration [22,23], equilibrium dialysis [24], microdialysis [25], magnetic beads [26–28]. Among which, magnetic ligand fishing has advantages of stable immobilized macromolecular targets and easy magnetic isolation [29], which have been successfully used to fish out ligands for HSA/BSA [26,30,31], acetylcholinesterase [32], acetylcholine [33], α-amylase [34], xanthine oxidase [35], COX-1 [36], maltase [37], SIRT6 [38,39], etc. To the best of our knowledge, no screening assay based on magnetic ligand fishing for COX-2 inhibitors has been previously reported.

In view of in continuation of an ongoing efforts aiming at rapidly and efficiently finding bioactive components from complex natural products [26,35,36], we report proof of principle for the first time of integration of magnetic ligand fishing and HPLC–DAD–MS^n for facile, specific screening and elucidation of COX-2 inhibitors from green tea. Six common catechins [(−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epigallocatechin-3-gallate (EGCG), (−)-gallocatechin-3-gallate (GCG), (−)-epicatechin-3-gallate (ECG)] and two rare catechins [(−)-epigallocatechin-3-(3″-*O*-methyl)-gallate (3″-*O*-methyl-EGCG), (−)-epicatechin-3-(3″-*O*-methyl)-gallate (3″-*O*-methyl-ECG)] were screened and identified, and their COX-2 inhibitory activities were evaluated.

2. Experimental

2.1. Chemicals and reagents

Human recombinant COX-2, arachidonic acid, prostaglandin E_2 (PGE_2), d_4-PGE_2, 3-aminopropyltriethoxysilane (APTES), tetraethyl orthosilicate (TEOS), and glutaraldehyde (25% (w/v) aqueous solution) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol and acetic acid (HPLC grade) were bought from Tedia Company Inc. (OH, USA). Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system from Millipore (Bedford, MA, USA). Phosphate buffer solution (10 mM, pH 7.4) was selected. Reference standards (purities > 99%), GC, EGC, EC, EGCG, GCG, ECG, and positive control sample, celecoxib, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Yunwu green tea (*C. sinensis*) was obtained from Tea Research Institute of Hunan province, China, and authenticated by Prof. Mijun Peng, Key Laboratory of Hunan Forest Products and Chemical Industry Engineering, Jishou University, Zhangjiajie, China.

2.2. Instrumental

Transmission electron microscopy (TEM) (JEM-2100F, JEOL, Japan) was used to observe morphology of microspheres. The FT-IR spectra (4000–400 cm^{-1}) were obtained via a Nicolet 6700 FT-IR spectrometer (Thermo Nicolet Co., Waltham, MA, USA). The magnetic property was measured at room temperature using vibration sample magnetometer (VSM7407, Lakeshore, USA). ^{1}H NMR experiments were performed on a VARIAN INOVA-400 (Varian Corporation, USA) NMR spectrometer.

Chromatographic separation was performed on an analytical SunFire™ C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Waters, Milford, MA) in tandem with a Phenomenex C_{18} guard cartridge (4.0 mm × 3.0 mm, Phenomenex, Torrance, CA). The eluent was delivered from an Agilent 1260 HPLC system and diode array detector system. The mobile phase for green tea extract analysis was consisted of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile) with a 60 min linear gradient elution from 4% to 25% B at a flow rate of 1.0 ml/min at 25 °C, while the chromatograms were monitored at 275 nm and collected by Agilent ChemStation software. Isocratic mobile phase consisted of water–methanol (15:85, v/v) with a flow rate of 0.8 ml/min was prepared for celecoxib analysis at 25 °C, and the chromatograms were acquired at 254 nm. All the mobile phases were prepared daily.

The high-resolution MS and MS^n experiments were performed on a Thermo Scientific Exactive orbitrap mass spectrometer with negative ion electrospray interfaced to Thermo Accela LC system (San Jose, CA, USA). HPLC conditions were the same as above mentioned. Nitrogen sheath gas was set at 50 arbitrary units and auxiliary gas at 10 arbitrary units. The instrument was operated in full-scan mode from *m*/*z* 100 to 800 with a resolution of 50,000. The ion source parameters included heated capillary temperature at 300 °C, capillary voltage and spray voltage at −52 V and −3.5 kV. An additional collision cell for high energy collision induced decomposition (HCD) experiments was incorporated, while the collection of HCD data used a fixed energy setting at 30 V.

2.3. COX-2 inhibition assay

COX-2 inhibitory assays were performed according to a previously described PGE_2 (a stable oxidation product resulting from COX-2 oxidation of arachidonic acid) detection method [40]. Briefly, hematin (100 μM, 2 μl) mixed with L-epinephrine (40 mM, 10 μl) and made up with buffer solution to a final volume of 150 μl. Then COX-2 solution (20 μl, 0.2 μg) was added and incubated at 30 °C for 2 min. After that, six different concentrations of COX-2 ligands (20 μl) from 0 μM to 100 μM were added and preincubated at 30 °C for 30 min. The COX-2 inhibition reaction was initiated by adding arachidonic acid (50 μM, 20 μl) and terminated by adding HCl (2.0 M, 20 μl). The concentration of product PGE_2 was detected by HPLC–MS/MS method by using [d_4]-PGE_2 as surrogate standard. The inhibitory activity was determined by comparing the amount of PGE_2 produced with that of a negative control (buffer solution). The COX-2 inhibitory activity was expressed as the half maximal inhibitory concentration (IC_{50}).

2.4. Preparation of Fe_3O_4@SiO_2–COX-2 nanoparticles

COX-2 was immobilized onto Fe_3O_4@SiO_2 solid support by a typical glutaraldehyde activation procedure as shown in Supplementary Scheme S1 [26]. Typically, $FeCl_3 \cdot 6H_2O$ (1.35 g), sodium acetate (3.60 g) and polyethylene glycol (1.00 g) were dissolved in ethylene glycol (40 ml), and the mixtures were stirred at room temperature for 60 min and heated at 200 °C in a teflon lined stainless steel autoclave for 8 h to prepare Fe_3O_4 nanoparticles (0.37 g). Then, Fe_3O_4 nanoparticles (120 mg) were dispersed in the mixture of ethanol (476 ml), water (139 ml) and ammonia aqueous solution (28 wt.%, 15 ml), followed by the addition of TEOS (6 ml). After stirring at room temperature for 8 h, Fe_3O_4@SiO_2 nanoparticles (140 mg) were achieved after being washed with water three times and dried in a vacuum oven at 50 °C. Fe_3O_4@SiO_2 nanoparticles