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Evidence that diclofenac and celecoxib are thyroid hormone receptor beta antagonists

Mire Zloh, Noelia Perez-Diaz, Leslie Tang, Pryank Patel, Louise S. Mackenzie *

Life and Medical Sciences, University of Hertfordshire, Hatfield AL10 9AB, UK

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ABSTRACT

Long term use of NSAIDs is linked to side effects such as gastric bleeding and myocardial infarction.
Aims: Use of in silico methods and pharmacology to investigate the potential for NSAIDs diclofenac, celecoxib and naproxen to bind to nuclear receptors.
Materials and methods: In silico screening predicted that both diclofenac and celecoxib has the potential to bind to a number of different nuclear receptors; docking analysis confirmed a theoretical ability for diclofenac and celecoxib but not naproxen to bind to TRβ.
Key findings: Results from TRβ luciferase reporter assays confirmed that both diclofenac and celecoxib display TRβ antagonistic properties; celecoxib, IC_{50} 3.6×10^{-6} M, and diclofenac IC_{50} 5.3×10^{-6} M, comparable to the TRβ antagonist MLS (IC_{50} 3.1×10^{-6} M). In contrast naproxen, a cardio-sparing NSAID, lacked TRβ antagonist effects. In order to determine the effects of NSAIDs in whole organ in vitro, we used isometric wire myography to measure the changes to Triiodothyronine (T3) induced vasodilation of rat mesenteric arteries. Incubation of arteries in the presence of the TRβ antagonist MLS000389544 (10^{-5} M), as well as diclofenac (10^{-5} M) and celecoxib (10^{-5} M) but not naproxen significantly inhibited T3 induced vasodilation compared to controls.
Significance: These results highlight the benefits of computational chemistry methods used to retrospectively analyse well known drugs for side effects. Using in silico and in vitro methods we have shown that both celecoxib and diclofenac but not naproxen exhibit off-target TRβ antagonist behaviour, which may be linked to their detrimental side effects.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDS) inhibit cyclooxygenase (COX), the enzymes that are responsible for prostaglandin production [1]. There are two isoforms, COX-1 which is constitutively expressed, and COX-2 which is inducible. NSAIDS are widely used for their analgesic, antipyretic and anti-inflammatory properties however despite their therapeutic effectiveness, their use has been widely scrutinized due to their tendency to produce side effects. Since prostaglandins protect the gastrointestinal tract and are important in platelet aggregation, NSAID reduction of prostanoid production increases the risk of gastrointestinal ulceration and bleeds. Due to the toxic effects of NSAIDs such as diclofenac on gastrointestinal mucosa, COX-2 selective drugs such as celecoxib were developed. Clinical trials revealed the side effects of both pan- and COX-1 sparing NSAIDs led to gastrointestinal damage and cardiovascular complications including myocardial infarction [2,3].

There are currently two conflicting models that explain the cardiovascular side effects of NSAIDs. The first model put forward by Cheng et al. states that under normal physiological conditions endothelial COX-2 drives the production of prostacyclins whilst platelet COX-1 drives the production of thromboxanes [4]. The model predicts that a balance between pro-thrombotic and antithrombotic state exist under normal physiological conditions. However, when an NSAID which inhibits COX-2 in endothelial cells is introduced, the balance is disrupted and a pro-thrombotic state develops [4].

Recent evidence has emerged that provides evidence that COX-2 is not expressed in endothelial cells [5,6], but is highly expressed in the renal medulla [7], indicating a need for a new model for what causes NSAID induced side effects to be developed. Loss or inhibition of COX-2 in mice and man leads to an increase in the production of endogenous eNOS inhibitor, asymmetric dimethyl arginine (ADMA) which suggests that specific pathways are altered by COX-2 inhibition [7].

While much debate about the side effects of NSAIDs has concentrated on the direct effects of NSAIDs on COX activity, we investigated the indirect side effects of celecoxib and diclofenac using computational chemistry methods. In silico modelling indicated a potential for both drugs to associate with thyroid hormone receptor β (TRβ), and further analysis using in vitro methods indicate that both celecoxib and diclofenac possess TRβ antagonistic properties. This nuclear receptor is of great interest, with clear relationships between hypothyroidism

* Corresponding author at: School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK.
E-mail address: l.mackenzie2@herts.ac.uk (L.S. Mackenzie).

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associated with increased heart muscle stiffness and an increased risk of myocardial infarction [8].

2. Materials and methods

2.1. In silico methods

Open Virtual ToxLab .5,21 [9] was used to predict toxic potential by predicting binding affinities to 10 off-target nuclear receptors, 4 cytochrome P450 enzymes, a transcription factor and a potassium ion channel and forecast endocrine and metabolic disruption, some aspects of carcinogenicity and cardiotoxicity. The default values of the software for the predictions of toxic potentials for diclofenac and celecoxib were used as described previously [9].

The Pharmmapper, freely available web server (http://59.78.96.61/pharmmapper), was used to predict potential target candidates for both drugs. The mol2 files for two molecules were submitted to the Pharmmapper server by using default settings and limiting the target set to human targets [10].

The shape and electrostatic similarity of the diclofenac and celecoxib to ligands of the thyroid hormone receptor was explored using vROCS [11,12] and EON [13,14] software packages. ROCS was used to align the three dimensional alignment of the drug conformers generated by OMEGA [15–17] with the ligands extracted from the crystal structures of the thyroid hormone receptor beta (PDB entries: 1Q4X, 1NQ1 and 2J4a), followed by calculation of electrostatic similarity score (ET_combo) using EON.

The ability of drugs to bind into TRβ active site was investigated using Glide (Small-molecule Drug Discovery Suite 2014-3: Glide, Version 6.4 Schrödinger, LLC, New York, NY (2014) with Maestro as a graphical user interface. The protein preparation wizard was utilized to adjust charges and protonation states of above mentioned protein data bank entries, as well as to correct problems with proteins target structures. Prepared protein structures were used to build energy grids with enclosing boxes of default sizes centred on co-crystalized ligands. The drug molecules, diclofenac and celecoxib, were docked flexibly using XP docking protocol; ligands were minimized onto OLSA-2005 non-bonded interaction grid, with all other parameters set to their default values.

2.2. TRβ reporter assays

Human TRβ reporter assay system was purchased from INDIGO Bioscience (State College, PA). Assays were performed according to the manufacturer's instructions for both agonist and antagonist activity. Briefly, TRβ reporter cells were dispensed into the wells of the assay plate and immediately dosed with L-triiodothyronine (T3), celecoxib, diclofenac and naproxen. Following 24 h incubation at 37 °C, treatment media was discarded and the Luciferase Detection Reagent added. Light emission from each sample well was quantified using a plate reading luminometer. In order to assess TRβ antagonistic activity the protocol was adjusted; TRβ Reporter Cells were exposed to a sub-maximal concentration of T3 (100 nM) while plating cells, prior to addition of naproxen, celecoxib and diclofenac to the wells.

2.3. Myography

Male Wistar rats (350–450 g) were housed in pairs, and killed by CO_2 asphyxiation. The care and use of the rats were carried out in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act of 1986 under PPL70/7732.

Mesenteric arteries were removed and prepared as described previously [18]. Briefly, artery segments were dissected in Krebs buffer (pH 7.4, NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25.0 mM and glucose 11.0 mM), and loaded onto isometric wire myographs. The bath solution was continuously bubbled with 95% O_2 and 5% CO_2. All vessels were allowed to equilibrate for 30 min prior to being set at a 'normalized' internal circumference $0.9.L_{100}$ estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100 mm Hg transmural pressure. This was calculated for each individual vessel on the basis of passive length-tension characteristics of the artery and the Laplace relationship [19].

Arteries were incubated with a thyroid hormone receptor antagonist MLS000389544 (10^{-5} M; MLS), 10^{-5} M diclofenac, 10^{-5} M celecoxib or 10^{-5} M naproxen for 30 min prior to addition of increasing concentrations of the thromboxane A_2 (TP) receptor agonist 9,11-Dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619; 10^{-9} M to 10^{-6} M). Arteries were washed four times with Krebs, and once tone had returned to basal levels, arteries were incubated with MLS, celecoxib and diclofenac for further 15 min. Arteries were then pre-contracted with 3×10^{-7} M U46619; once plateau was achieved, vasodilation in response to increasing concentrations of L-triiodothyronine (10^{-10} to 3×10^{-7} M; T3) was measured.

2.4. Materials

All chemicals and reagents were obtained from Sigma Aldrich unless otherwise stated. Drugs were dissolved in water, except for U46619, celecoxib and diclofenac which were dissolved in DMSO up to 10^{-2} M, and then water for further dilutions.

3. Results

3.1. In silico modelling

Using VirtualTox screening programme, structures for diclofenac and celecoxib were assessed for the potential binding to a series of target protein known to be correlated with the side effects, and a normalized toxicity potential was calculated (Table 1). The results suggest that both drugs can potentially bind all nuclear receptors, albeit with various affinities. Both drugs exhibited no affinity with CYP enzymes, arylhydrocarbon (AhR), and **h**uman ***E**ther-à-go-go-***R**elated **G**ene (hERG K). The overall predicted toxicity potentials were 0.56 and 0.57 for diclofenac and celecoxib respectively. These values indicate that they have potential to induce side effects to similar extent as chlomazone and bisphenol B.

The results indicated that the initial assumption that NSAIDs may interact with nuclear receptors was correct and warranted further investigation. The potential targets were looked at in light of side effects, and as a proof of principle we have taken TRβ for further consideration. The predicted binding affinities of drugs to this protein were not the most favourable, but were still in nano molar range. A possibility that

Table 1
Prediction model; data shows the potential binding of celecoxib and diclofenac to 10 nuclear receptors; androgen receptor (AR), oestrogen receptor α (ERα), oestrogen receptor β (ER β), glucocorticoid receptor (GR), liver X receptor (LXR), mineralocorticoid (MR), PPARγ, progesterone (PR), thyroid hormone α receptor (TRα) and thyroid hormone receptor β (TRβ). Binding potential is indicated by molar concentration and ToxPot is a measure of a toxic potential, a normalized binding affinities in respect to series of protein models with known adverse effects.

Protein	Diclofenac	Celecoxib
AR	8.62×10^{-6}	4.14×10^{-6}
ERα	1.31×10^{-5}	6.22×10^{-8}
ERβ	5.70×10^{-5}	1.64×10^{-7}
GR	4.12×10^{-8}	5.42×10^{-6}
LXR	1.79×10^{-7}	1.58×10^{-7}
MR	2.47×10^{-6}	4.21×10^{-8}
PPARγ	2.96×10^{-8}	6.14×10^{-8}
PR	4.96×10^{-7}	8.57×10^{-7}
TRα	1.80×10^{-7}	3.59×10^{-6}
TRβ	1.05×10^{-7}	3.24×10^{-7}