Evidence that Diclofenac and Celecoxib are thyroid hormone receptor beta antagonists

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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 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 6.4Schrödinger, 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 hour 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 (100nM) while plating cells, prior to addition of naproxen, celecoxib and diclofenac to the wells.

2.3 Myography

Male Wistar rats (350-450g) 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 118mM, KCl 4.7mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $CaCl_2$ 2.5mM, $NaHCO_3$ 25.0mM and glucose 11.0mM), 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 minutes prior to being set at a 'normalised' internal circumference $0.9.L_{100}$ estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100mmHg 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 homrone receptor antagonist MLS000389544 (10^{-5}M; MLS), 10^{-5}M diclofenac, 10^{-5}M celecoxib or 10^{-5}M naproxen for 30 minutes 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 minutes. Arteries were then pre-contracted with 3×10^{-7}M U46619; once plataeu was acheived, vasodilation in response to increasing concetrations of L-Triiodothyronine (10^{-10} to 3×10^{-7}M) 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 potenital 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 *Ether-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.

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}

Table 1. Prediction model; data shows the potential binding of celecoxib and diclofenac to 10 nuclear receptors; Androgen Receptor (AR), Estrogen receptor α (ERα), Estrogen receptor β (ER β), Glucocorticoid receptor (GR), Liver X receptor (LXR), Mineralcorticoid (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.

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 diclofenac and celecoxib could bind to this receptor was further investigated by using the reverse docking platform Pharmmapper. Diclofenac and celecoxib structures were submitted to the webserver and the ability to bind to TRβ was demonstrated as a list of possible protein data bank files in whose active sites these two drugs could favourably bind (Table 2).

Drug	Pharma Model	Num Feature	Fit	Norm Fit	Num Hydrophobic	Num HB Acceptor	Num HB Donor	Num +ve	Num -ve	Num Aromatic
Diclo	2j4a_v	6	3.20	0.533	4	1	0	0	1	0
Diclo	1nq1_v	7	3.34	0.477	4	1	1	0	1	0
Diclo	1nax_v	7	3.08	0.440	4	1	1	0	1	0
Diclo	1nq2_v	7	3.01	0.429	4	1	1	0	1	0
Diclo	1nq0_v	7	2.99	0.428	4	1	1	0	1	0
Diclo	1r6g_v	8	3.26	0.408	5	1	1	0	1	0
Diclo	1q4x_v	9	4.01	0.445	6	2	0	0	1	0
Cele	1q4x_v	9	2.96	0.329	6	2	0	0	1	0

Table 2. The ranked list of hit target pharmacophore models for diclofenac (Diclo) and celecoxib (Cele) obtained using Pharmmapper webserver. The list is sorted by fit score and filtered to show diclofenac and celecoxib hits for TRβ.

Although initial analysis indicates that the celecoxib should have lower probability to bind to TRβ, the celecoxib has considerable shape similarity to several TRβ ligands, with highest similarity to ligand extracted from PDB entry 1NQ1 ([4-(4-hydroxy-3-iodo-phenoxy)-3,5-diiodo-phenyl]-acetic acid) with Tanimoto Combo index of 0.68 and EON_ShapeTanimoto index of 0.54 (Figure 1), and high similarity to T3 with Tanimoto Combo index of 0.52 and EON_ShapeTanimoto index of 0.42.

The docking analysis was carried out using three TRβ crystal structure with three different ligands; diclofenac (Figure 2A) could bind to active sites of all three structures, while celecoxib (Figure 2B) could bind only into active site of 1Q4X PDB entry, as the ligand of this complex ([4-(3-benzyl-4-hydroxybenzyl)-3,5-dimethylphenoxy]acetic acid is in a similar size as celecoxib (Figure 2C). This observation indicates that the flexibility of the protein and induced fit has to be taken into account. The two NSAIDs, diclofenac and celecoxib, share similar interaction patterns with TRβ binding site, specifically with residues I276, A317, M313, L330, L346. Furthermore R282 and N331 are involved in hydrogen bonding formation with carboxylic and sulphonamide groups of diclofenac and celecoxib respectively. On the contrary, naproxen has fewer interactions with residues due to its

smaller size and planar shape. Importantly, its carboxylic group forms hydrogen bonds with R300 rather than R22 and N313, that could be one of the main reason for the absence of the antagonistic activity against TRβ.
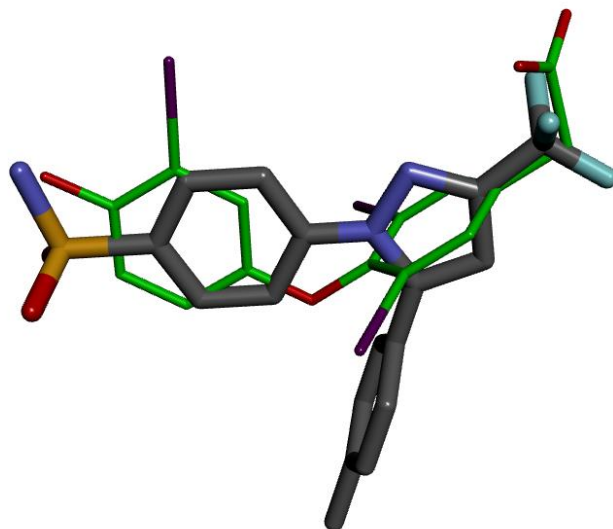

Figure 1. Overlay of the celecoxib (depicted with thick bonds with grey carbon-carbon bonds) and a ligand extracted from the PDB entry 1NQ1 entry (depicted with thin green carbon- carbon bonds). Hydrogen atoms are not shown for clarity.
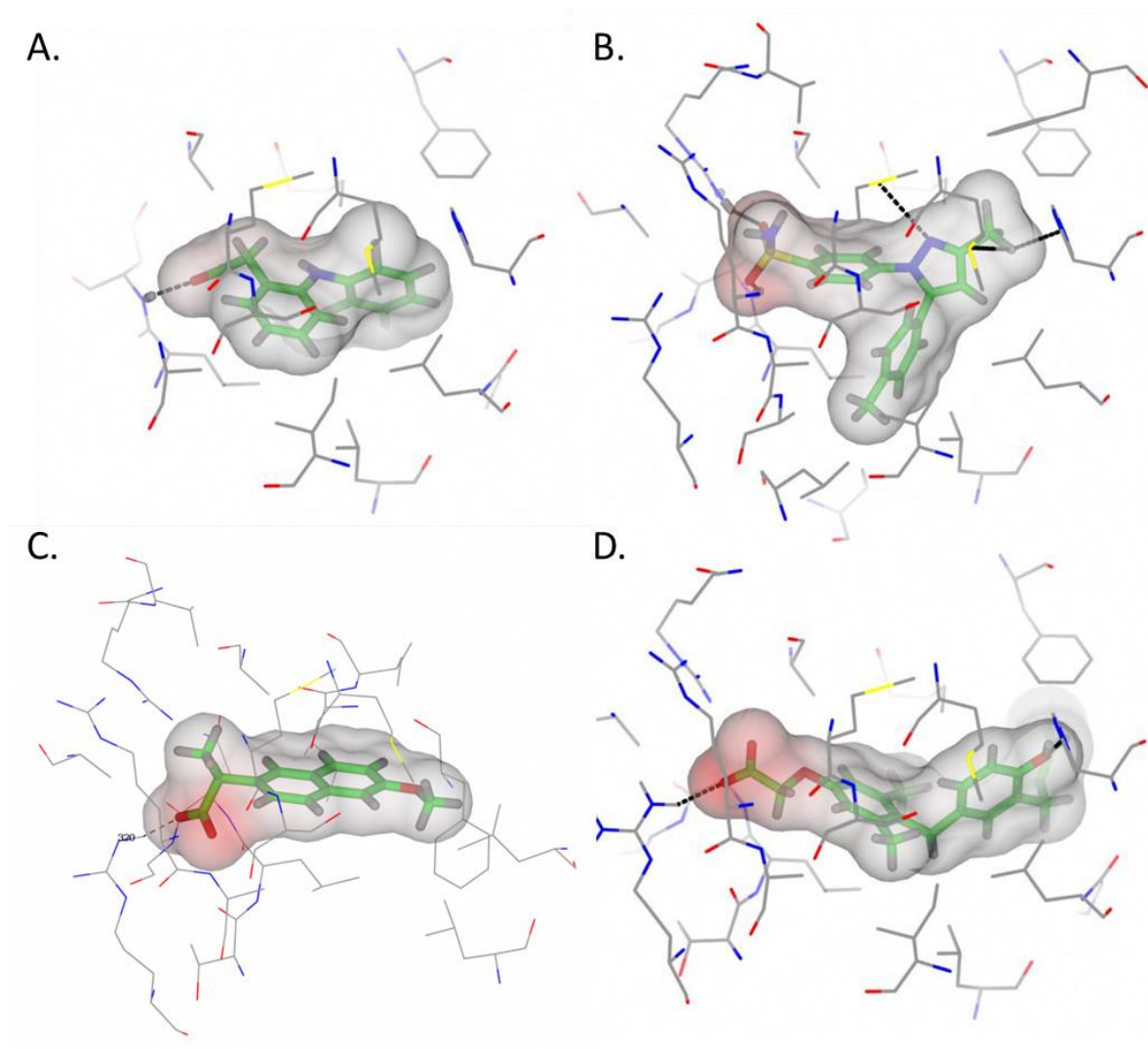
Figure 2. Docking analysis using three TRβ crystal structure with three different ligands; **A.** diclofenac; **B.** celecoxib; **C.** naproxen and **D.** original ligand present in 1Q4X. Surface shows active site of PDB 1Q4X entry; ligands are represented by ball-and-stick, side-chain residues of TRβ are represented by cylinders. Dashed lines represent hydrogen bond interactions to amino acid residues in TRβ. Transparent surfaces are coloured by electrostatic charges. Side-chain residues shown are within 4Å of bound ligand

3.2 TRβ Reporter assays

In silico data indicated a potential for celecoxib and diclofenac to bind to TRβ, although neither compound elicited agonist properties using the TRβ luciferase reporter assay (data not shown). Some reporter assays were run using an antagonist protocol, incubating reporter cells with 100nM Triiodothyronine (T3; a TRβ agonist) and then increasing concentrations of celecoxib and diclofenac. Celecoxib inhibited T3 induced TRβ luciferase activity with an IC_{50} 3.6×10^{-6}M, and diclofenac IC_{50} 5.3×10^{-6}M (Figure 3), comparable to the TRβ antagonist MLS (IC_{50} 3.1×10^{-6}M). In contrast naproxen lacked any effect on T3 reporter assay (Figure 3C).
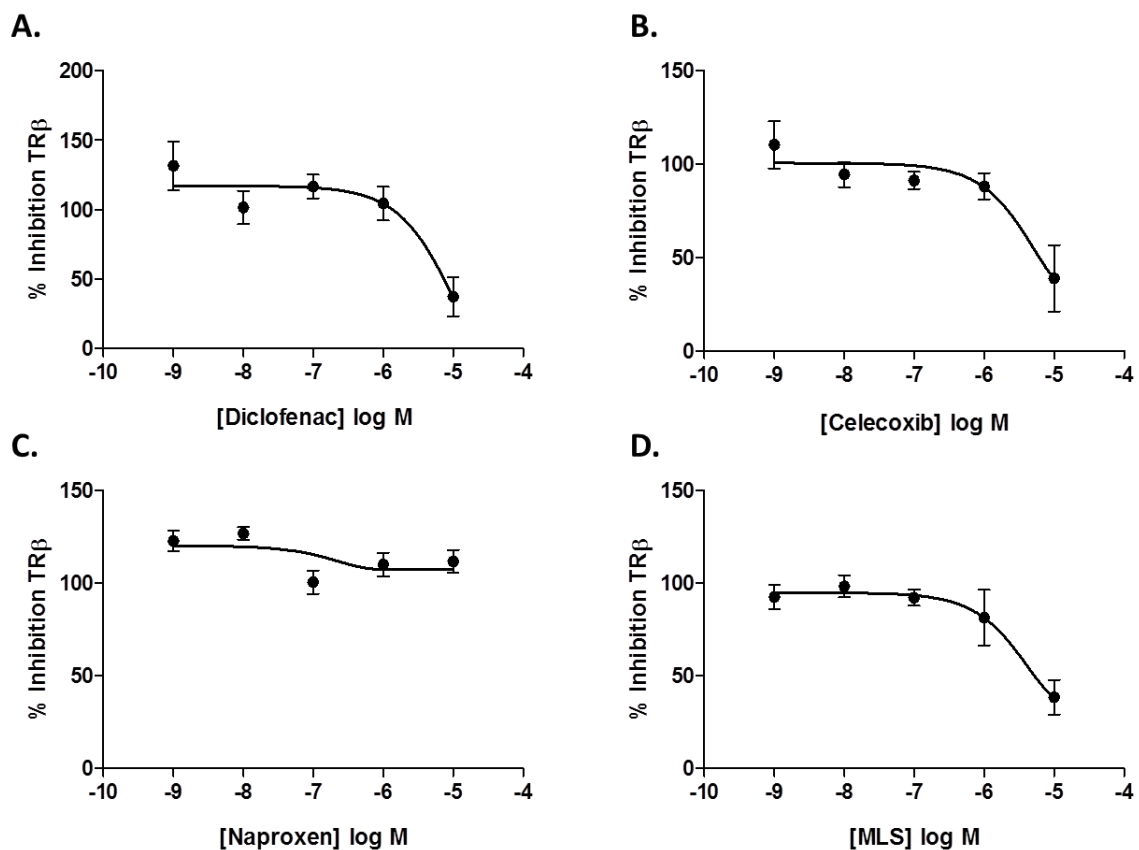

Figure 3. Luciferase reporter gene assay of TRβ antagonism. Reporter cells were activated with 100nM L-triiodothyronine (T3), and TRβ antagonist activity measured following incubation with increasing concentrations of **A.** Diclofenac, **B.** Celecoxib, **C.** Naproxen and **D.** MLS for 24 hours; n=6-9 from 2-5 separate experiments.

3.3 Myography

In order to determine whether celecoxib and diclofenac antagonism of TRβ has any effect in a whole organ system, we performed isometric wire myography of rat mesenteric arteries. T3 induces full vasodilation of mesenteric arteries pre-contracted with U46619 (Figure 4), which is significantly inhibited by pre-incubation with diclofenac (Figure 4A), celecoxib (Figure 4B) and the TRβ antagonist MLS (Figure 4 D); naproxen had no effect on T3 dilaiton (Figure 4C).

The presence of 10^{-5}M diclofenac significantly reduced U46619 mediated contraction (Figure 5A), in contrast to incubation with 10^{-5}M celecoxib or 10^{-5}M naproxen which significantly increased U46619 mediated contraction MLS has no effect on U46619 mediated contraction (Figure 5D).
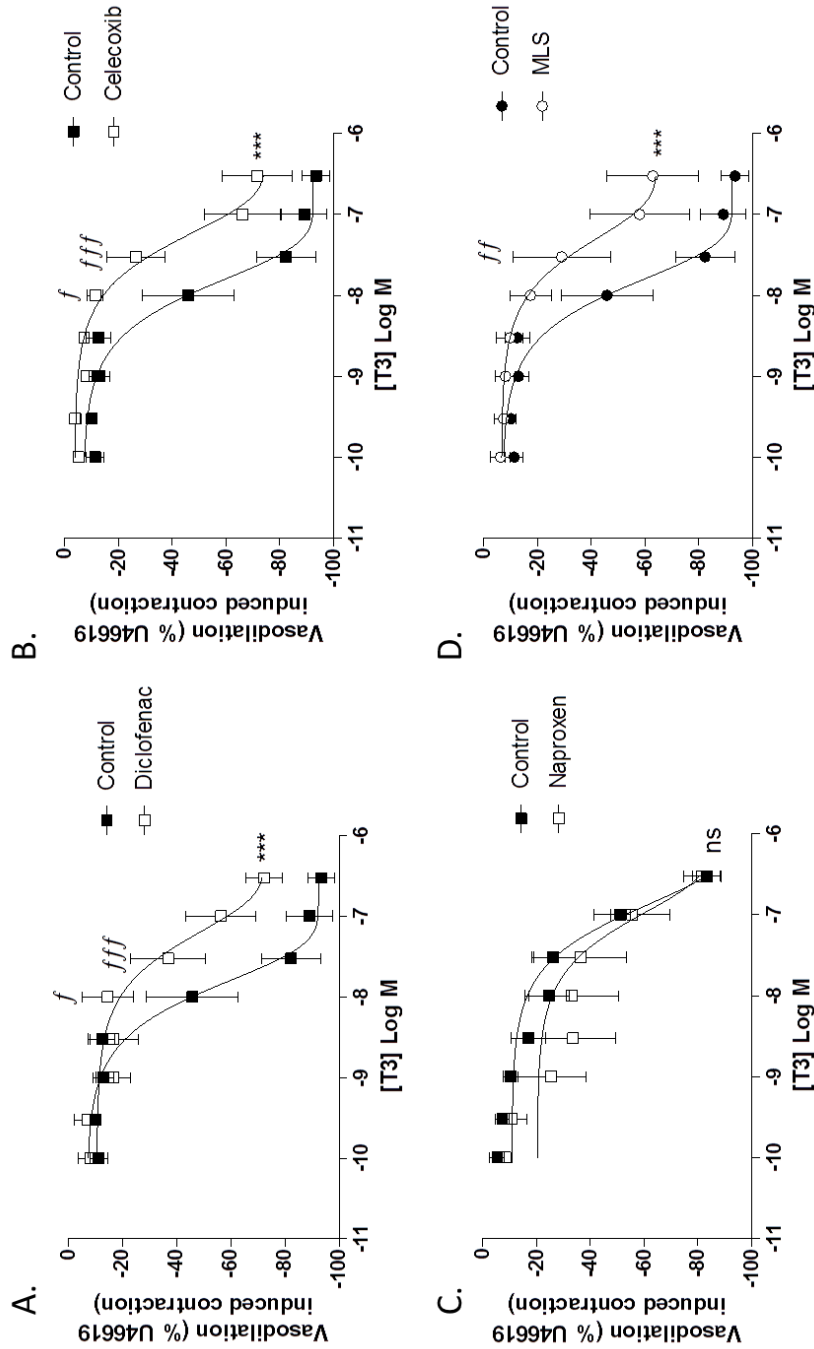
Figure 4 L-Triiodothyronine (T3) induced vasodilation in the presence of NSAIDs. Arteries were incubated with **A.** 10^{-5}M diclofenac, **B.** 10^{-5}M celecoxib, **C.** 10^{-5}M naproxen and **D.** 10^{-5}M MLS; following 30 minutes arteries were pre-contracted with EC_{80} U46619 (3×10^{-7}M) and dilation to T3 determined. Data is presented as mean ± SEM, and significance is represented as *** p=<0.001 by two way ANOVA and significance compared to control at each concentration by Bonferroni *post hoc* test *f* =p<0.05, *ff* =p<0.01 and *fff* =p<0.001.
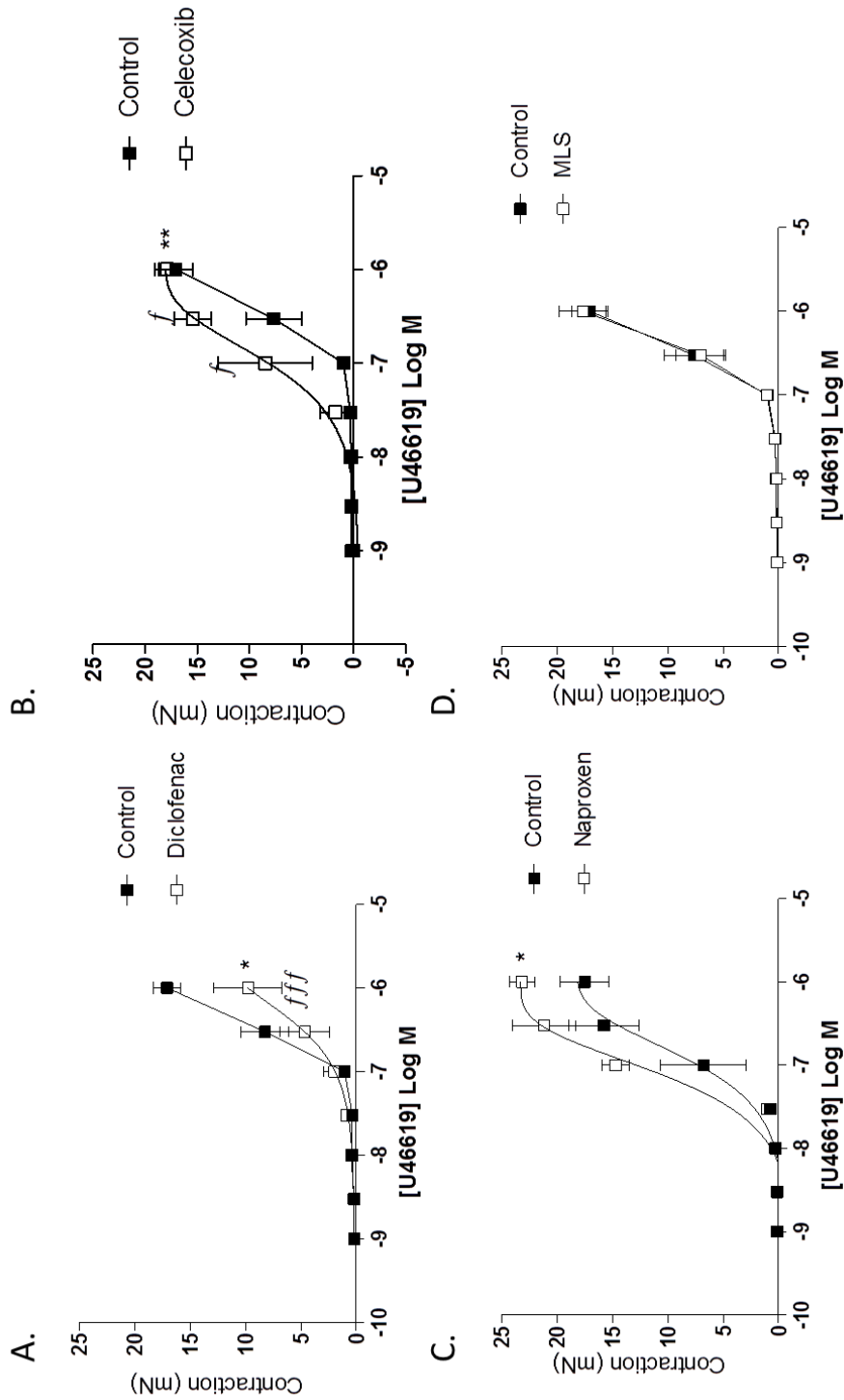
Figure 5 The effects of A. 10^{-5}M diclofenac, B. 10^{-5}M celecoxib, C. 10^{-5}M naproxen and D. 10^{-5}M MLS

on U46619 mediated contraction. Data is presented as mean ± SEM, and significance is represented

as *** p=<0.001 by two way ANOVA and significance compared to control at each concentration by

Bonferroni *post hoc* test *f* =p<0.05, *ff* =p<0.01 and *fff* =p<0.001.

4 Discussion

Our data is the first to demonstrate that T3 induces vasodilation of rat mesenteric arteries, which can be significantly reduced by the TRβ antagonist MLS. Moreover, diclofenac and celecoxib have the capacity to bind to and antagonise TRβ and TRα receptors.

Thyroid hormones have been shown to act directly on rat aortic artery smooth muscle cells [20], rat skeletal muscle resistance arteries[21] and rat mesenteric arteries[22] to induce vasodilation in a short time frame, indicating a non-genomic mechanism of action[23]. Reports vary in opinion on T3 mediated dilation, which has been shown to be endothelium independent [22], partially endothelium independent [21] or endothelial cells mediated via the interaction with eNOS [23]. The diversity of types of responses may be linked to the size of the artery[22] and role and location of the different isoforms of thyroid hormone receptors[23]. There are two main thyroid hormone receptors: TRα and TRβ, to which T3 binds. While TRβ was not found in human umbilical vein endothelial cells (HUVECs) or bovine aorta endothelial cells (BAECs), TRα was found to be highly expressed in HUVECs and BAECs and cross-couples to the phosphoinositol 3-kinase (PI3-kinase) pathway [23]. In arterial endothelial cells, activation of PI3 kinase leads to eNOS activation, which results in production of NO and thus induces full dilation. Other members of the steroid hormone superfamily have been shown to cross couple with the PI3-kinase/Akt pathway, including oestrogen [24], vitamin D [25], PPARβ/δ [26] and glucocorticoid receptors [27], indicating that non-genomic control of steroidal receptors play an important role in vascular homeostasis. In contrast, studies in rat arteries indicate that T3 mediates dilation in endothelium denuded mesenteric arteries[22], femoral arteries[22] and skeletal muscle resistance arteries[21], and is not significantly reduced by inhibition with antagonists of the nitric oxide pathway, potassium channels, L-Type calcium channels, calcium sensing receptor, G proteins or protein kinases, and so the actual underlying TRβ T3 dilation remains elusive[22].

The side effects of NSAIDs have been extensively investigated, and numerous models postulated to explain the direct effects of NSAIDs and link to cardiovascular complications. This study

has used *in silico* modelling to investigate whether some of the effects may also be attributed to off-target effects with nuclear receptors. Virtualtox screening returned several potential positive relationships, one of which is the interaction between NSAIDs and PPARγ which has previously been reported [28], and others which have so far not been investigated, such as a possible interaction between diclofenac and celecoxib with LXR. The nuclear receptor that was initially chosen and studied was TRβ due to two main reasons: 1) the predicted potency of binding was similar for diclofenac and celecoxib ($1.05x\ 10^{-7}$M diclofenac and $3.24x10^{-7}$M celecoxib) and 2) there are well established links between TRβ antagonism and cardiovascular complications.

As proof of concept we chose the potential interaction between celecoxib and diclofenac with TRβ, and show for the first time that both celecoxib and diclofenac have the potential to directly bind to and inactivate TRβ signalling. Previous reports have indicated a link between NSAIDs and the lowering of serum thyroid hormone concentrations [29], although this is the first report that indicates a potential for diclofenac and celecoxib to interfere with the TRβ receptor at physiological concentrations. Alterations in thyroid hormone activity is linked to cardiac function, in particular hypothyroidism leads to impaired systolic and diastolic function [30], as well as increased vascular stiffness and endothelial dysfunction [31], symptoms often associated with NSAID usage.

The normal physiological range of total T3 in the human plasma is between 1 to 2.9×10^{-9}M, concentrations which did not induce vasodilation in our study. However, a prominent feature of prolonged hyperthyroidism on the vascular system is a reduction in peripheral resistance, which may be due in part to the direct activity of T3 on vascular smooth muscle cell tone.

Further studies investigating the effects of longer term incubation of diclofenac and celecoxib on TRβ activity are required to fully understand whether the concentrations at which these drugs mediate TRβ antagonism in a meaningful way are required. In this study we used a concentration of 10^{-5}M diclofenac, naproxen and celecoxib, concentrations which are regularly used in previously published studies [32] and which are close to the maximum concentration (Cmax) ranges

found in human plasma. The concentration of diclofenac can reach 1347ng/ml in the plasma; diclofenac has 100% bioavailability and having a pKa 4.0 it is absorbed mostly in the stomach within 30 minutes [33]. The pharmacokinetic parameters of celecoxib dffer to diclofenac, having a greater tmax of 2.4 hours and Cmax 842μg/L [34], and naproxen reaches 60μg/ml in the plasma within 1.5 to 3 hours [35]. Not accounting for plasma binding, the equivalent molarity that is calculated for each NSAID would be 440μM diclofenac, 2.21μM celecoxib and 260μM naproxen in the plasma, concetrations a lot higher or lower than the 10μM used in this study.

T3 mediated dilation of rat mesenteric arteries is significantly inhibited by the thyroid hormone antaongonist MLS, as well as NSAIDs diclofenac and celecoxib. The chemical structure of celecoxib and diclofenac has similarities in shape, and differ in comparison to small, flat naproxen. Diclofenac and celecoxib shared binding profiles with other known TRβ binding molecules, and exhited TRβ antagonistic behaviours in the reporter assay as well as inhibiting T3 mediated vasodilation. These effects were not apparent with naproxen, which lacked effect on the TRβ reporter assay and had no effect on T3 mediated dilation. This is the first report to show the effects of diclofenac and celecoxib on T3 dilation, and raises insight in the NSAID structure required to avoid unwanted TRb antagonism.

MLS had no effect on U46619 mediated contraction, indicating that TRβ has no effect on TP receptor mediated contraction. On the other hand, diclofenac significantly reduced U46619 mediated contraction, and the NSAIDs celexoxib and naproxen significantly increased U46619 contraction, results which can be explained by the inhibition of contractile and dilatory prostanoids that contribute to U46619 contraction[36].

It is clear that the majority of side effects induced by long term usage of NSAIDs are due to the inhibition of downstream prostanoids, in particular prostacyclin. Not all of the effects can be attributed to direct changes to the prostanoids profile, and we demonstrate here that diclofenac and

celecoxib have the potential to bind to and inactivate THβ receptor. Our study indicates that on the short term this may have an effect on vascular homeostasis by altering the direct effects of T3 on vascular responses, and suggests that longer term effects akin to hypothyroidism are a potential risk from NSAID usage.

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5 Disclosure

The authors declare that there is no conflict of interest regarding the publication of this article