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# Pharmacological assessment of ibuprofen arginate on platelet aggregation and colon cancer cell killing

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, are amongst the most commonly used medications and produce their anti-inflammatory and analgesic benefits by blocking cyclo-oxygenase (COX)-2. These drugs also have the potential to prevent and treat cancer and some members of the class including ibuprofen can produce anti-platelet effects. Despite their utility, all NSAIDs are associated with increased risk of cardiovascular side effects which our recent work suggests could be mediated by increased levels of the endogenous NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) leading to reduced endothelial NOS activity and associated endothelial cell dysfunction. ADMA is a cardiotoxic hormone and biomarker of cardiovascular risk whose effects can be prevented by L-arginine. The ibuprofen salt, ibuprofen arginate (Spididol®) was created to increase drug solubility but we have previously established that it not only effectively blocks COX-2 but also provides an arginine source able to reverse the effects of ADMA *in vitro* and *in vivo*. Here we have gone on to explore whether the formulation of ibuprofen with arginine influences the potency and efficacy of the parent molecule using a range of simple *in vitro* assays designed to test the effects of NSAIDs on (i) platelet aggregation and (iii) colon cancer cell killing. Our findings demonstrate that ibuprofen arginate retains these key functional effects of NSAIDs with similar or increased potency compared to ibuprofen sodium, further illustrating the potential of ibuprofen arginate as an efficacious drug with the possibility of improved cardiovascular safety.



## 1. Introduction

Ibuprofen is a relatively old member of the nonsteroidal anti-inflammatory drug (NSAID) class of medications that all work by blocking cyclooxygenase (COX) enzymes. COX has two isoforms; a house-keeping form, COX-1, which is constitutively expressed throughout the body and COX-2, which is an inducible isoform rapidly expressed at the site of inflammation [1] and in cancer [2,3]. As such, COX-2 is the primary therapeutic target for NSAIDs in the treatment of pain, inflammation and cancer. However, COX-2 is also expressed constitutively in discreet regions of the body [4,5] where its inhibition by NSAIDs causes the much-reported cardiovascular side effects.

Initially the cardiovascular side effects caused by NSAIDs were thought to be associated with COX-2 selective NSAIDs [6], such as Vioxx (rofecoxib), Bextra (valdecoxib) and Celebrex (celecoxib), introduced in the early 2000s to spare the gastrointestinal side effects caused by older style drugs. However, since all NSAIDs work by blocking COX-2 [7], we and others suggested that cardiovascular toxicity is a class effect [8], an idea that is now universally accepted [9] and most recently fully corroborated by the publication of two large clinical cardiovascular outcome studies, SCOT [10] (Europe) and PRECISION [11] (USA), showing that traditional NSAIDs including ibuprofen, particularly at higher daily doses, carry at least

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as great a cardiovascular risk as COX-2 selective drugs including celecoxib. Despite this ibuprofen remains the only conventional NSAIDs listed in WHO Model List of Essential Medicines 2015 [12]. Clearly, then because ibuprofen usage is so firmly embedded within health care systems neither it's over the counter status nor its global availability will likely be affected by the reporting of side effects it causes. This remains a curious situation within the COX-2 field since cardiovascular side effects caused by other members of the NSAID class, particularly the COX-2 selective drugs, have been the subject of intense media coverage including, in 2004, the events surrounding the dramatic withdrawal of rofecoxib.

The mechanisms associated with the cardiovascular side effects caused by blocking COX-2 with NSAIDs are incompletely understood but are thought to involve the inhibition of cardio-protective prostanoids such as prostacyclin [7,13]. It is now clear that COX-1 is the dominant driver of prostacyclin production in the systemic circulation [4,14,15] including under conditions of low-grade inflammation such as occurs in atherosclerosis [16]. With this in mind attention is increasingly turning to the kidney [5,17], where COX-2 is constitutively expressed [5,17,18], to explain how NSAIDs cause cardiovascular side effects. This idea is supported by a new understanding of the origin of urinary metabolites of prostacyclin which are well established to be COX-2 driven and which our recent work shows can originate exclusively from the kidney [19]. Further to this our recent work shows that COX-2 in the kidney regulates plasma levels of asymmetric dimethylarginine (ADMA) [17]. ADMA is a well-recognised biomarker of cardiovascular risk that competes with L-arginine to inhibit the cardioprotective production of vascular NO via endothelial nitric oxide synthase (eNOS). As such the cardiovascular compromise caused by ADMA can be prevented by arginine supplementation.

Considering the popularity of ibuprofen and what we now know of its side effect profile, it is of particular importance to revisit what we understand of the mechanisms of action of this drug in all its formulations. Ibuprofen acid is relatively insoluble in aqueous solutions. To overcome this problem, by allowing for more rapid absorption from the stomach and hence faster pain relief, ibuprofen has been synthesised in a number of aqueous-soluble salt formulations. These include ibuprofen sodium and ibuprofen lysine, which are available worldwide, and ibuprofen arginate, which is available in South America, Italy, Spain and China but not in Northern Europe or North America. This latter formulation is particularly relevant as our recent work shows that following dissociation in aqueous solutions ibuprofen arginate effectively blocks COX-2 and, through the delivery of arginine, reverses eNOS compromise and endothelial dysfunction *in vitro* and *in vivo* [20]. At maximum recommended dosing ibuprofen arginate is predicted to deliver ≈2 g of arginine per day, which is sufficient according to one study, to cause beneficial cardiovascular effects in man [21]. Based on this study and on our preclinical observations we have suggested that ibuprofen arginate may have an improved cardiovascular profile compared to other preparations of the drug [20]. Whilst our bold claim needs to be tested in clinical studies the pharmacology of ibuprofen arginate compared to other formulations remains incompletely understood. Here in this study we have compared the potency and efficacy of ibuprofen sodium and ibuprofen arginate in two relevant *in vitro* pharmacological bioassays. Specifically we have investigated the effects of ibuprofen formulations on (i) inhibition of platelet aggregation and (ii) human colon cancer cell death.

## 2. Materials and methods

### 2.1. Materials

Zambon (Italy) provided ibuprofen arginate. Unless stated otherwise all other drugs were obtained from Sigma Aldrich, UK.

### 2.2. Solubility studies

Ibuprofen formulations (0.01–10 mM) were dissolved in 50 mM Tris buffer at pH 3 or pH 7 in 96-well plates. Solutions were incubated for 10 min at 37 °C before absorbance was measured at 570 nm using a spectrophotometer (Infinite®F50; Tecan, Switzerland).

### 2.3. Cell culture studies

The human epithelial colorectal adenocarcinoma cell line, Caco-2 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured using Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal bovine serum (LabTech, UK), 2 mM L-glutamine, nonessential amino acids (Invitrogen, UK) and penicillin-streptomycin at 5% $CO_2$ and 37 °C. For drug treatments cells were plated in arginine free media at $1 \times 10^3$/well in 96 well plates and cultured for 96 h before cell death was measured using Alamar Blue viability assays according to manufacturer's instructions.

### 2.4. Platelet aggregation studies

This study was approved by the NHS St. Thomas' Hospital Research Ethics Committee (reference 07/Q0702/24). All volunteers gave written informed consent before entering the study. Blood was collected by venepuncture into tri-sodium citrate (3.2% w/v final) from healthy volunteers who had abstained from NSAIDs for two weeks. Platelet aggregation in response to 1 mM arachidonic acid or 10 μM U46619 (Cayman Chemical Company) was measured in platelet rich plasma using a 96-well plate platform as we have described previously [22,23].

### 2.5. Statistical analysis

Data is mean ± S.E.M for data from n = separate experiments/donors. For solubility experiments each n-value represents separate incubations. For cell based experiments drug treatment data was derived from separate individual wells and for control (vehicle) data derived from means of duplicate incubations on each experimental day. All statistical tests were performed using GraphPad Prism v5 (GraphPad Inc., UK) and are defined in figure legends. Statistical significance (*) was assumed when $p < 0.05$.

## 3. Results

### 3.1. Solubility of ibuprofen formulations

As expected, ibuprofen acid was relatively insoluble in aqueous solutions at neutral (pH 7) or acidic (pH 3) pH, corresponding to blood and gastric fluid, respectively. However, ibuprofen sodium was relatively soluble at pH7 but not pH3 whilst ibuprofen arginate was readily soluble in aqueous solutions at either pH tested (Fig. 1).

### 3.2. Effects of ibuprofen salts on human colon cancer cell death

It is now well accepted that COX-2 is an oncogene and that a broad range of NSAIDs kill cancer cells *in vitro*. The case for NSAIDs as chemotherapeutic agents is most established for celecoxib in colon cancer where in prospective clinical trials patients on the drug had around 50% less tumours than those on placebo [24]. Indeed celecoxib was licensed in Europe for the prevention of colon cancer until 2011 when it was withdrawn due to safety concerns [3]. Here we have used the human colon cancer cell line, Caco-2, to assess the effects of ibuprofen salts on cell death. Celecoxib and the

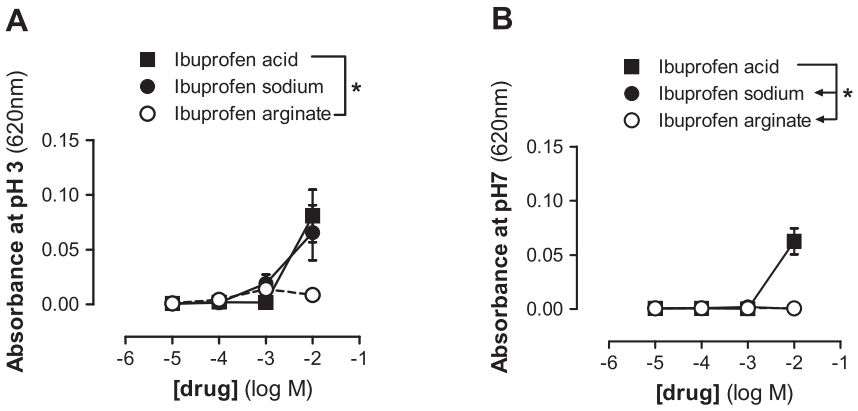


**Fig. 1.** Solubility of ibuprofen formulations at pH3 (A) and pH7 (B). Data are mean ± SEM from n = 3 separate experiments. Statistical significance was determined by two-way ANOVA with repeated measures and shown as *p < 0.05 when compared to ibuprofen acid.

cancer killing drug doxorubicin were used as positive controls. Doxorubicin and celecoxib induced concentration dependent reductions in cell viability which reached a maximum of >90% cell death at 3 μM and 100 μM respectively (Fig. 2). Ibuprofen arginate had relatively weak effects on cell viability with no response seen at concentrations up to 100 μM reflecting its lower potency as a COX-2 inhibitor compared to celecoxib [25]. However, at 1 mM, ibuprofen arginate but not ibuprofen sodium, caused a small but statistically significant reduction in Caco-2 cell viability (Fig. 2). L-arginine at concentrations calculated to be equivalent to those delivered in fully dissociated ibuprofen arginate, arginine increased survival at 15–150 μM ($p < 0.001$) with no effect seen at 0.5 mM (one-way ANOVA followed by Dunnett's test; $p > 0.05$).

### 3.3. Effects of ibuprofen salts on platelet aggregation

Traditional NSAIDs such as ibuprofen can act as inhibitors of COX-1 and COX-2 [26]. In the case of ibuprofen, which has higher affinity for COX-1 than COX-2, this means that at the therapeutic doses required to block COX-2 by ≈ 80% [25] COX-1 will also be strongly inhibited. COX-1 in platelets is responsible for thromboxane release, which activates platelets to aggregate.

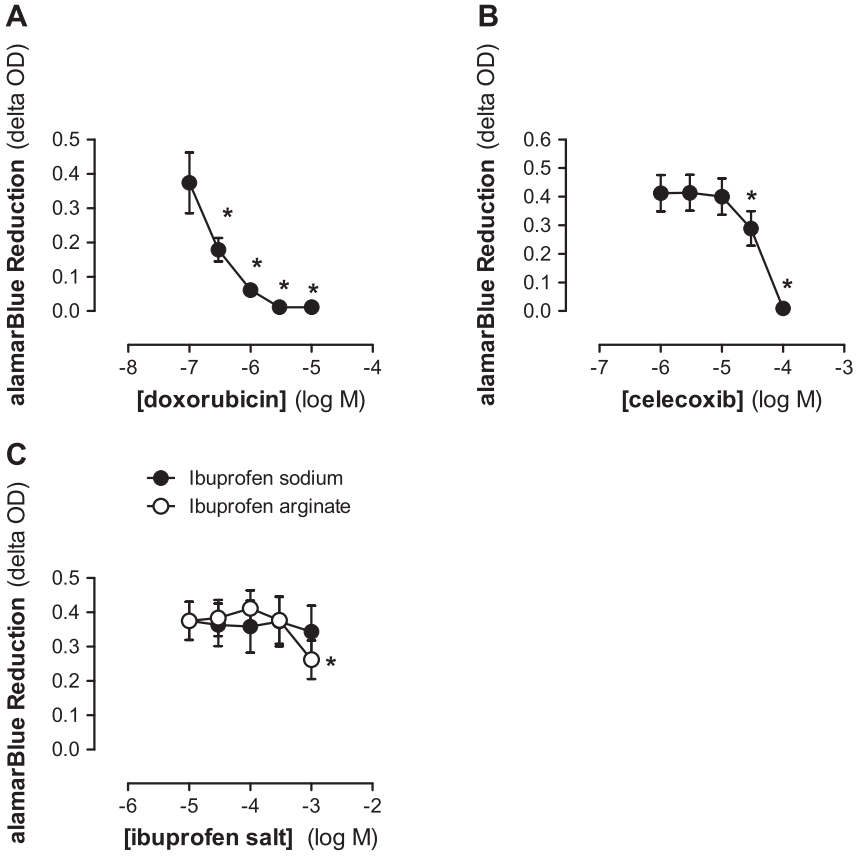


**Fig. 2.** Cancer killing effects of doxorubicin (A), celecoxib (B) and ibuprofen salts (C). Data are mean ± SEM from n = 6–8. Statistical significance was determined by either a one-way ANOVA with a Dunnets post-hoc test.

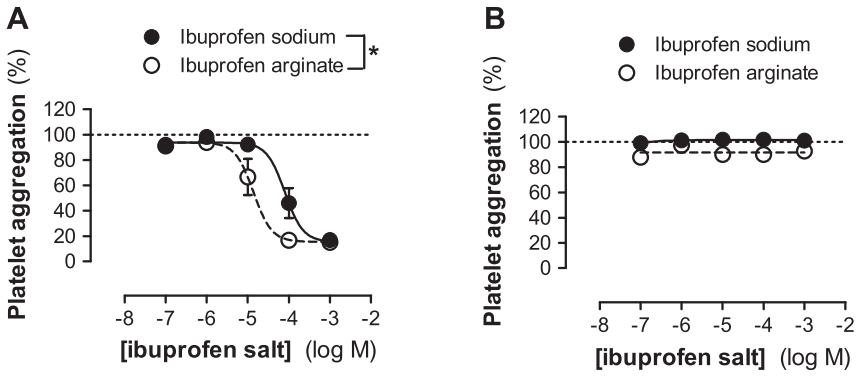


**Fig. 3.** Effect of Ibuprofen salts on platelet aggregation stimulated with arachidonic acid (A) or U46619 (B). Data are mean ± SEM from n = 4. Statistical significance was determined by two-way ANOVA with repeated measures and shown as *p < 0.05 when compared to ibuprofen sodium.

Consequently, blocking COX-1 in platelets inhibits aggregation induced by agents that work via the release of thromboxane such as arachidonic acid without affecting aggregation induced by exogenous thromboxane mimetics such as U46619. In man at therapeutic doses, the anti-platelet effects of NSAIDs are limited by their pharmacokinetics. Therefore the greatest anti-platelet effects are seen with irreversible (aspirin) and slowly metabolised (naproxen) NSAIDs with milder, but demonstrable effects of more rapidly metabolised drugs including ibuprofen [27,28] and diclofenac. In this study we found that both ibuprofen sodium and ibuprofen arginate caused concentration dependent reductions in platelet aggregation induced by arachidonic acid but not by U46619 *in vitro* (Fig. 3). Interestingly, whilst both salts of ibuprofen were effective, ibuprofen arginate was significantly more potent than ibuprofen sodium as an inhibitor of platelet aggregation (Fig. 3). L-arginine at concentrations calculated to be equivalent to those delivered in fully dissociated ibuprofen arginate (up to 0.5 mM) had no effect on platelet aggregation induced by either arachidonic acid or U46619 (one-way ANOVA followed by Dunnett's test; $p > 0.05$).

## 4. Discussion

The cardiovascular side effects associated with NSAIDs are of major clinical concern. Whilst the specific mechanisms are unclear our previous work has identified ADMA as a potential biomarker and mediator of cardiovascular compromise associated with NSAID usage and consequent COX-2 blockade. ADMA competes with arginine in the vasculature leading to reduced eNOS activity which can be prevented by supplementation with the substrate L-arginine. Most recently we have shown that the arginine salt of ibuprofen, which is currently available as an over-the-counter drug, provides in one formulation an effective COX-2 inhibitor and substrate for eNOS.

In addition to treating pain and inflammation, NSAIDs have other beneficial properties that for drugs like ibuprofen either (i) do not reach clinical significance or (ii) have not been tested in prospective clinical trials but remain important and relevant to assess. With this in mind, here we have extended the pharmacological assessment of ibuprofen arginate to include two relevant activities of NSAIDs (i) inhibition of platelet aggregation and (iii) cancer cell killing. Importantly, in addition to finding that ibuprofen arginate is effective at killing cancer cells, we found it to be more potent than the sodium salt as an inhibitor of platelet aggregation. Whilst these studies rely on *in vitro* assessments they provide promising pre-clinical evidence that ibuprofen arginate is not pharmacologically limited (compared to other salt formulations). This is crucial when considering the issue of cardiovascular side effects caused by NSAIDs because platelet activation and thrombosis are thought to be key underlying factors. Moreover, because it has recently been suggested that the anti-cancer effects of NSAIDs include two components, (i) inhibition of COX-2 in tumour cells and (ii) inhibition of platelet activation via blocking COX-1 in platelets [29,30], ibuprofen arginate may not only represent a superior anti-thrombotic to ibuprofen sodium but also in large populations may be better at preventing cancer. This work supports the idea that arginine formulations of NSAIDs should be further investigated for efficacy and for their potential to spare the cardiovascular system by supplying arginine. It should be noted however that arginine supplementation in some forms of cancer may not be advisable and may be linked to progression.

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## Transparency document

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