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REVIEW

Limitations of drug concentrations used in cell culture studies for understanding clinical responses of NSAIDs

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Abstract

In this review, the in vitro cellular effects of six nonsteroidal anti-inflammatory drugs (NSAIDs), salicylate, ibuprofen, naproxen, indomethacin, celecoxib and diclofenac, are examined. Inhibition of prostanoid synthesis in vitro generally occurs within the therapeutic range of plasma concentrations that are observed in vivo, consistent with the major action of NSAIDs being inhibition of prostanoid production. An additional probable cellular action of NSAIDs has been discovered recently, viz. decreased oxidation of the endocannabinoids, 2-arachidonoyl glycerol and arachidonyl ethanolamide. Many effects of NSAIDs, other than decreased oxidation of arachidonic acid and endocannabinoids, have been put forward but almost all of these additional processes are observed at supratherapeutic concentrations when the concentration of albumin, the major protein that binds NSAIDs, is taken into account. However, one exception is salicylate, a very potent inhibitor of the neutrophilic enzyme, myeloperoxidase, the inhibition of which leads to reduced production of the inflammatory mediator, hypochlorous acid, and inhibition of the inflammation associated with rheumatoid arthritis.

Keywords Cyclooxygenase · 2-Arachidonoyl glycerol · Arachidonoyl ethanolamide · Non-steroidal anti-inflammatory drug · Protein binding · Myeloperoxidase · Albumin · Therapeutic plasma concentrations · Enantiomer · Racemate · Salicylate · Ibuprofen · Naproxen · Indomethacin · Celecoxib · Diclofenac

Abbreviations

COX	Cyclooxygenase
NSAID	Non-steroidal anti-inflammatory drug
2-AG	2-Arachidonoyl glycerol
AEA	Arachidonoyl ethanolamide
AA	Arachidonic acid
FBS	Foetal bovine serum
RSK-2	Ribosomal protein S6 kinase alpha-3
PG	Prostaglandin
PGM	Prostaglandin M
PGE_2	Prostaglandin E_2
2-PG	2-Prostaglandin glycerol
PGEA	Prostaglandin ethanolamide
PMA	Phorbol myristate acetate
AMPK	5′ Adenosine monophosphate-activated protein kinase
PPAR	Peroxisome proliferator-activated receptor
LPS	Lipopolysaccharide
BAEC	Bovine aortic endothelial cells

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Introduction

There is considerable concern about the extent to which some of the principles of pharmacology are either treated inadequately or, in many cases, not considered at all in papers in which the in vivo mechanisms of action of drugs are inferred from the effects of the drugs in vitro. In our view, studies on the mechanisms of action of drugs are all too often conducted without regard for the comparative drug concentrations in vitro and in vivo. However, a principle of clinical pharmacology is that the activity of reversibly acting drugs is controlled by the plasma concentrations of the

drug, particularly those of the unbound drug. This is a significant aspect in the analysis of the therapeutic and adverse effects of NSAIDs. Further, we consider that for a proposed mechanism of action to be accepted, particularly with well-established therapies such as nonsteroidal anti-inflammatory drugs (NSAIDs), these mechanisms must be relevant to the observed in vivo clinical effect.

Repurposing marketed drugs for new possible clinical indications is now a major aspect of modern pharmacology. Many studies contain claims that a new mechanism of action of a drug has been discovered when the studies were conducted in vitro at much higher concentrations than achieved in plasma by therapeutic dosage. The use of supratherapeutic concentrations of drugs is now so pervasive in in vitro studies that it can be argued that current drug research is impaired significantly by the neglect of the basic principle of pharmacology that in vitro and in vivo effects should occur at similar unbound concentrations. This is not a new concept but is still highly relevant to ensuring the validity of conclusions that are drawn about the pharmacology of NSAIDs and many other drugs.

There are a number of reasons why active drug concentrations in vitro and in vivo require careful consideration. These include:

- The effects of a drug may be mediated by competitive binding of an agonist or stimulant of the system and, therefore, depend upon the relative concentration of the agonist. A high concentration of an agonist may require a high concentration of an antagonist for effective blockade.
- The activity of most enantiomeric drugs depends upon the active optical isomer. The activities of individual optical isomers should be compared. This is particularly important in understanding the pharmacology of ibuprofen and naproxen.
- Most NSAIDs are bound strongly to plasma proteins, particularly albumin, as well as to their cellular receptors. The result is that a competitive interaction may be established between binding to the receptor and plasma albumin. Accordingly, the response to NSAIDs in vitro should decrease with increasing levels of added albumin.
- It is generally accepted that the activity of NSAIDs in vitro is dependent on their cellular concentrations. The cellular uptake of NSAIDs has been considered to be passive and related to their lipid solubilities. However, the cellular uptake of several NSAIDs, such as salicylate, ibuprofen, celecoxib and diclofenac (Emoto et al. 2002; Novakova et al. 2014) can be facilitated by carrier-mediated transporters.
- NSAIDs and fatty acids are physicochemically similar. Both exist at physiological pH as ions, with minor proportions as lipid-soluble unionised forms. Both are strongly bound to plasma albumin. The mechanism of cellular uptake of fatty acids is unclear but it has been suggested that they are taken up in albumin-bound forms (Burczynski et al. 2001). It is possible that NSAIDs may also be taken up in the albumin-found forms.
- The potency of drugs in vitro may be dependent upon cell density. The drug or its metabolite may be taken up avidly, even held covalently, by cells in in vitro incubations. Consequently, the inhibitory activity may depend on the ratio of drug to cell density. A higher cell density may lead to lower in vitro potency because of lesser availability of the drug per cell. Examples include the in vitro actions of the gold drug, auranofin, which is bound strongly to neutrophils. Its activity in inhibiting the oxidative burst of neutrophils decreases from approximately 70 to 20% when the density of neutrophils is increased from 200,000 to 800,000 cells/mL (Rudkowski et al. 1990). This phenomenon is also seen with cell-penetrating peptides where: “At a given cell number, doubling of the incubation volume increased intracellular peptide concentration to a similar extent as the doubling in incubation concentration.” (Hallbrink et al. 2004). Thus, a critical aspect of pharmacological research in vitro may be the ratio of added drug concentration to cell density. A classic example of the influence of the amount of drug per cell is the goldfish experiment in which the toxicity to goldfish of a solution of chlorpromazine increases with increasing volume despite keeping the concentration constant (Gillette 1965). Essentially, the explanation is that the availability of chlorpromazine to the goldfish is greater from larger than smaller volumes in the goldfish bowl.
- Binding of drugs to glass and plastic equipment may limit the apparent efficacy of drugs in vitro. An example is Taxol, the concentration of which decreases by 73% in protein-free culture medium in polystyrene plates (Song et al. 1996). The decrease in culture medium is prevented by 9% FBS. Chloroquine also binds to glass containers (Geary et al. 1983). Again, this binding is prevented by serum protein. This phenomenon has not been studied widely but is potentially significant in pharmacological studies in vitro, particularly when dilute drug solutions are made up in albumin-free aqueous solutions.
- The metabolism of drugs should be considered when examining the pharmacology of drugs. Activation or inhibition of drug actions may be a particular problem in vitro in cells or isolated tissues containing cytochrome P450 enzymes or myeloperoxidase. The chemical properties of drug metabolites should also be considered. A very well-known example is aspirin, which has pharmacological properties per se as does its metabolite, salicylate.

Many pharmacological effects of NSAIDs are described in this review but, it should be noted that a comprehensive review of these effects has not been attempted. However, studies on inhibition of prostanoid synthesis is a major aspect of NSAID pharmacology that is considered. NSAID effects on oxidation of the endocannabinoids, 2-arachidonoyl glycerol (2-AG) and arachidonyl ethanolamide (AEA) are also examined as is NSAID-mediated inhibition of myeloperoxidase. The in vitro studies that have been reviewed here are largely limited to results from incubations of intact cells. NSAIDS often have little activity in broken cell preparations and NSAID concentrations in such studies generally do not correlate with plasma concentrations (Mitchell et al. 1993).

The view presented in this review is that inhibition of prostanoid or endocannabinoid synthesis in vitro is generally relevant to the mechanism of action of NSAIDs as these effects are produced at unbound levels that are similar to those achieved in plasma by therapeutic dosage. Non-prostanoid effects of NSAIDs are, for the most part, not relevant to their mechanism of action as the concentrations used are generally too high to be clinically relevant.

Binding to plasma proteins

As mentioned above, the influence of protein binding should be considered in any in vitro study investigating the mechanism of action of a drug. Some examples of the influence of protein-binding on in vitro pharmacology of non-NSAIDs are shown in Table 1. Incubation media commonly contain 10% foetal bovine serum (FBS) and as a result, these media have less capacity to bind drugs than full serum. An experimental example is dipyridamole, a coronary vasodilator, which averaged 1.9–3.5% unbound in human plasma but 75–100% unbound in 10% FBS at the same total drug concentration (Table 1). As a result, to achieve equivalent unbound concentrations in cell culture as those measured in the therapeutic concentration range in plasma, would require only 1/25th–1/55th of the total concentration measured in plasma.

NSAIDs are strongly bound to serum albumin in vivo. However, in experimental pharmacological studies, it is common for cells to be incubated in vitro in media containing little or no plasma protein. The question is "Are the concentrations of bound or unbound NSAIDs that are used in experimental studies relevant to the in vivo actions of NSAIDs on prostaglandins (PGs)?" This review is therefore largely focused on correlations between responses in vitro, particularly with respect to unbound concentrations under low serum conditions and the unbound plasma concentrations that are required for therapeutic responses in vivo. These principles are examined in experimental studies on six NSAIDs: salicylate, ibuprofen, naproxen, indomethacin, celecoxib and diclofenac. Tables 2, 3, 4, 5, 6 and 7 summarise in vitro experimental results and contain comments on comparisons with bound and unbound plasma levels of these NSAIDs in vivo. In this regard, it should also be noted that the pharmacokinetics of NSAIDs

Table 1 Examples of the effect of plasma protein on in vitro actions of drugs

Protein in medium	Experimental system	Active concentrations	Comments	Reference
Human plasma or 10% FBS	In vitro equilibrium levels of free drug at therapeutic concentrations determined	Range of dipyridamole concentrations studied. 2–10 mM in plasma, 0.08–5.0 mM in 10% FBS	24- to 55-fold higher total drug concentrations required in plasma to achieve equivalent free drug concentrations seen in 10% FBS	Szebeni and Weinstein (1991)
10–50% human serum	Inhibition replication of human immunodeficiency virus type 1 (HIV-1) in MT-4 cells	Potency decreases with increasing % of human serum	Binding increases and effect decreases with increasing protein. Increasing lipid solubility of drugs increases potency of drugs	Baba et al. (1993)
Ovarian cancer cell line in 2.5–10% FBS	Cytotoxicity of experimental marine natural products	Cytotoxicity decreases with increasing levels of FBS	Follows general pattern of effects of protein binding on drug activity	Tognon et al. (2004)
Ovarian cancer cell line in 1 and 10% FBS	Cytotoxicity of experimental insecticides	Cytotoxicity decreases with increasing FBS	Follows general pattern of effects of protein binding on drug activity	Bayoumi et al. (2003)
F11 sensory neuron cell line No extracellular protein In 10% FBS	Viability of flavanols	Proliferation at 0.1 ng/mL and IC_{50} (cell death) ≈ 10 µg/mL No effect at 100 µg/mL Total inhibition at 1 mg/mL	Binding to albumin in FBS decreases potency	Fujii et al. (2019)

Table 2 Active concentrations of salicylate in experimental systems in vitro

Cellular system and protein in medium	Inhibited system	Active concentrations of salicylate	Comments	Reference
Prostanoid synthesis and related				
Platelets in:				
0.35 mg/mL BSA (equivalent to ~ 1% serum)	COX-1	IC_{50} 16 μM	Relevant	Warner et al. (2006)
3.5 mg/mL BSA (equivalent to ~ 10% serum)	COX-1	IC_{50} 85 μM	Relevant	
Blood (100% serum)	COX-1	IC_{50} 300 μM	Relevant	
Ca^{++} ionophore-stimulated A549 cells in:				
0.35 mg/mL BSA	COX-2	IC_{50} 20 μM	Relevant	Warner et al. (2006)
3.5 mg/mL BSA	COX-2	IC_{50} 130 μM	Relevant	
Blood	COX-2	IC_{50} 190 μM	Relevant	
Homogenised guinea pig lung	PGE_2 synthesis Protein present from blood and tissue in homogenised lung	≈ 40% inhibition at 440 μM	Relevant	Vane (1971)
Human pulmonary epithelial cell line A549 in 10% FBS	PGE_2 synthesis	Salicylate IC_{50} at AA 0–10 μM ≈ 35 μM	Relevant	Mitchell et al. (1997)
Human endothelial cells in whole blood	COX-1 (whole blood assay) COX-2 (method 1) COX-2 (method 2)	IC_{50} ≈ 5 mM IC_{50} = 34 mM IC_{50} = 482 μM	Not relevant Potency on COX-2 depends upon method	Warner et al. (1999)
Bovine aortic endothelial cells (BAEC) in 10% FBS	COX-1 activity COX-2 activity	IC_{50} 220 μM IC_{50} 620 μM	Relevant Relevant	Mitchell et al. (1993)
Endotoxin-stimulated intact murine macrophages (J774.2) in 10% FBS	Broken cells	IC_{50} > 6 mM	Much less active in broken cells than in intact cells	
Inflamed tissue in protein-free medium	PGE_2 synthesis	IC_{50} 0.07 mM	Relevant	Higgs et al. (1987)
Murine osteoblast cell line (MC3T3E1) stimulated by TNF-α and IFN-γ in 10% FBS	Expression of COX-2 protein	IC_{50} ≈ 10 mM but some apparent effect at 500 μM. Uncontrolled observational Western blot data	Not relevant. High level	Chae et al. (2004)
Human pulmonary epithelial adenocarcinoma cell line A549 in 10% FBS	IL-1β (10 ng/mL) induced expression of COX-2 protein or mRNA or activation of NFκB	No inhibition at 0.6 mM for COX-2 induction and 1 mM for NF-κB activation	Not relevant	Mitchell et al. (1997)
PMA-stimulated human foreskin fibroblasts in 0.5% FBS	Expression of COX-2 reduced incompletely	IC_{50} ≈ 10 μM due to inhibition of COX-2 mRNA	Relevant	Xu et al. (1999)
PMA-stimulated human fibroblasts in 10% FBS	Expression of COX-2 reduced incompletely	IC_{50} ≈ 10 μM	Relevant	Cieslik et al (2005)
Rat islets in 0.2% FBS, 22.2 mM glucose stimulated with IL-1β (5 ng/mL) for 24 h	IL-1β—induced COX-2 and EP3 mRNA expression	1.25 mM	Not relevant	Tran et al. (2002)
Non-prostanoid effects				
Isolated enzyme. No serum albumin	Isolated myeloperoxidase	IC_{50} 9.4 μM	Relevant. Potent effect	Kettle and Winterbourn (1991)

Table 2 (continued)

Cellular system and protein in medium	Inhibited system	Active concentrations of salicylate	Comments	Reference
Human monocyte cell line in serum albumin-free medium	Expression of several inflammatory cytokines including IL-1β and TNF-α	Inhibition 5–20 mM	Not relevant, high level	Housby et al. (1999)
Rat liver, kidney and brain mitochondria. No serum albumin	Oxidative phosphoryl-ation	IC_{50} 1–5 mM	Not relevant. High level. Author points out possible relevance to salicylate overdose	Brody (1956)
Cartilage slices and liver mitochondria. No serum albumin	Oxidative phosphoryl-ation	Inhibition by 40% in cartilage at 2.5 mM and in liver mitochondria at 0.5 mM salicylate	Possibly relevant Possible toxic effect	Whitehouse (1964)
Cell-free [purified rat liver AMP-activated protein kinase (AMPK)] HEK 293 cells (10% FBS) and in vivo (mouse)	Activation of AMPK	IC_{50} 1.0 mM Increase at 0.5 mM 1–30 mM 2.4 mM plasma concentration	Not relevant Authors' caveat is that salicylate concentrations may be supratherapeutic	Hawley et al. (2012)
LPS-stimulated THP-1 human monocyte cell line in 10% FBS	Activation of AMPK phosphorylation	5 mM	Not relevant. Supratherapeutic	Bao et al. (2018)
Mouse patella in physiological saline In 100% serum	Sulphate uptake by patella	5–10 mM No effect at 1–2 mM	Not relevant. High level	de Vries et al. (1986)
Breast cancer cell line in 10% FBS Breast cancer cell line in protein–free medium	Cell proliferation Urokinase plasminogen activator	IC_{50} 15–20 μM IC_{50} 5 mM	Not relevant. High level Not relevant. High level	Madunic et al. (2017)
Mouse epidermal cell line in 5% FBS	TPA-induced transformation	≈ 30% inhibition at 0.1 mM. 90% inhibition at 1 mM	Possibly relevant	Dong et al (1997)
Ribosomal S6 kinase (RSK2)	Purified protein			Stevenson et al. (1999)
Blood-derived human monocytes	LPS-stimulated RSK2 kinase activity	20 mM	Not relevant. High level	Stevenson et al. (1999)
Serum-starved murine fibroblasts (NIH3T3 cells)	TPA-stimulated RSK2 kinase activity EGF-stimulated RSK2 activity TPA-induced ERK activity	5 mM 20 mM 20 mM	Not relevant. High level	Stevenson et al. (1999)
Rat islets in 0.2% FBS stimulated with IL-1β (5 ng/mL) for 24 h	IL-1β-mediated suppression of glucose-induced insulin secretion	45 min pre-treatment with 12.5 pM–1.25 mM Partial inhibition No dose response	Relevant	Tran et al. (2002)
Rat islets in 0.2% FBS, 22.2 mM glucose stimulated with IL-1β (5 ng/mL) for 1 h	IL-1β activation of NF-κB	45 min pre-treatment with 1.25–2.5 mM Apparent qualitative partial inhibition	Not relevant	Tran et al. (2002)

By comparison, therapeutic concentrations of salicylate are: Peak total plasma concentrations of salicylate are ~0.33 mM (45 mg/L) after the administration of 650 mg aspirin, a commonly used analgesic or antipyretic dose (Rowland et al. 1972). Average steady state plasma salicylate concentrations following dosing of rheumatoid arthritis patients with aspirin (3.6 g/day) are ~0.53 mM (73.4 mg/L) total concentration and 0.06 mM (8.5 mg/L) unbound concentration (Günsberg et al. 1984)

Table 3 Active concentrations of ibuprofen in experimental systems in vitro

Cells and protein in medium	Inhibited system	Active concentrations	Comments	Reference
Prostanoid synthesis and related				
Human platelets in plasma	Synthesis of thromboxane A_2	R-ibuprofen IC_{50} 380 µM S-ibuprofen IC_{50} 88 µM	Not relevant Relevant S-ibuprofen more potent than R-ibuprofen	Villanueva et al. (1993)
Homogenised insect cells. No added albumin	COX-1 activity COX-2 activity	RS-ibuprofen COX-1 IC_{50} 3.3 µM COX-2 IC_{50} 38 µM	Not relevant	Gierse et al. (1995)
COX-1 and COX-2 activity in whole blood	COX-1 (ionophore-stimulated whole blood) COX-2 (aspirin-treated, LPS-stimulated whole blood) COX-2 (Il-1β-stimulated A549 cells in whole blood)	RS-ibuprofen IC_{50} 7.6 µM IC_{50} 7.2 µM IC_{50} 20 µM	Relevant Relevant Potency dependent on method	Warner et al. (1999)
Isolated COX-1 and COX-2. No plasma protein	COX-1 activity COX-2 activity COX-1 activity COX-2 activity	R-Ibuprofen IC_{50} 1175 µM 1 mM S-Ibuprofen 37 µM 1 mM	Contrasting effects with other studies on R-Ibuprofen	Jaradat et al. (2001)
Platelets in whole blood	Concentration of thromboxane B2 produced	IC_{50} mean plasma unbound concentration 0.048 µM	Relevant	Evans et al. (1991)
Isolated COX-2 in albumin-free medium	Oxidation of 50 µM AA Oxidation of 50 µM 2-AG	RS-Ibuprofen IC_{50} 7 µM IC_{50} 20 nM	Supra-therapeutic concentration Relevant	Duggan et al. (2011)
Isolated COX-2 in albumin-free medium	Oxidation of 5 µM 2-AG Oxidation of 50 µM 2-AG	R-Ibuprofen IC_{50} 10 µM IC_{50} 18 µM	Supra-therapeutic concentrations	Duggan et al. (2011)
Dorsal root ganglion cells in 10% FBS	Oxidation of AA	R-ibuprofen Inactive	Probably relevant	Duggan et al. (2011)
Bovine aortic endothelial cells in 10% FBS Endotoxin-stimulated murine macrophage cell line (J774.2) in 10% FBS. Whole cells Broken cells	COX-1 COX-2	RS-ibuprofen IC_{50} 5 µM IC_{50} 73 µM IC_{50} 780 µM	Relevant Activity of ibuprofen much less in broken cells and isolated COX-1 and COX-2 than in intact cells	Mitchell et al (1993)
Non-prostanoid effects				
Human neutrophils in albumin (0.005%) in saline	Superoxide production	R-ibuprofen IC_{50} 430 µM S-ibuprofen IC_{50} 500 µM	Not relevant Not relevant	Villanueva et al. (1993)
Neuroblastoma cells in 5% FBS	Growth of cells	S-ibuprofen $IC_{50} \geq 500$ µM	Not relevant	Ikegaki et al. (2014)
Strains of S aureus	Bacterial growth	RS-ibuprofen MIC 500–2000 mM	Not relevant	Oliveira et al. (2019)
Human colon carcinoma cell line in 10% FBS	Cell survival	400–1000 µM R and S-ibuprofen	Author's conclusion Not relevant Effects not due to inhibition of COX-1 or COX-2	Janssen et al. (2006)
Reporter-transfected H4IIEC3 rat hepatoma cells in 10% FBS	Stimulation of peroxisome proliferator-activated receptor-α (PPARα)	S-ibuprofen EC_{50} approx. 100 µM R-ibuprofen approx. tenfold less active	Lower activity than inhibitory potency on COX-1 or COX-2 Not relevant	Jaradat et al. (2001)
Reporter-transfected monkey kidney cells (CV1) in 10% FBS	Activation of PPARα and PPARγ Activation of PPARα Activation of PPARγ	R-Ibuprofen Little activity $EC_{50} > 1$ mM S-Ibuprofen $EC_{50} \sim 100$ µM $EC_{50} \sim 100$ µM	Not relevant Not relevant Not relevant	Jaradat et al. (2001)

By comparison, at a single dose (800 mg), peak total plasma concentrations of S-ibuprofen and R-ibuprofen are approximately 28 mg/L (135 µM) and 23 mg/L (111 µM) respectively. Unbound peak concentrations are approximately 200 µg/L (0.97 µM) and 100 ug/L (0.48 µM) respectively (Evans et al. 1989)

Table 4 Active concentrations of naproxen in experimental systems in vitro

Cells and protein in medium	Inhibited system	Active concentrations	Comments	Reference
Prostanoid synthesis and related				
COX-1 and COX-2 activities in whole blood	COX-1 (ionophore-stimulated whole blood) COX-2 (aspirin-treated, LPS-stimulated whole blood) COX-2 (Il-1b-stimulated A549 cells in whole blood)	S-Naproxen IC_{50} 9.3 µM IC_{50} 28 µM IC_{50} 35 µM	Relevant Supratherapeutic concentrations Potency dependent on method	Warner et al. (1999)
Platelets in:				
0.35 mg/mL BSA (equivalent to ~ 1% serum) 3.5 mg/mL BSA (equivalent to ~ 10% serum) Blood (100% serum)	COX-1 COX-1 COX-1	S-Naproxen IC_{50} 1.7 µM IC_{50} 5.5 µM IC_{50} 17 µM	Relevant Supratherapeutic concentrations although IC_{50} values in predicted order	Warner et al. (2006)
Ca^{++} ionophore-stimulated A549 cells in:				
0.35 mg/mL BSA 3.5 mg/mL BSA Blood	COX-2 COX-2 COX-2	S-Naproxen IC_{50} 14 µM IC_{50} 18 µM IC_{50} 50 µM	Supratherapeutic concentrations although IC_{50} values in predicted order	Warner et al. (2006)
Bovine aortic endothelial cells in 10% FBS Endotoxin-stimulated murine macrophage cell line (J774.2) in 10% FBS	COX-1 COX-2	S-Naproxen IC_{50} 9.6 µM IC_{50} 5.6 µM	Not relevant Not relevant	Mitchell et al. (1993)
Synovial cells in 10% FBS	Expression of prostaglandin endoperoxide synthase-1 RNA	S-Naproxen Activity at 200–400 µM	Not relevant concentrations of S-naproxen >> effective concentrations	Wang et al. (2019)
Isolated COX-1 Isolated COX-2	Oxidation of 10 µM AA Oxidation of 10 µM AA	S-Naproxen IC_{50} 0.78 µM IC_{50} 4.2 µM	Concentrations of S-naproxen >> unbound concentrations in plasma	Jaradat et al. (2001)
Dorsal root ganglion cells in 10% FBS	Oxidation of 5 µM 2-AG Oxidation of 50 µM AA	R-Naproxen IC_{50} 8.9 µM IC_{50} 11.8 µM	Probably relevant	Duggan et al. (2011)
Non-prostanoid effects				
MDCK (canine renal cell line) in protein-free medium	Protection of MDCK cells (measured as RNA in supernatant)	S-Naproxen IC_{50} 16 µM	Not relevant Concentration >> unbound in plasma	Lejal et al. (2013)
Primary culture of osteoblasts in:				
20% FBS Osteoblast cell line in 10% FBS	Inhibition of expression gene of vascular endothelial growth factor (VEGF)	10 µM, 90% inhibition 10 µM, 70% inhibition	Similar effect shown by paracetamol and non-selective NSAIDs. Not relevant	Manzano-Moreno et al. (2018)
Reporter-transfected monkey kidney cells (CV1) in 10% FBS	Activation of PPARα Activation of PPARγ	No activity, EC_{50} ~ 20 µM	See also effects of indomethacin, R and S-Ibuprofen Not relevant	Jaradat et al. (2001)
Human osteoclasts in 10% FBS	Inhibition of aminopeptidase activity	Weak inhibitor at about 125 µM	Also, weak inhibition by indomethacin and other NSAIDs	Lucena et al. (2016)

By comparison, peak concentrations of S-naproxen in plasma are approximately 3 µM (Davies et al. 2000). The % unbound is about 2.5 (Paulson et al. 1999) making the peak unbound concentration approximately 80 nM

are controlled largely by their binding to plasma proteins (Lin et al. 1987).

The tabulated data is divided into results showing NSAID inhibition of the production of prostanoids [PGs and thromboxane A_2 (TXA_2)] and non-prostanoid effects of NSAIDs. Conclusions on the relevance of in vitro studies are summarised as:

Relevant, where the effects of NSAIDs in in vitro studies are apparent within either total or unbound plasma concentrations observed in vivo during treatment with NSAIDs;

Not relevant, where the results of studies on NSAIDs in media in which the unbound or total NSAID concentrations are clearly greater than those which may be relevant in vivo.

Inhibitory NSAID concentrations in the experimental media are noted and categorised as either clinically relevant or not relevant as shown by the following examples.

- To be relevant, results from in vitro incubations in media containing no added serum or less than 0.5% serum must be comparable to the unbound concentrations in plasma during therapy. Villanueva et al. (1993) recorded that superoxide production by neutrophils was inhibited by S-ibuprofen, but only when the cells were incubated in saline at drug concentrations that are orders of magnitude greater than the unbound concentrations in plasma. This effect is classified as not relevant (Table 3).
- To be relevant, effects from in vitro incubations containing 100% serum or high concentrations of the binding protein during treatment must be comparable to total (bound and unbound) therapeutic plasma concentrations. Villanueva et al. (1993) recorded effects of S-ibuprofen on platelet TXA_2 in plasma at concentrations below peak total plasma concentrations. This effect is classified as relevant (Table 3).

Many cellular studies on NSAIDs are conducted in media containing 10% FBS and have been compared with therapeutic NSAID concentrations in patient serum. Information is therefore required on the binding of NSAIDs by FBS to understand the clinical significance of such in vitro studies on NSAIDs. Theoretically, comparisons should be made after correction for the lower binding of NSAIDs in 10% FBS relative to plasma. However, the only comparison on the binding of an NSAID in the literature comes from data of Beaven and Bayer (1980) on indomethacin. Literature searches, such as 'foetal bovine serum' and 'protein binding' of 'a particular NSAID' by Medline or Embase, have not yielded any other result. Binding of NSAIDs to intact plasma is usually available but binding to FBS is not. In the one exceptional case, Beaven and Bayer show that indomethacin is about 32% unbound in 10% FBS compared to 10% unbound in 100% serum. In general, the percentage unbound at any concentration is expected to be higher in 10% FBS than the same level in pure plasma or serum.

IC_{50} values

The following sections compare the in vitro inhibitory concentrations (IC_{50} values) of the six NSAIDs with their plasma concentrations during therapeutic dosage (Tables 2, 3, 4, 5, 6, 7). By definition IC_{50} values are concentrations producing a substantial effect, namely 50% of maximal effect. However, data in Tables 2, 3, 4, 5, 6 and 7 show that many in vitro IC_{50} values are well above the therapeutic or toxic plasma concentrations in vivo and the clinical effect should be very low.

IC_{50} quantifies the relative inhibitory potency of drugs in a defined experimental system. Unlike quantitatively constant terms, such as a dissociation constant for a binding interaction between a drug and its target, an IC_{50} is dependent on the experimental conditions in which it is measured and can thus change when these conditions are altered. IC_{50} is very useful in determining the relative potency of drugs in any one particular system. However, it is not appropriate to extrapolate findings in one in vitro system to another without taking obvious differences in experimental conditions into account. The following numerical example predicts very low therapeutic relevance of an IC_{50} measured in vitro when the effect of the known protein-binding capacity of a drug is taken into account. The IC_{50} for inhibition of superoxide production by S-ibuprofen is 500 µM in a medium containing very little albumin (0.005% albumin). By comparison, C_U, the peak unbound concentration in plasma during treatment is 0.09 µM (Table 3) (Villanueva et al. 1993). Thus, it follows that at therapeutic dosage, inhibition of superoxide production by S-ibuprofen is extremely low and not predicted to be achieved by a therapeutic dosage. From the classical concentration/effect relationship, the fractional effect of S-ibuprofen at the therapeutic C_U is given by:

$$I/I_{\max} = C_U/\left(\mathrm{IC}_{50} + C_U\right) = 0.09/(0.09 + 500) = 0.00036.$$

Another numerical example is the predicted effect of indomethacin from its IC_{50} for inhibition of COX-2 (0.36 µM) in whole blood. This concentration is well within the therapeutic range (up to 7 µM in whole blood; Table 5) (Patrignani et al. 1994). The in vitro result is therefore assessed as relevant to the clinical pharmacology of indomethacin.

Table 5 Active concentrations of indomethacin in experimental systems in vitro

Cells and protein in medium	Inhibited system	Mean active concentrations	Comments	Reference
Prostanoid synthesis and related				
Platelets in:				
0.35 mg/mL BSA (equivalent to ~ 1% serum)	COX-1	IC_{50} 0.15 µM	Relevant	Warner et al. (2006)
	COX-1	IC_{50} 0.074 µM	Relevant	
3.5 mg/mL BSA (equivalent to ~ 10% serum)	COX-1	IC_{50} 0.11 µM	Relevant	
Blood (100% serum)				
Ca^{++} ionophore-stimulated A549 cells in:				
0.35 mg/mL BSA	COX-2	IC_{50} 0.41 µM	Relevant	Warner et al. (2006)
3.5 mg/mL BSA	COX-2	IC_{50} 0.84 µM	Relevant	
Blood	COX-2	IC_{50} 0.24 µM	Relevant	
COX-1 and COX-2 activity in whole blood	COX-1 (ionophore-stimulated whole blood)	IC_{50} 0.013 µM	Relevant	Warner et al. (1999)
	COX-2 (aspirin-treated, LPS-stimulated whole blood)	IC_{50} 1 µM	Relevant	
	COX-2 (Il-1β-stimulated A549 cells in whole blood)	IC_{50} 0.13 µM	Relevant	
Supernatant of lung homogenate of guinea pig	Synthesis of PGF_{2a}	0.75 µM	Relevant Albumin present in lung homogenate	Vane (1971)
Perfused dog spleen	Prostanoid synthesis	< 1 µmol/L	Relevant	Ferreira et al. (1971)
Bovine endothelial cells (BAEC) in 10% FBS	COX-1	IC_{50} 25 nM	Relevant	Mitchell et al. (1993)
	COX-2	IC_{50} 1.5 µM	Relevant	
Endotoxin-stimulated murine macrophage cell line (J774.2) in 10% FBS				
Homogenised insect cells in albumin-free medium	hCOX-1 activity	IC_{50} 100 nM	Relevant	Gierse et al. (1995)
	hCOX-2 activity	IC_{50} 9 µM	Not relevant. High concentration	
Whole blood	COX-1	IC_{50} 0.7 µM	Relevant	Patrignani et al. (1994)
	COX-2	IC_{50} 0.36 µM		
Isolated COX-2 in albumin-free medium	Oxidation of: 50 µM AA	IC_{50} 180 nM	Relevant	Duggan et al. (2011)
	50 µM 2-AG	IC_{50} 30 nM		
Interface membranes from loose prostheses in 10% FBS	PGE_2 production	90–99% reduction by 1.4 µM indomethacin	Relevant By comparison the same concentration of indomethacin had inconsistent effects on cytokines and metalloproteinases	Syggelos et al. (2007)
Bovine endothelial cells in 10% FBS	COX-1 activity	IC50 250 µM	Not relevant	Mitchell et al. (1993)
	COX-2 activity	IC50 720 µM	Very high levels	
Murine macrophage cell line in 10% FBS				
Isolated COX-1	COX-1 activity	IC_{50} 0.68 µM	Relevant	Jaradat et al. (2001)
Isolated COX-2	COX-2 activity	IC_{50} 4.2 µM	Not relevant	
Oesophageal adenocarcinoma cells in 10% FBS	COX-1 activity	IC_{50} 0.28 µM	Relevant	Aggarwal et al. (2000)
	COX-2 activity	IC_{50} 1.7 µM		
Non-prostanoid effects				
Oesophageal adenocarcinoma cells in 10% FBS	Apoptosis	100 µM	Not relevant. High concentration	Aggarwal et al. (2000)
Human gastric cells in 1% FBS	Calcium uptake by cells	100 µM	Not relevant	Kokoska et al. (1998)
	Cell damage	250 µM	Cell uptake and damage potentiated by pre-incubation with indomethacin	

Table 5 (continued)

Cells and protein in medium	Inhibited system	Mean active concentrations	Comments	Reference
Human glioma cell line in 10% FBS	Cell viability	≥ 250 μM	Not relevant	Chang et al. (2018)
Human colon cancer cells (RKO and DLD1) in 10% FBS	Cell viability	RKO 50 μM 20% decrease at 200 μM DLD1 50% decrease at 200 μM	Not relevant	Foreman et al. (2009)
Hepatocellular carcinoma (HepG2) cells in 10% FBS	Cytotoxicity of doxorubicin	Cytotoxicity increased at 1 and 5 μM	Expression of P-glycoprotein and MRP1 decreased. Though relevant concentrations, effect not related to inhibition of PGE_2	Ye et al. (2011)
Reporter-transfected monkey kidney cells (CV1) in 10% FBS	Activation of PPARα Activation of PPARγ	No activity, 1 mM Little activity, 1 mM	Not relevant	Jaradat et al. (2001)
Human osteoclasts in 10% FBS	Inhibition of aminopeptidase activity	IC_{50} values 50–140 μM	Not relevant. Also, weak inhibition by naproxen and other NSAIDs	Lucena et al. (2016)

By comparison, therapeutic concentrations of indomethacin are: at a dose of 50 mg three times daily, peak total plasma concentrations of indomethacin are approximately 7 μM (2.5 mg/L) with an unbound concentration of 0.7 μmol/L (0.25 mg/L). The percentage of indomethacin unbound in 10% FBS is ≈ 30% making the relevant unbound concentration ≈ 2 μM in 10% FBS

Salicylate

Salicylate is well known as the major active metabolite of aspirin, but sodium salicylate has been widely used per se as an analgesic and anti-inflammatory agent in the treatment of rheumatoid arthritis and rheumatic fever. Salicylate has been very useful in determining some principles of clinical pharmacology. To a significant extent this is due to the large tolerated doses in vivo and ease of assay by quantitation of the purple colour that develops when ferric salts are added to plasma or urine or extracts of these fluids containing salicylate. Additionally, salicylate is fluorescent, allowing assay of low concentrations.

The mechanism of action of salicylate has been controversial because it has no significant actions against purified COX-1 and COX-2 protein (Mitchell et al. 1993). Salicylate also does not decrease COX-1 or COX-2 actions in broken cell preparations despite inhibitory actions in the same intact cells (Table 2) (Mitchell et al. 1993). However, inhibition of the in vitro synthesis of PGE_2 has been reported in several studies on intact cells at concentrations which are within the therapeutic range (Table 2). This includes inhibition of PGE_2 in the original study by Vane (1971), who showed that aspirin, salicylate and indomethacin decreased the production of PGE_2 (Vane 1971). It is of note that this study indicated that salicylate inhibits prostanoid synthesis at unbound therapeutic concentrations (Table 2). This work earned John Vane the shared award of a Nobel prize for the discovery of the mode of action of NSAIDs in blocking the synthesis of prostanoids, which are mediators of pain, fever and inflammation. Later discovery of the inhibition of platelet aggregation by aspirin led to its use in the prevention of clotting.

Overall inhibition of prostanoid synthesis in vivo can be monitored by inhibition of excretion of the metabolite, prostaglandin M (PGM), the major urinary metabolite of series-1 and series-2 PGs. Salicylate has inconsistent effects on the urinary output of PGM, being reduced in a study in two male subjects (Hamberg 1972) but not reduced in another study of seven female subjects using similar doses (Rosenkranz et al. 1986). Inhibition of the urinary excretion of PGM is, however, shown consistently by aspirin, indomethacin, ibuprofen and celecoxib (Hamberg 1972; Rosenkranz et al. 1986; Seyberth et al. 1976).

In agreement with principles of pharmacology, increased values of IC_{50} were recorded with increasing serum albumin concentrations (0.35–3.5 mg/mL) in vitro with still higher values after incubations in whole blood (Table 2). This pattern of decreasing efficacy with increasing serum albumin was expected but is not seen clearly with several other NSAIDs, including indomethacin and celecoxib (Warner et al. 2006).

Several non-COX effects of salicylate, particularly inhibition of cell growth, are shown in cellular systems in vitro. As outlined above, many cellular in vitro studies on salicylate are conducted in media containing either no serum or 10% FBS, where the percentage unbound should be higher than in 100% plasma. However, IC_{50} levels of salicylate in non-COX studies are of the order of 5 mM, which is higher than the total therapeutic plasma concentrations (Table 2). Consequently, the IC_{50} values of unbound salicylate in cellular

Table 6 Active concentrations of celecoxib in experimental systems in vitro

Cells and protein in medium	Experimental findings	Active concentrations	Comments	Reference
Prostanoid synthesis and related				
Platelets in:				
0.35 mg/mL BSA (equivalent to ~ 1% serum)	COX-1	IC_{50} 79 µM	Not relevant	Warner et al. (2006)
3.5 mg/mL BSA (equivalent to ~ 10% serum)	COX-1	IC_{50} 26 µM	Not relevant	
Blood (100% serum)	COX-1	IC_{50} 72 µM	Not relevant	
Ca^{++} ionophore-stimulated A549 cells in:				
0.35 mg/mL BSA	COX-2	IC_{50} 7 µM	Not relevant	Warner et al. (2006)
3.5 mg/mL BSA	COX-2	IC_{50} 18 µM	Not relevant	
Blood	COX-2	IC_{50} 24 µM	Not relevant	
COX-1 and COX-2 activity in whole blood	COX-1 (ionophore-stimulated whole blood)	IC_{50} 1.2 µM	Relevant	Warner et al. (1999)
	COX-2 (aspirin-treated, LPS-stimulated whole blood)	IC_{50} 0.83 µM	Relevant	
	COX-2 (Il-1β-stimulated A549 cells in whole blood)	IC_{50} 0.34 µM	Potency dependent on method	
Homogenised insect cells. No added protein	PGE_2 synthesis	COX-1 15 µM COX-2 40 nM	Not relevant Relevant	Kawamori et al. (1998)
Lung cancer cell line in FBS (1%)	PGE_2 synthesis	IC_{50} 18 nM	Relevant	Williams et al. (2000)
Cholangiocarcinoma cell line in 10% FBS	PGE_2 synthesis, growth and apoptosis	Inhibition of PGE_2 synthesis 26–74% at celecoxib ranging from 10 to 40 µM	Not relevant, high concentration	Wu et al. (2003)
Isolated COX-2 in albumin-free medium	Oxidation of AA (50 µM)	IC_{50} 80 nM	Relevant	Duggan et al. (2011)
	Oxidation of 2AG (50 µM)	IC_{50} 95 nM	Potent inhibitor of oxidation of endocannabinoids	
Non-prostanoid effects				
Prostate and ovarian cancer cell lines in 10% FBS	Growth	> 30 µM	Not relevant Reduced % growth Rate at cell densities > 5000/well	Vital-Reyes et al. (2006)
Colon cancer cell line in 1–10% FBS	Increased cell death	50 µM and 100 µM	Not relevant Activity of 50 µM abolished by 10% FBS	Tuynman et al. (2008)
Several cell types in 10% FBS	Apoptosis related to increased release Ca^{++} from internal stores and increased cell influx depending on celecoxib level	> 10 µM	Not relevant, high levels	Johnson et al. (2002)
Prostate cell line (PC3) in FBS (10%)	Increased intracellular Ca^{++} concentration	IC_{50} 9 µM	Not relevant Effect on Ca^{++} not seen with other selective COX-2 inhibitors	Wang et al. (2012)
Lung cancer cell line in FBS (1%)	Decreased viability due to apoptosis	Viability decreased at ≥ 20 µM	Not relevant	Williams et al. (2000)
Multiple epithelial cell lines (COX-2 positive) multiple haematopoietic cell lines (COX-2 negative) all in 10% FBS	Decreased viability due to apoptosis	Viability reduced ≈ 45% at 30 µM Viability reduced ≈ 55% at 30 µM	Not relevant Apoptosis not influenced by presence of COX-2	Waskewich et al. (2002)
Inhibition of carbonic anhydrase II (CAII) in albumin-free medium	IC_{50} of celecoxib 410 nM Acetazolamide IC_{50} 7.5 nM		Much less potent than acetazolamide	Knudsen et al. (2004)

By comparison, peak concentrations of celecoxib in plasma are approximately 1200 µg/L (Davies et al. 2000) equivalent to about 3 µM. The % unbound is about 2.5 (Paulson et al. 1999) making the peak unbound concentration approximately 80 nM

incubations must be far higher than that in total therapeutic plasma levels. Results of such in vitro studies are not clinically significant (Table 2). As an example, Stevenson et al. report inhibition of the 90 kD ribosomal S6 kinase RSK2, which activates cellular proliferation through phosphorylation of signalling pathways. However, high concentrations

Table 7 Active concentrations of diclofenac in experimental systems in vitro

Cells and protein in medium	Experimental measure	Active concentrations	Comments	Reference
Prostanoid synthesis and related				
Platelets in:				
0.35 mg/mL BSA (equivalent to ~ 1% serum)	COX-1	IC_{50} 0.041 mM	Relevant	Warner et al. (2006)
3.5 mg/mL BSA (equivalent to ~ 10% serum)	COX-1	IC_{50} 0.36 μM	Not relevant	
Blood (100% serum)	COX-1	IC_{50} 0.86 μM	Relevant	
Ca^{++} ionophore-stimulated A549 cells in:				
0.35 mg/mL BSA	COX-2	IC_{50} 0.5 μM	Not relevant	Warner et al. (2006)
3.5 mg/mL BSA	COX-2	IC_{50} 2.9 μM	Not relevant	
Blood	COX-2	IC_{50} 0.84 μM	Relevant	
Whole bovine endothelial cells in 10% FBS	COX-1	IC_{50} 1.7 μM	Not relevant, high level	Mitchell et al. (1993)
Whole murine macrophage cell line in 10% FBS	COX-2	IC_{50} 1.2 μM	Not relevant, high level	
Non-prostanoid effects				
Cancer cell lines, (HCT116 and SW480) in 10% FBS	Loss of viability of cells	Time-dependent effect at 400 μM	Not relevant, high level	Arisan et al. (2018)
Primary culture of osteoblasts in:				
20% FBS	Inhibition of expression of gene of VEGF	10 μM, 80% inhibition	Similar effect shown by paracetamol and non-selective NSAIDs	Manzano-Moreno et al. (2018)
Osteosarcoma cell line in 10% FBS		10 μM, 60% inhibition	Not relevant, high level	
HL-60 (human promyelocytic cell line) in 10% FBS containing myeloperoxidase-activating system	Cell death	Cell survival at 600 μM decreased by 50%	Effect due to active hydroxyl metabolites Not relevant, high concentration	Morgan et al. (2017)
U937 monocyte cell line in 10% FBS	Reduction of lactonase activity	Significant effect at 1.69 mM	Not relevant, high level	Avcıkurt and Oğuzhan Korkut (2018)
Acetoacetate treated LLC-PK_1 kidney cells in 3% FBS	Oxygen consumption rate	IC_{50} 710 μM	Not relevant, high level	Denoon et al. (2020)
Normal epidermal keratinocytes, serum-free medium	Effects on sulfur mustard-induced cytokine production	IL-6 decreased by diclofenac (15–150 μM). IL-8 and TNFa increased by diclofenac	Contrasting effects on IL-6 and IL-8 or TNFa Not relevant, high level	Wagner et al. (2019)

By comparison, peak concentrations of diclofenac in plasma are approximately 4 μM after 50 mg oral dosage (Willis et al. 1981). The % unbound concentration is about 0.05% (Chan et al. 1987) making the peak unbound concentration approximately 20 nM. However, higher peak concentrations occur after parenteral dosage

are required to inhibit purified RSK2 (~ 15 mM) and stimulated RSK2 kinase activity in cells (5–20 mM), well above therapeutic concentrations.

A notable effect of salicylate is down-regulation of COX-2 synthesis. In two studies, therapeutic salicylate concentrations decreased PMA-mediated stimulation of COX-2 levels (Cieslik et al. 2005; Xu et al.1999; Table 2). Relative down-regulation of COX-2 could lead to decreased levels of prostanoids. However, a much higher IC_{50} for inhibition of TNF-α and IFN-γ-mediated induction of COX-2 synthesis was reported in an osteoblast line (Chae et al. 2004; Table 2) while Mitchell et al. (1997) found no effect of salicylate (at 0.62 mM) on IL-1β-mediated up-regulation of COX-2 synthesis or on NF-κB activation (at 1 mM salicylate).

A notable non-COX effect of salicylate is its inhibition of purified myeloperoxidase, an enzyme in neutrophils which converts chloride and hydrogen peroxide to hypochlorous acid. The IC_{50} for this inhibition is 9.4 μM, which is well within the therapeutic range of unbound salicylate (Table 2). Inhibition of myeloperoxidase may contribute to salicylate's

mechanism of action as hypochlorous acid promotes oxidative stress, a mediator of chronic inflammation in rheumatoid arthritis (Stamp et al. 2012).

A non-COX effect of salicylate that is of note is its activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) (Hawley et al. 2012; Bao et al. 2018; Table 2), which is an important regulator of glucose metabolism. Increased activity of AMPK leads to decreased blood glucose and may be an important mechanism of action of metformin, a major treatment of Type II diabetes. This discovery about salicylate was presented in the major journal, Science, and was also the subject of a commentary in the same journal. However, the authors of the original paper, Hawley et al. (2012), were concerned that the required concentration of salicylate may be supratherapeutic, but this caveat was not discussed in the commentary (Shaw and Cantley 2012). Salicylate (5 mM, a supratherapeutic level), on upregulation of AMPK phosphorylation in LPS-treated THP-1 monocyte cells is accompanied by induction of apoptosis, reduced cell proliferation and increased secretion of inflammatory factors, IL-1β and TNF-α via an AMPK-dependent mechanism and AMPK-independent suppression of LPS-induced IL-6 (Bao et al. 2018; Table 2).

In the 1950s–1960s, several studies on the effects of salicylate on intermediary metabolism showed that 0.5–5 mM salicylate decreased the production of acidic intermediates in the tricarboxylic acid (Krebs) cycle. However, the formation of ATP is inhibited to a greater degree i.e. there is uncoupling of oxidative phosphorylation. Oxidative phosphorylation in slices of cartilage is inhibited substantially by 2.5 mM salicylate (Whitehouse (1964), Table 2). Inhibition of oxidative phosphorylation and increased levels of acids in mitochondria may be a major cause of the hyperthermia and acidosis produced by overdoses of aspirin, or sodium salicylate and aspirin.

In summary, the effects of salicylate on prostanoid synthesis in intact cells are produced at levels consistent with binding to serum albumin i.e. greater potency with decreasing albumin. By contrast non-COX effects are generally produced at supratherapeutic levels of salicylate.

R- and S-ibuprofen

Ibuprofen is a well-known NSAID which, like several drugs, is a racemate made up of two optical isomers (enantiomers) (for review see Rainsford 2015). Unusually, after its dosage to human subjects, the R-enantiomer is partially (70%) converted to the S-enantiomer (Lee et al. 1985). S-ibuprofen is a much more potent inhibitor of prostanoid synthesis than the R-enantiomer (Table 3). Further, the IC_{50} value for inhibition of prostanoid synthesis by S-ibuprofen is generally within its range of plasma concentrations (Table 3), making it likely that S-ibuprofen inhibits prostanoid synthesis in vivo. By contrast, the IC_{50} values of R-ibuprofen exceed plasma concentrations making it unlikely that R-ibuprofen inhibits prostanoid synthesis in vivo (Table 3). Furthermore, the presence of plasma protein markedly affects the IC_{50} values of racemic ibuprofen enantiomers in vivo and in vitro when their activities are due to inhibition of prostanoid synthesis (Table 3). The cerebral uptake of racemic ibuprofen has been measured in protein-free perfusing solution and is slowed significantly by the addition of albumin to the perfusate (Parepally et al. 2006).

Although R-ibuprofen is a weak inhibitor of prostanoid synthesis by COX-2, it does decrease the oxidation of endogenous endocannabinoids, 2-AG and AEA (Table 3) and, consequently, decreases the formation of the corresponding prostaglandin analogues, 2-prostaglandin glycerol (2-PG) and prostaglandin ethanolamide (PGEA), the function of which is not known. Studies on the actions of R-ibuprofen on the oxidation of endocannabinoids have largely been conducted with purified COX-2 protein. However, R-ibuprofen does inhibit the oxidation of endocannabinoids by a cellular system, although the concentration of R-ibuprofen (IC_{50} 10 µM) appears too high for clinical significance (Table 3).

The clinical pharmacological effects of R-ibuprofen are unclear because of its conversion to S-ibuprofen, which resembles other non-selective NSAIDs in its analgesic, anti-inflammatory and antiplatelet actions. The in vivo actions associated with the R-configuration are often concluded from experimental and clinical properties of R-flurbiprofen, which is metabolised to the S-enantiomer only to a very small degree. The anti-inflammatory activity of R-flurbiprofen is weaker than that of S-flurbiprofen with less gastrointestinal damaging effects. At doses of 1 mg/kg, R-ibuprofen reduces carrageen-induced oedema in the rat by approximately 15%, significantly less than the same dose of S-flurbiprofen (approximately 55% inhibition) (Geisslinger et al. 1993). However, the two enantiomers have almost identical potencies in the Randall-Selitto test, which compares pain from pressure on inflamed and untreated rat paws (Geisslinger et al. 1993). Both enantiomers of flurbiprofen reduce pain from an experimental procedure in man (Geisslinger and Schaible 1996). R-flurbiprofen has therefore been suggested to be an improved NSAID.

Like other non-selective NSAIDs, racemic ibuprofen inhibits the excretion of PGM (by about 50%) (Stichtenoth et al. 1996).

Naproxen

Naproxen, like ibuprofen, is a phenylpropionate, which can exist as two enantiomers. The S-enantiomer is easily separable from the R-enantiomer and consequently, naproxen in

therapeutic products is essentially pure S-enantiomer. S-naproxen is usually simply termed naproxen without specification of its stereochemistry, but the inclusion of R-naproxen in Table 4 has led to specification of the labelling of the enantiomer in this review. The pharmacological properties of the two enantiomers have only been compared with respect to oxidation of arachidonic acid (AA) and endocannabinoids. S-naproxen inhibits oxidation of AA more than R-naproxen, but the R-enantiomer does inhibit oxidation of the endocannabinoid, 2-AG (Table 4). S-naproxen inhibits COX-1 and COX-2 and is, therefore, considered to be a conventional NSAID. Not surprisingly, S-naproxen has analgesic, anti-inflammatory, anti-pyretic and anti-platelet actions similar to other non-selective NSAIDs. Nevertheless, R-naproxen should be considered as a potential clinically-active compound with actions on the central nervous system.

Indomethacin

Indomethacin is an old NSAID, which is widely considered as a model non-selective NSAID. Inhibition of prostanoid synthesis has been shown in many studies at therapeutic or near therapeutic levels of indomethacin (Table 5). It is well known that therapeutic dosage of indomethacin inhibits prostanoid synthesis in vivo both in clinical and in experimental animal studies. Indomethacin was identified as an inhibitor of prostanoids in the original studies by Vane (1971) (Table 5). Its pharmacological properties, particularly its inhibition of prostanoid synthesis, have been reviewed widely (Lucas 2016). Many experimental studies analysing the physiology and pharmacology of prostanoids have utilised indomethacin as an inhibitor of prostanoid synthesis. Indomethacin also decreases non-COX pathways, but only at supratherapeutic levels, which are not considered relevant to its clinical effects (Table 5). Indomethacin is, however, a very potent inhibitor of oxidation of the endocannabinoid, 2-AG (Table 5) but, unlike ibuprofen, indomethacin is a symmetrical compound. Consequently, inhibition of AA oxidation to prostanoids and also oxidation of 2-AG to prostaglandin derivatives must be mediated by the same molecular structure. The classical analgesic and anti-inflammatory actions of indomethacin would, therefore, appear to the combination of these two primary actions.

Indomethacin inhibits prostanoid synthesis in all cellular systems examined (Table 5). An unexpected aspect of the anti-prostanoid efficacy of indomethacin, however, is the lack of a significant difference in effect when indomethacin is incubated with whole blood, 0.35% serum albumin and 3.5% serum albumin (Table 5) (Warner et al. 2006). Indomethacin is approximately 10 times less potent when incubated with purified COX-1 and COX-2 proteins than with intact cells (Mitchell et al.1993). As is the case with racemic ibuprofen, the uptake of indomethacin by the brain, however, follows the pattern predicted by binding to serum protein. Indomethacin is taken up rapidly from a protein-free infusion into the common carotid artery, but the addition of albumin slows uptake by the brain (Parepally et al. 2006).

Celecoxib

Celecoxib is a selective COX-2 inhibitor, which has been widely studied for other cellular effects in vitro (Table 6). Celecoxib inhibits prostanoid synthesis at submicromolar concentrations, consistent with the inhibition of COX-2 in cellular systems in vitro (Table 6). However, there is one report of IC_{50} values for celecoxib in the range of 7–24 μM in a monocyte cell line at various albumin levels (Table 6) (Warner et al. 2006). van Wijngaarden et al. (2007) have also reported that 10 μM celecoxib potentiates the cytotoxic activity of doxorubicin in a cellular system in the absence of added albumin. This activity is well above the unbound concentration.

Inhibition of prostanoid synthesis in vivo is confirmed by reduced urinary excretion of the metabolite, PGM. This effect is shown clearly in smokers with elevated levels of PGM (Duffield-Lillico et al. 2009). Celecoxib also increases the urinary levels of leukotriene E_4, particularly when PGM levels are high. This change is an indicator of shunting of AA to leukotrienes when prostanoid synthesis is blocked by celecoxib, with increased potential for pulmonary inflammation. Inhibition of the urinary recovery of PGM has also been utilised in physiological studies on renal function (Stichtenoth et al. 2005). This study in healthy female volunteers treated with either celecoxib or indomethacin demonstrated that "Renin-release in healthy humans with normal salt intake is COX-2 dependent. While COX-1 is critical for renal and systemic PGE(2) production, renal prostacyclin synthesis is apparently COX-2 dependent". Measurement of PGM has been included in several clinical trials on the combination of celecoxib and cytotoxic agents (Mutter et al. 2009; Edelman et al. 2017).

Celecoxib is a potent inhibitor of the oxidation of both AA and 2-AG (Table 6). Like indomethacin, celecoxib is a symmetrical molecule, and the single molecular structure must inhibit the oxidation of both AA and 2-AG. Celecoxib binds well to carbonic anhydrase, with X-ray crystallography demonstrating a close fit of celecoxib to the three-dimensional structure of type II carbonic anhydrase (Weber et al. 2004). However, oral administration of celecoxib does not result in the characteristic bicarbonate diuresis or hyperchloremic metabolic acidosis that results from administration of the major carbonic anhydrase, acetazolamide (Alper et al. 2006). However, oral celecoxib does reduce the intraocular pressure of rabbits with glaucoma (Weber et al. 2004). The

contrast between some of the in vivo effects of celecoxib and acetazolamide correlates with the in vitro IC_{50} values of purified type II carbonic anhydrase (CAII). The IC_{50} of celecoxib (410 nM) is much greater than that of the classical inhibitor, acetazolamide (7.5 nM), consistent with the contrasting clinical effects of the two drugs (Knudsen et al. 2004; Table 6). Celecoxib may, however, inhibit other isozymes of carbonic anhydrase, although details are lacking (Knudsen et al. 2004).

Diclofenac

Diclofenac is a widely used moderately selective non-selective NSAID which is available as its sodium salt. While diclofenac interacts with both COX-1 and COX-2, it has a moderate preference for COX-2 (Pantziarka et al. 2016). As with other NSAIDs, diclofenac inhibits prostanoid synthesis by intact cells at near therapeutic concentrations, but inhibits non-COX functions at concentrations 2.5- to 422-fold higher than peak therapeutic plasma levels (Table 7).

The principle seen with salicylate (Table 2) and S-Naproxen (Table 4) for both COX-1 and COX-2 and celecoxib for COX-2 (Table 6) that the potency of the inhibitory effect on COX activity decreases as the serum protein concentration increases is seen with diclofenac for COX-1 activity in platelets and is also seen in a cellular assay for COX-2, but the drug shows ~ threefold greater potency in a whole blood COX-2 assay than in the cellular assay with added BSA simulating ~ 10% serum. It should be noted also that all in vitro cell assays of COX activity shows effectiveness at ~ 100-fold higher concentrations than the unbound concentration of diclofenac (20 nM), while in the whole blood assays diclofenac is effective at > fourfold lower concentrations than the total peak plasma concentration (4 mM).

Effects on cell viability in cancer cell lines in the presence of 10% serum occur at concentrations 100-fold higher than peak plasma concentrations. Though apparently more potent in inhibiting expression of VEGF gene expression in primay osteoblast cultures, effective concentrations ar still higher than peak plasma concentrations and 500-fold higher than unbound concentrations in plasma. Diclofenac concentrations are also substantially higher than theraputically relevant concentrations in diverse endpoint assays including survival of HL-60 cells, reduction of lactonase activity in U937 cells, reducing oxygen consumption in kidney cells and modulating sulfur-mustard-induced cytokine production (Table 7).

Conclusion

It is now evident that in vitro cell culture effects of NSAIDs that are relevant to their analgesic and anti-inflammatory actions in vivo are consistent with the well-characterised inhibition of prostanoid synthesis at therapeutic doses. In addition, many in vitro actions of the NSAIDs on prostanoid synthesis are decreased by increased plasma albumin in the medium. A related action of NSAIDs is their inhibition of the oxidation of the endocannabinoids, 2-AG and AEA. Effects of NSAIDs on the endocannabinoids are also related to their plasma concentrations. Inhibition of the oxidation of 2-AG and AEA occurs at therapeutic or near therapeutic concentrations and should be considered as a second mechanism of action of NSAIDs in vivo. The only other example of a clinically significant non-prostanoid effect of an NSAID is the inhibition of myeloperoxidase by salicylate.

NSAIDs commonly have cellular effects at supratherapeutic concentrations which are not due to inhibition of synthesis of prostanoids. An important general question is: "Is it worthwhile to conduct detailed studies on NSAIDs, or other drugs, when preliminary work indicates that activity is shown only at supratherapeutic concentrations?" This is not a new question but is still highly relevant to valid conclusions about the pharmacology of NSAIDs and many other drugs. The data compiled here provides compelling evidence that, for the well-studied NSAIDs, these alternative mechanisms are unlikely to have relevance in patients.

For the observed non-COX effects of NSAIDs to be clinically relevant we are required to postulate that NSAIDs are actively accumulated in target cells. Mechanistically, this could be achieved through active transport as shown by Burczynski et al. who found uptake of protein-bound NSAIDs as seen with fatty acids. The lack of a sensitive and consistent method to measure the concentration of NSAIDS in cells has made these potential mechanisms difficult to evaluate. Recent advances in mass spectrometry that improve sensitivity to analyte quantitation, now make it possible to measure drug concentrations in single cells (Bensen et al. 2021). Application of this approach to establish intracellular NSAID concentration in cell cultures treated with drug at low serum conditions and in tissues would be of benefit to relate these findings to the clinical pharmacology of NSAIDs.

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