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A Review On Biological Screening Of Anti-Inflammatory Drugs

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Abstract:

The competitive inhibitors of cyclooxygenase (COX), the enzyme that facilitates the bioconversion of arachidonic acid to inflammatory prostaglandins (PGs), are non-steroidal anti-inflammatory medicines (NSAIDs). The usage of them is linked to side effects like renal and gastrointestinal toxicity. While the undesirable side effects of NSAIDs result from the suppression of COX-1 activity, the therapeutic anti-inflammatory impact is provided by the inhibition of COX-2. Therefore, it was assumed that side effects would be lower with more specific COX-2 inhibitors. As safer NSAIDs with an enhanced stomach safety profile, a variety of selective COX-2 inhibitors (rofecoxib, celecoxib, valdecoxib, etc.) were developed. Nonetheless, the recent withdrawal of some COXIBs from the market, including rofecoxib, because of their detrimental effects on the cardiovascular system obviously motivates researchers to investigate and assess substitute templates has inhibiting action on COX-2. Research on the development of selective COX-2 inhibitors is still being drawn by the discovery of novel applications for these drugs in cancer treatment and neurological conditions including Parkinson's.

Key words: Coxibs, SAR, NSAIDs, Cyclooxygenase, and Selective COX-2 Inhibitors

Introduction:

- **Inflammation**- The term ‘Inflammation’, derived from ‘flame’ which means warmth or redness. The cardinal features of inflammation had observed by Aulus Cornelius (*ca.*25 BC to *ca.* 50 AD) i.e. pain (dolor), redness (rubor), warmth (calor), and swelling (tumor).

- **Anti- inflammatory drugs**- A drug or substance that reduces inflammation (redness, swelling, and pain) in the body. Anti-inflammatory agents block certain substances in the body that cause inflammation.

Classification of Anti-inflammatory drugs:

I. Non selective COX inhibitors-
   A Salicylates
   e.g. Aspirin
   B. Propionic acid derivatives
   e.g. Ibuprofen, Naproxen, Ketoprofen, Flubiprofen
   C. Fenamates
   e.g. Mephenamic acid
   D. Enolic acid derivatives

e.g. Piroxicam, Tenoxicam

E. Acetic acid derivatives

e.g. Ketorolac, Indomethacin, Nabumetone

F. Pyrazolone derivatives

e.g. Phenylbutazone, Oxyphenbutazone, Propyphenazone

II. Preferencial COX 2 inhibitors

e.g. Nimesulide, Diclofenac, Aceclofenac, Meloxicam, Etorolac

III. Selective COX 2 inhibitors

e.g. Celecoxib, Etoricoxib, Parecoxib

IV. Analgesic antipyretic activity with poor anti-inflammatory action

A. Para aminophenol derivative

e.g. Paracetamol (Acetaminofen)

B. Benzoxazocine derivative

e.g. Nefopam

Researchers have postulated that the anti-inflammatory benefits of non-steroidal anti-inflammatory medicines (NSAIDs) are produced by a distinct mechanism than the often reported adverse effects of these treatments since the identification of a second isoenzyme of cyclooxygenase (COX), COX-2. They include suppression of platelet aggregation, renal function, and impairment of the stomach's cyto- protection. The constitutive COX-1 isoenzyme, referred to as a "housekeeping" enzyme that is present in most tissues under normal settings, whereas COX-2 production is elevated, especially during inflammatory processes. It has been suggested that the important target for the anti-inflammatory benefits of non-steroidal anti-inflammatory medicines is COX-2 inhibition, whereas the negative effects of these drugs on the stomach and kidneys are caused by inhibition of COX-1.

To compare and characterize the relative inhibitory activities of non-steroidal anti-inflammatory medications against COX-1 and COX-2, several in vitro tests have been established. The ratios of the IC50 values for COX-1 and COX-2 are used to indicate the indices of selectivity, whereas the inhibitory activities are expressed as IC50 values, or the doses which inhibit activity by 50%. Numerous systems have been created, which has led to a wide range of IC50 values and ratios as well as occasionally unclear comparisons.

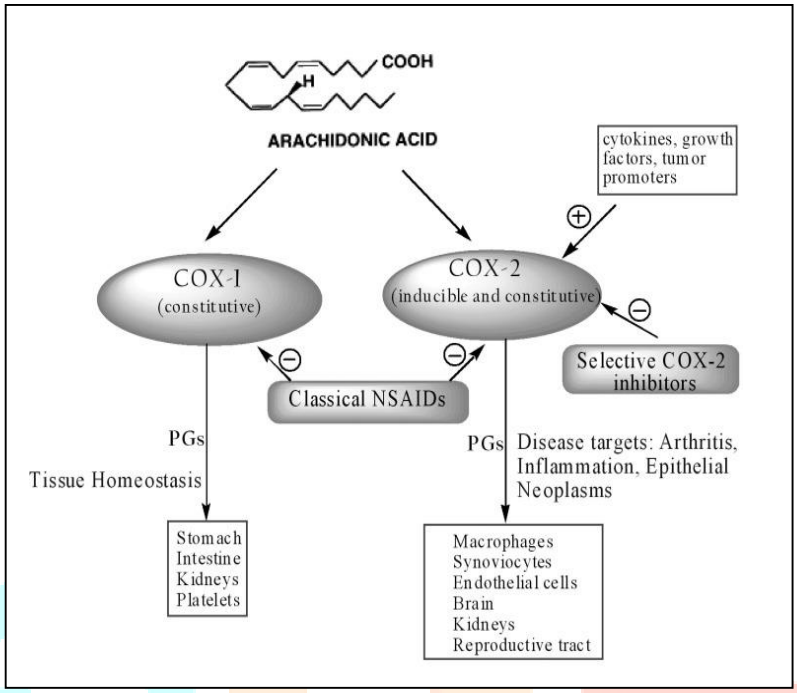
This review seeks to highlight test interpretation difficulties by offering a critical examination of the existing in vitro tests. Furthermore, the pharmacokinetic characteristics of every molecule will be taken into account, along with an analysis of the in vivo significance of the in vitro results. Outcomes pertaining to human pharmacy Studies that have looked at the differentiating inhibition of prostanoid production in vivo in distinct tissues will be contrasted with COX-2 selectivity findings obtained in vitro.

The COX isozymes:

Even though NSAIDs have been used extensively over the past century, their exact mechanism of action was unknown until Vane discovered the COX enzyme in 1971, which serves as their molecular target. A second isoform (COX-2) that was different from the previous one and subsequently dubbed COX-1 was found in the early 1990s. Two isoenzymes are COX-1 and COX-2. Given that isoenzymes are genetically distinct proteins, the human genes corresponding to the two enzymes are found on distinct chromosomes and exhibit dissimilar characteristics. Numerous organs express COX-1 constitutively, and the PGs it produces mediate "housekeeping" tasks such platelet aggregation, renal blood flow control, and gastric mucosa cytoprotection. On the other hand, COX-2 expression is quickly activated but is not seen in the majority of normal tissues.

Increased synthesis of prostaglandins (PGs) in inflammatory and neoplastic tissues due to stimuli like proinflammatory cytokines (IL-1b, TNFα), lipopolysaccharides, mitogens and oncogenes (phorbol esters), growth factors (fibroblast growth factor, FGF; platelet-derived growth factor, PDGF; epidermal growth factor, EGF), hormones (luteinizing hormone, LH), and abnormalities of water-electrolyte hemostasis. Thus, pathogenic processes including inflammation and several forms of cancer have been linked to the inducible isozyme .

Recent research has revealed that there is more complexity in the relationship between the two isoforms. Indeed, COX-1 may have a role in inflammatory processes, whereas COX-2 is constitutively produced in a number of tissues and organs, including the kidneys and the brain and reproductive tract.



Structure of Enzymes:

The endoplasmic reticulum (ER) contains membrane-bound enzymes known as the COX isoenzymes. The crystal structures of human and murine COX-2 were soon after, having been originally described in 1994 together with the three-dimensional structure of the ovine COX-1. As a homodimer, COX tries to form monomeric species, but all it has produced are inactive enzymes. The overall structures of COX-1 and COX-2 are extremely conserved, and the crystal structures of the COX isoforms are quite structurally homologous and consistent with a high sequence identity (around 60%). Three structural domains make up the COX monomer: an N-terminal domain that resembles epidermal growth factor (EGF), a membrane binding domain (MBD) that is roughly 48 amino acids long and attaches the protein to one leaflet of the lipid bilayer, and a huge C-terminal globular catalytic domain including the peroxidase active site, which has the heme cofactor, and the COX active site, which can hold the substrate or inhibitors. Although these locations are separate, they are related architecturally and functionally.

Evaluation of COX-2 inhibitory efficacy in vitro:

Enzyme immunoassay was used to assess COX-2 inhibition in vitro. By following the manufacturer's instructions and utilizing an enzyme immunoassay (EIA) kit (Catalogue No. 560131, Cayman Chemical, Ann Arbor, MI, USA), the test compound's ability to inhibit COX-2 (human recombinant) was examined. After dissolving the test chemical in DMSO, a solution was obtained with a final concentration of 10 µM. Ten microliters of the 10-µM test drug solution were added to a reaction buffer solution (960µl, 0.1M Tris-HCL, pH-8, including 5 mM EDTA and 2 mM phenol) that included COX-2 enzymes (10 µl) and heme (10 µl). These solutions were then incubated at 37°C for 10 minutes. Following that, 10 µl of After adding the AA solution, the COX reaction was stopped by adding 50 µl of 1 M HCL. Cyclooxygenase catalyzes the conversion of arachidonic acid (AA) to PGH2, which is then reduced to PGF2α by stannous chloride. By reducing PGH2 with 100 µl of stannous chloride, PGF2α was generated, and its level was assessed using an enzyme immunoassay technique. The fight for the restricted quantity of PG antiserum between PGs and PG-acetyl cholinesterase conjugation (PG tracer) served as the basis for this. Since the concentration of PGs tracer is maintained constant while the concentration of PGs fluctuates, the quantity of PG tracer that can bind to the PG antiserum is inversely proportional to the concentration of PGs in the well. This combination of antibodies and PG binds to monoclonal antibodies against mice that have been previously affixed to the well. After washing the plate to get rid of any unattached chemicals, the well is filled with Ellman's reagents, which include the acetylcholine esterase substrate. The amount of PG tracer bound to the well and the amount of free PGs present in the well during the incubation are inversely proportional, and the product of this enzymatic reaction produced a distinct yellow color, which was measured spectrophotometrically (using a Micro titre Plate reader) at 412 nm: Absorbance α [PG tracer bound] α 1/PG units. By comparing the chemical treated by the control incubations, the percentage inhibition was computed.

In vitro human whole blood assay:

This assay uses whole blood that has been clot to measure COX-1 activity and whole blood that has been stimulated with lipopolysaccharide to measure COX-2 activity. There are several benefits to the human whole-blood assay. The target cells for the anti-inflammatory effects (monocytes) and side effects (platelets) of nonsteroidal anti-inflammatory medications (NSAIDs) are intact human cells. Additionally, the complete blood's plasma proteins provide a more accurate depiction of interactions that occur in vivo when nonsteroidal anti-

inflammatory medications are present. To enable a direct comparison of the outcomes from each assay, the same volunteer (or patient) provides the entire blood required for both tests at the same time. Lastly, blood from volunteers (or patients) who have been given nonsteroidal anti-inflammatory medication treatment in the past (ex vivo assay), enabling a comparison of the applicability of in vitro findings in vivo.

The primary disadvantage is that distinct incubation periods are required for COX-1 and COX-2 since COX-2 must be stimulated. Furthermore, target cells for the therapeutic or deleterious effects of nonsteroidal anti-inflammatory medicines would be more typical of cell types other than platelets and monocytes, such as stomach mucosal cells and synoviocytes.

Table below presents a summary of the findings from various laboratories that used the human whole blood assay. With a few notable exceptions, the COX-2 selectivity rank order is consistent across laboratories. On both isoenzymes, standard non-steroidal anti-inflammatory medications work about equally well. Diclofenac has the best profile among non-steroidal anti-inflammatory medications that are commonly used. Etodolac, nimesulide, and meloxicam are examples of compounds that selectively (ratio 3 to 30) inhibit COX-2; in contrast, flosulide, DuP-697, NS-398, L-745, 337, and SC 58125 exhibit COX-2 selectivity.

This overall pattern is consistent with the outcomes of utilizing human recombinant enzymes. The COX-1/COX-2 selectivity ratios achieved with various non-steroidal anti-inflammatory medicines utilizing human whole-blood test or human recombinant enzymes are shown in Fig. 2. The bars show the range of ratios that various laboratories were able to generate using the same model. This illustration clearly shows a parallel pattern. Compared to the whole-blood experiment, the selectivity ratio range is larger when whole cell recombinant enzymes are utilized. Furthermore, compared to recombinant enzymes, the variations in COX-2 selectivity amongst the drugs are less pronounced in the whole blood experiment. Variations in the concentrations of proteins could be connected to this.

As of right now, new in vitro assays are being created. Human cells used in these test systems include synoviocytes, chondrocytes, and gastric mucosa cells, which are targets for the anti-inflammatory or side effects of nonsteroidal anti-inflammatory medications. Complete validation of these models still requires the establishment of standardized circumstances. Nevertheless, the drugs evaluated showed a similar trend for ranking COX-2 selectivity, as seen with human recombinant enzymes and the human whole blood testing [30–32]. It should be emphasized, nonetheless, that it will be challenging to Figure 2 shows selectivity ratios for a number of non-steroidal anti-inflammatory medications achieved with human recombinant enzymes in a whole cell assay (dark gray bars) [19, 20, 22], in a microsomal assay (light gray bars) [16, 17, 19], or in a black bar assay for human whole blood [24–27, 36]. Only estimates were used to determine the comparatively low values for L745,337 and SC58125 (hatched bars) in the whole blood assay. Because of issues with solubility at high doses, IC50 values for cyclooxygenase-1 inhibition could not be obtained, making exact ratios impossible to calculate. Use these models to simulate drug binding to proteins as nearly as possible to a human whole-blood experiment.

In vivo assay:

Carrageenan-induced rat paw edema assay: Using carrageenan-induced rat paw edema, the selected samples demonstrating promising average (activity in all solvents) COX-2 selective activities were assessed for in vivo anti-inflammatory research. animal model of edema. The assay ran according to the preceding description.45 In short, 20 µl of carrageenan (1 % w/v) in 0.9% saline was subplantarly injected to cause edema on the right hind paw. One hour prior to carrageenan injection, the extract of the chosen samples was made in 1% w/v gum acacia and given orally at doses of 100 mg/kg and 250 mg/kg. A standard group received indomethacin (20 mg/kg, p.o.) and a control group was given a vehicle alone. After induction, the volume of the injected and contralateral paws was measured one, three, and five hours later.

A plethysmometer (Orchid Scientific Laboratory) to measure the degree of inflammation. The value was given as the percentage of volume reduction at various time intervals relative to the control group.

In Vivo Wistar rat model study:

Live an overabundance of anesthetic was administered intraperitoneally to induce unconsciousness in male Wistar rats (220 ± 250 g; Tuck, Rayleigh, U.K.) using thiobutabartibal sodium (Inactin; 120 mg kg71, i.p.; RBI, Natick, U.S.A.). Rats were given specific medications on a minimum of four separate research days. A homoeothermic blanket that was attached to a rectal probe was used to keep the patient's body temperature at 37 °C. Ventilation was facilitated by cannulating the trachea. A pressure transducer (type 4-422-0001, Transamerica Instruments) was cannulated into the right carotid artery and attached to monitor systemic blood pressure, which was displayed on a Graphtec Linearcorder. In order to facilitate medication injections and saline infusions, the jugular vein was also cannulated. After surgery, the animals were kept for 30 minutes to stabilize before time (t=760) at which a control plasma sample was extracted. An intravenous bolus of aspirin (20 mg kg71; n = 4), diclofenac (3 mg kg71; n = 4), L-745,337 (30 mg kg71; n = 5), nimesulide (15 mg kg71; n = 4), sodium salicylate (20 mg kg71; n = 4), or sulindac (10 mg kg71; n = 5) was given to the rats one hour later (t=0). The carotid artery was used to draw 300 ml of blood at t=760, 5, 60, 120, 180, 240, 300, and 360 minutes. The samples were placed in a 12000 g centrifuge for two minutes at 48 degrees Celsius. The plasma was extracted and then snap-frozen in liquid nitrogen with heparin (15 U ml71, National Veterinary Supplies, Stoke on Trent) added. Following each blood draw, an intravenous injection of 300 milliliters of warm saline was given. By the conclusion of

Cell culture:

When exposed to IL-1b, the human epithelial carcinoma cell line Cell culture A549 (ECACC Ref. No. 86012804) expresses COX-2 (Mitchell et al., 1994). This cell line's ability to produce PGE2 makes it a useful indicator of COX-2 activity. A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, Poole, U.K.) supplemented with 10% fetal bovine serum (FBS; Sigma, Poole, U.K.) and kept in a humidified environment of 5% CO2-95% air at 378C. Prior to use in the experimental methods, cells were grown to confluence in 96-well plates using seeding. The cells were cultured in fresh DMEM supplemented with 10% FBS and 10 mg/ml of IL-1b for 24 hours in order to promote COX-2 expression. The media was changed out with new DMEM prior to the experiment: Ca2+- free modified Krebs-Ringer solution (see below) (4 : 1, v/v) at 37° C.

Washed platelets: The thromboxane (TX) B2 generation by platelets served as a COX-1 activity index. Blood was drawn by venepuncture into plastic tubes covered with 0.1% porcine gelatine in water, at 378C for 1 ± 3 hours. The subjects were healthy and had not used NSAIDs for at least two weeks. The tubes also contained 3.15% trisodium citrate (1: 9, v/v) trisodium citrate. To create platelet rich plasma, the blood was centrifuged at 2006g for 7 minutes (PRP). After that, 300 ng ml71 of prostacyclin was added to the PRP, and then % bovine serum albumin. To sediment the platelets, the pellet was gently resuspended and then 300 ng of prostacyclin (ml71) was added by centrifugation at 10006g for 15 minutes. After that, the supernatant was discarded and replaced with the same volume of modified KrebsRinger solution that was free of Ca2+ at

37° C (10 mM NaHCO3, 20 mM HEPES, 120 mM NaCl, 2 mM Na2SO4, and 4 mM KCl). 0.1 glucose, 0.1%. After being pelleted once more, the platelets were resuspended at 37°C in a modified Krebs Ringer buffer free of Ca2+, matching half of the original plasma volume. After 30 minutes, the platelet solution was diluted 1: 5 in DMEM with 10% FBS added, and it was then plated into 96-well plates with gelatin coating (100 ml well 71). Analyzing the effects of NSAIDs on COX-1 and COX-2 Ten milliliters of plasma were introduced to a medium containing either preinduced A549 cells or washed platelets in order to measure the activity of NSAIDs in the plasma obtained from the rats. Concentration response curves for L-745,337 (n=5), nimesulide (n=4), sulindac (n=4), or sulindac sulphide, aspirin (n=4) or salicylate (n=4) (0.1 nM to 1 mM), diclofenac (n=4)

On the same culture plates used to evaluate the appropriate plasma series, 0.1 mM) were also generated. The cells or platelets were incubated for a further 15 minutes at 378C with the addition of calcium ionophore A23187 (50 mM) during a 30-minute incubation period. After the incubation period, the platelet suspension-containing plates were centrifuged for five minutes at 15006g (48C), with the supernatant being collected and snap frozen until radioimmunoassay analysis. A549 plate medium was also taken out and frozen. Two sets of control wells were included in each plate containing cells and platelets; one set was treated with a vehicle (0.1% dimethyl sulfoxide), while the other set received control plasma that was removed at t=760. The calculation of test chemicals' or plasma samples' inhibition of COX-1 and COX2 was proportion of the activity that was recorded in the matching control wells. At least four distinct experimental days were required to determine the effects of the vehicle, plasma series, and NSAIDs on COX activity in at least three different determinations (wells). On more than one trial day, different sets of plasma samples or medication dilutions were not used.

Materials: All of the medications were dissolved in a 5% bicarbonate - 2.5% glucose booster in water for the in vivo technique, and the mixture was sonicated until a clear solution or a very fine suspension was achieved. One milliliter of the needed dosage was dissolved and injected over the course of one hundred seconds. All of the medications were dissolved in DMSO to create stock solutions for the in vitro tests, ranging from 0.1 to 1 M, after which it was further diluted in DMEM. Unless otherwise noted, all chemicals were purchased from Sigma (Poole, U.K.). A contribution of L-745,337 was given by Merck Frosst in Canada. We bought sulindac sulphide from Aniti (Exeter, U.K.). Antisera to PGE2 and TXB2 were procured from Sigma (Poole, U.K.) for the radioimmunoassay; [3 H]-PGE2 and [3 H]-TXB2 were acquired from Amersham (Little Chalfont, U.K.). Genzyme provided the IL-1b (Kings Hill, U.K.).

Analyzing data:

The findings are shown as mean+s.e.m. The concentration response curves were fitted with a variable slope sigmoidal regression.

ANOVA, a two-way analysis of variance, was employed to assess statistical differences among dosage response curves. For unpaired observations, the t-test was used to compare IC50 values. The one-sample t-test was used to identify significant variations from
standardized controls. A statistically significant P value was defined as one that was less than 0.05. To do all analyses and regressions, GraphPad Prism (GraphPad Software, San Die) was used.

The main drawback is that since COX-2 has to be induced, different incubation times are used for COX-1 and COX-2. In addition, cell types other than platelets and monocytes, such as gastric mucosal cells and synoviocytes, would be more representative of target cells for the thera peutic or adverse effects of nonsteroidal anti-inflammatory drugs.

Results obtained by different laboratories, using the human whole blood assay, are summarized in Table 4. The rank order for COX-2 selectivity is reproducible between laboratories with a few exceptions. Standard non-steroidal anti-inflammatory drugs are approximately equally effective on both isoenzymes. Among standard non-steroidal anti- inflammatory drugs, diclofenac has the most favorable profile. Compounds such as etodolac, nimesulide, and meloxicam inhibit COX-2 preferentially (ratio 3 to 30), while flosulide, DuP-697, NS-398, L-745,337, and SC 58125 are selective for COX-2.

This general trend is in agreement with the results obtained using human recombinant enzymes illustrates the COX-1/COX-2 selectivity ratios obtained with several non-steroidal anti-inflammatory drugs, using either human recombinant enzymes or the human whole- blood assay. The bars indicate the range of ratios obtained with the same model by different laboratories. A parallel trend is obvious from this representation. However, the range of selectivity ratios is wider when whole cell recombinant enzymes are used than in the whole-blood assay. In addition, the differences in COX-2 selectivity between the compounds are less marked in the whole

blood assay than with recombinant enzymes. This may be related to differences in protein concentrations. New in vitro assays are currently being developed. These test systems use human cells such as gastric mucosa cells, chondrocytes or synoviocytes which are target cells for the anti-inflammatory or adverse effects of nonsteroidal anti- inflammatory drugs. Standardized conditions still have to be established to validate these models completely. However, a similar trend for ranking COX-2 selectivity, as seen with human recombinant enzymes and the human whole blood assay, was found for the compounds tested

Conclusion:

The most pertinent models show a similar trend in non-steroidal anti-inflammatory drug selectivity, notwithstanding the multitude of in vitro assays that have been established and the variations among these systems. Non-steroidal anti-inflammatory medicines can be categorized as nonselective, COX-2 preferential, or COX-2 selective based on the outcomes of experiments conducted using human recombinant enzymes in whole cells or in the human whole-blood assay.

While in vitro systems are valuable for assessing activity, it is crucial to carefully consider the therapeutic implications of these data. It is not possible to anticipate the degree of COX-1 and COX-2 inhibition in vivo at a particular dose solely based on in vitro studies. Each compound's pharmacokinetic characteristics, such as its plasma levels, distribution, and ability to bind to plasma proteins, must be considered.

Numerous In pertinent in vitro test systems, drugs have consistently demonstrated preferential or selective suppression of COX-2. Using indicators such as urine excretion of PGE2, serum TXB2 levels, or platelet aggregation, human pharmacological trials also demonstrated a COX-1 sparing effect for some of these drugs. To fully understand how these substances affect the creation of prostaglandins in other organs, like the synovium and stomach mucosa, more research is required. Nevertheless, head-to-head comparisons in extensive clinical studies are required to provide the final assessment of the therapeutic significance of COX-2 selectivity.

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