The Side Effects of Non-steroidal Anti-inflammatory Drugs: Are They Attributed to the Selective Inhibition of Different Isoforms of Cyclo-oxygenase ?

Pravit Akarasereenont, M.D., Ph.D.*
Supornchai Kongpatanakul, M.D.*
David A. Henry, M.B., Ch.B.**
Christoph Thiemermann M.D., Ph.D.***

Abstract : **Cyclooxygenase (COX) exists as two isoforms. COX-1 is present under physiological conditions and COX-2 is induced by inflammatory stimuli. Previous studies *in vitro* have suggested that the side-effects of non-steroidal anti-inflammatory drugs (NSAIDs) correlate with their ability to inhibit COX-1, while the anti-inflammatory effects are due to their ability to inhibit COX-2. In order to strengthen this hypothesis, we examined the correlation between the inhibitory effects of eight NSAIDs (ibuprofen, aspirin, diclofenac, sulindac, naproxen, indomethacin, tolmetin and piroxicam) on the activity of COX isoforms *in vitro* and the range of relative risks of gastrointestinal (GI) complications that have been reported with individual NSAIDs in a meta-analysis. Analysis by Spearman rank correlation test demonstrated that IC_{50} values of NSAIDs on COX-1 activities in intact cells had a negative correlation with relative risk of GI complications ($rs = -0.74, p < 0.05$). A similar analysis using the IC_{50} of NSAIDs on the activity of COX-2 did not demonstrate any significant correlation ($rs = -0.59, p = 0.12$). The ratio of COX-2/COX -1 did not appear to have any significant correlation with the risks of clinical GI side-effects ($rs = 0.5, p = 0.21$). Further analyses of *in vitro* data from other sources are encouraged to validate the usefulness of this model in predicting the GI side effects of each particular NSAID.**

เรื่องย่อ : **ฤทธิ์ของยาระงับปวดลดไข้และต้านการอักเสบที่แตกต่างกันต่อเอนซัยม์ cyclooxygenase isoform มีผลต่อการเกิดอาการข้างเคียงหรือไม่ ?**

ประวิทย์ อัครเสรีนนท์ พ.บ., ปร. ด.*, สุพรชัย กองพัฒนากูล พ.บ.,* **David A. Henry M.B., Ch.B.,** ** **Christoph Thiemermann M.D., Ph.D.*****

*ภาควิชาเภสัชวิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร ๑๐๗๐๐,
**Discipline of Clinical Pharmacology, Faculty of Medicine and Health Sciences, University of Newcastle, ***The William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ, U.K.

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'Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Bangkok 10700, "Discipline of Clinical Pharmacology, Faculty of Medicine and Health Sciences, University of Newcastle, "'The William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ, U.K.
C. Thiemermann is a Senior Fellow of The British Heart Foundation (FS/96/015).

เอนซัยม์ cyclooxygenase (COX) เป็นเอนซัยม์ตัวแรกที่ใช้ในกระบวนการสร้าง prostaglandins (PGs). ปัจจุบัน COX มีอยู่อย่างน้อย ๒ ชนิด (isoforms) คือ constitutive isoform (COX-1) ซึ่งพบในภาวะปรกติของร่างกาย ทำหน้าที่เกี่ยวข้องกับสรีรวิทยาของร่างกาย และ inducible isoform (COX-2) ซึ่งพบในภาวะที่ร่างกายมีพยาธิสภาพ. ยาระงับปวดลดไข้และต้านการอักเสบ (nonsteroidal anti-inflammatory drugs; NSAIDs) มีฤทธิ์ยับยั้ง COX แต่ละ isoform ไม่เท่ากัน และมีสมมติฐานว่าการออกฤทธิ์ ในการรักษาการอักเสบเป็นผลจากการยับยั้ง COX-2 ในขณะที่ผลข้างเคียงจากการรักษา, โดยเฉพาะ gastrointestinal bleeding และ renal toxicity เป็นผลจากการยับยั้ง COX-1. เพื่อพิสูจน์สมมติฐานดังกล่าว, คณะผู้วิจัยจึงได้ศึกษาความสัมพันธ์ระหว่างฤทธิ์ยับยั้ง COX isoform ของ NSAIDs (50 percent inhibitory concentration dose or IC_{50} ของ ibuprofen, aspirin, diclofenac, sulindac, naproxen, indomethacin, tolmetin และ piroxicam) ในห้องปฏิบัติการกับอัตราเสี่ยง (relative risk) ของผลข้างเคียงทางระบบทางเดินอาหารจากการใช้ NSAIDs ดังกล่าวในทางคลินิก.

ผลการศึกษาโดยใช้ Spearman rank correlation test พบว่า, ค่า IC_{50} ของ NSAIDs ในการยับยั้ง COX-1 ในหลอดทดลอง มีความสัมพันธ์ผกผันกับอัตราเสี่ยงของผลข้างเคียงทางระบบทางเดินอาหาร (rs = -0.74, $p < 0.05$), ในขณะที่ ค่า IC_{50} ของ NSAIDs ในการยับยั้ง COX-2 ในหลอดทดลอง ไม่มีความสัมพันธ์ (rs = -0.59, $p = 0.12$). นอกจากนี้ยังพบว่าอัตราส่วนของ COX-2IC_{50} ต่อ COX-1IC_{50} (COX-2/COX-1 ratio) ก็ไม่มีความสัมพันธ์กับอัตราเสี่ยงของผลข้างเคียงทางระบบทางเดินอาหาร ($rs = 0.5$, $p = 0.21$).

ดังนั้น การศึกษาเพิ่มเติมถึงความสัมพันธ์ระหว่างฤทธิ์ยับยั้ง COX isoform ของ NSAIDs (IC_{50}) ในห้องปฏิบัติการ กับอัตราเสี่ยงของผลข้างเคียงทางระบบทางเดินอาหารจากการใช้ NSAIDs ในทางคลินิก, อาจจะเป็นประโยชน์ในการพยากรณ์ถึงผลข้างเคียงทางระบบทางเดินอาหารจากการใช้ NSAIDs แต่ละชนิดในทางคลินิก.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and those recently developed, are among the most widely prescribed group of drugs world wide. As such, even small changes in the risk of side-effects may have major public health implications. NSAIDs are a large group of drugs with diverse structures and mechanisms of action. However, inhibition of cyclo-oxygenase (COX) activities, and subsequently prostaglandin (PG) production, is the common basis for their therapeutic benefits.[1] The most common side effects of NSAIDs are related to the gastrointestinal (GI) tract, and include dyspepsia, ulcer, haemorrhage and perforation. This injury represents the single most important reaction to this medication.[2] Like their therapeutic actions, the side effects of NSAIDs can be attributed to inhibition of COX, as PGs, such as prostacyclin (PGI_2) and PGE_2 are cytoprotective.[1,3]

Although most NSAIDs share basic mechanisms of action, there is little rigorous information to indicate whether individual NSAIDs are associated with variable risks of serious upper GI toxicity.[4,5] Differences in the response to NSAIDs could be due not only to the different types of NSAIDs, but also to intersubject variation[6] in drug handling and response, and varying drug utilization patterns including dose and duration among different NSAIDs.[7] Determining the comparative risks of serious GI toxicity with the different NSAIDs has proven to be difficult owing to the large sample size required. However, a recent meta-analysis on studies of NSAID-induced GI toxicity has provided relative risk data for major GI complications with individual drugs.[8]

The hypothesis that COX exists in multiple isoforms was first made by Flower and co-workers.[9] In their study, the authors demonstrated that COX activity from different organs has a different sensitivity to inhibition by aspirin, indomethacin and paracetamol. Recently, the hypothesis was confirmed by the demonstration that COX exists in two well-characterised isoforms. COX-1 is present constitutively, under physiological conditions, whereas COX-2 is induced by mitogen or pro-inflammatory cytokines including endotoxin.[10-14] We have recently

shown that COX-1 and COX-2 are differentially inhibited by a selection of commonly used NSAIDs.[15,16] These observations give rise to the hypothesis that the therapeutic benefits of NSAIDs are due to their ability to inhibit COX-2, whereas their side effects are due to inhibition of COX-1.[15]

In this study, we examined the correlation between the inhibitory effects of eifht NSAIDs (ibuprofen, aspirin, diclofenac, sulindac, naproxen, indomethacin, tolmetin and piroxicam) on the activity of COX isoforms *in vitro* [15,16] (using a cell model of bovine aortic endothelial cells for COX-1 and endotoxin-activated J774.2 macrophages for COX-2), and the range of relative risks of major GI toxicity that have been reported with individual NSAIDs in a meta-analysis.[8] Specifically, we wished to determine whether the results from the *in vitro* model are correlated with the clinical findings in terms of tendency to major gastrointestinal complication with each NSAID.

METETIALS AND METHODS

Measurement of the inhibitory effects of NSAIDs on the activity of COX isoforms in vitro.

The inhibitory effects of eifht NSAIDs (ibuprofen, aspirin, diclofenac, sulindac, naproxen, indomethacin, tolmetin and piroxicam) on the activity of COX isoforms have been assessed by using a cell model of COX-1 (bovine aortic endothelial cells; BAEC) and COX-2 (endotoxin-activated J774.2 macrophages; J774) as previously described.[15,16]

1. Cell Culture Murine macrophages (J774.2; The European Collection of Animal Cell Culture, Salisbury, UK) were grown in 96-well culture plates with Dulbecco's Modified Eagle's Medium supplemented with 10 percent foetal calf serum and L-glutamine (4 mM). BAEC were cultured from fresh bovine aortae, as previously described,[5] and seeded onto 96-well culture plates.

2. Measurement of COX-1 activity

BAEC were incubated for 30 minutes with a NSAID (up to 1 mg/ml). Arachidonic acid (30 μM) was then added and the cells incubated for a further 15 minutes at 37°C. The medium was then removed and radioimmunoassay used to measure the formation of 6-oxo $PGF_{1\alpha}$.

3. Measurement of COX-2 activity

Cultured J774.2 macrophages were treated with endotoxin (1 μg/ml) for 12 hours to induce COX-2. The culture medium was then changed and one of the NSAIDs was added for 30 minutes at 37°C. Arachidonic acid (30 μM) was then added and the cells incubated for a further 15 minutes at 37 °C. The medium was removed and 6-oxo- $PGF_{1\alpha}$ measured by radioimmunoassay, as above. The inhibitory effects of NSAIDs on COX activity were measured in at least nine separate determinations (wells) on at least three different experimental days.

Assessment of relative risks of major gastrointestinal toxicity with individual NSAIDs.

The methods used in the meta-analysis of epidemiological studies have been described in full elsewhere.[8] Only brief details will be provided here. Medline, published original articles and reviews, were searched to find controlled epidemiologic studies of the relationship between the use of NSAIDs and peptic ulcer complications. We selected studies that had found a relationship between the use of NSAIDs and hospitalisation with GI complications. Analysis, in the main, was confined to studies that provided comparative data for ibuprofen and other drugs of interest. Data on the relative risks (RR) of GI complications with use of individual NSAIDs were extracted by two individuals with differences resolved by consensus. We pooled RR, using ibuprofen as the reference, and used a novel ranking procedure to find a rank order that best summarised the sequence of relative risks that were observed in the studies.[8] The pooled relative risks and ranked relative risks were correlated with COX-1 and COX-2 activities using the method described below.

Statistical analysis

The relationships between the inhibitory effects of eight NSAIDs on the activity of COX isoforms *in vitro*, and the pooled and ranked relative risks of GI complications obtained from the meta-analysis were examined using the Spearman rank correlation test. The analyses were conducted separately using COX-1 activity, COX-2 activity and separately with the ratio of COX-2/COX-1 activities. The *P*-value <0.05 was considered statistically significant.

RESULTS

The inhibitory effects of NSAIDs on COX-1 and COX-2 activity in *in vitro* model and the range of relative risks of GI complication by individual NSAIDs were shown in tables 1 and 2, respectively.

Analysis by Spearman rank correlation test

Table 1. IC_{50} values (μM) of 8 NSAIDs on COX-1 and COX-2 activity in intact cells*.

NSAID	IC_{50} (μM) COX-1	COX-2	Ratio
Ibuprofen	4.8	72.7	15
Aspirin	1.6	277	173
Diclofenac	1.5	1.1	0.7
Sulindac	1.1	112	102
Naproxen	8.7	5.1	0.6
Indomethacin	0.028	1.6	57
Tolmetin	0.126	22.2	176
Piroxicam	0.0015	0.45	300

Note : The ratio of the IC_{50} values for the NSAIDs on COX-2 relative to COX-1 are given in the final column of the table. * Modified from Mitchell et al., 1993, *Proc. Natl. Acad. Sci. USA.*, **90,** 11693-11697. and Akarasereenont et al., 1994, *Br. J. Pharmacol.*, **112,** 183P.

Table 2. Results of the meta-analysis presented as ranked RR or as pooled RR, with use of ibuprofen as the reference*.

NSAID	Pooled RR	Ranked RR	Number of studies
Ibuprofen	1	1	11
Aspirin	1.6	3	7
Diclofenac	1.8	2	9
Sulindac	2.1	4	5
Naproxen	2.2	5	12
Indomethacin	2.4	6	12
Tolmetin	3	8	2
Piroxicam	3.8	7	11

Note : * Modified from Henry et al., 1996, *Br. Med. J.*, **312,** 1563-1566.

Table 3. Correlation coefficient (P value) between pooled RR and IC_{50} values of 8 NSAIDs on COX-1, COX-2 activity and ratio of COX-2/COX-1 (n=8, all).

IC_{50} (μM)	Spearman test
COX-1	- 0.74 (0.04)
COX-2	- 0.59 (0.12)
Ratio of COX-2/COX-1	0.5 (0.21)

Table 4. Correlation coefficient (P value) between ranked RR and IC_{50} values of 8 NSAIDs on COX-1, COX-2 activity and ratio of COX-2/COX-1 (n=8, all).

IC_{50} (μM)	Spearman test
COX-1	- 0.67 (0.07)
COX-2	- 0.36 (0.38)
Ratio of COX-2/COX-1	0.57 (0.14)

demonstrated that IC_{50} values of NSAIDs on COX-1 activities in intact cells had a reverse correlation with the pooled relative risk of GI complications (rs = -0.74; P = 0.04). When the same analysis was carried out with the ranked RR values, the correlation coefficient was smaller and not statistically significant (rs = -0.67; P = 0.07). A similar analysis using the IC_{50} of NSAIDs on the activity of COX-2 did not demonstrate any significant correlation for pooled and ranked relative risk (rs = -0.59, P = 0.12 and -0.36, P = 0.38, respectively; tables 3 and 4). Neither did an analysis of the ratio of IC_{50} of COX-2/COX -1 and the risks of clinical GI side-effects using either pooled relative risk (rs = 0.5; P = 0.21, table 3) or ranked relative risk (rs = -0.57; P = 0.14, table 4).

DISCUSSION

Our results strongly suggest that the potency of NSAIDs on the activity of COX-1 in intact cells has a reverse correlation with risk of GI toxicity. Furthermore, although the potency of NSAIDs on the activity of COX-2 and the ratio IC_{50} of COX-2/COX-1 does not demonstrate any significant correlation with the risk of GI toxicity, the data demonstrates trends that these two parameters may indicate the existence of a relationship with the potency of NSAIDs. The ratio IC_{50} of COX-2/COX -1, in fact, has been advocated as a predictor of GI toxicity.[15]

NSAIDs are effective therapies in a wide range of conditions. Their efficacy has made them the most widely prescribed drugs in the world, with a wide range of propietary preparations. The known efficacy of these drugs for the treatment of inflammatory conditions has always been hampered by problems of their side-effects, particularly GI toxicity. There is now evidence for two isoenzymes of cyclo-oxygenase. COX-1 is expressed constitutively and is the "housekeeping" enzyme which is responsible for gastric mucosal effects, and for maintaining renal blood flow and acting on platelets, while the inducible COX-2 is responsible for the inflammatory nature of prostaglandins.[12-14] The discovery of an isoform (COX-2) of COX induced by inflammatory mediators and present in inflammatory situations[3] allows a re-interpretation and refinement of the general theory that inhibition of COX activity explains the therapeutic effects and the side-effects of the aspirin-like anti-inflammatory drugs.[1] Consequently, NSAIDs which have a higher activity against COX-2 than COX-1, e.g., selective COX-2 inhibitors, cause potential benefits in reducing side effects whilst maintaining efficacy.

Comparison of the data on existing drugs which have differences in COX-1 and COX-2 activities with differences in their GI toxicity is one of the logical steps to support the hypothesis. In fact, it would help validate the *in vitro* experiments. There are now several studies which have shown that NSAIDs can be ranked in terms of the frequency of their side-effects.[17,18] Similarly, from a variety of *in vitro* experiments, it has been possible to derive a rank order of the selectivity of a certain NSAID for COX-1/COX-2. Can then the ratios from the laboratory findings given in our model (table 1) be correlated with the adverse drug events in clinical settings? In this study, putting together the epidemiological data with the *in vitro* data, we show that there is some agreement on rank order of selectivity and toxicity.

The correlation coefficient (table 3 and 4) indicated that NSAIDs with higher potency on COX-1 activity would be more likely to cause gastric damage. Overall, this prediction is supported by other clinical studies. In different reports, aspirin, indomethacin, and tolmetin produced the highest incidence of ulcers in patients taking NSAIDs for

arthritis[11]; in another report using a "toxicity index" to standardize the severity of different side-effects in patients taking NSAIDs, indomethacin, tolmetin, piroxicam and sulindac proved to be among the most toxic drugs analysed.[17] Most recently, Langman et al.[18] confirmed the greater toxicity of aspirin, indomethacin and piroxicam. All these NSAIDs showed a ratio of more than 50 in table 1.

As for the correlation between two other laboratory parameters (COX-2 activities and IC_{50} of COX-2/COX-1) and clinical findings, the calculation did not demonstrate any significant results. However, there are trends in both cases that support the hypothesis that those two parameters may be useful as predictors of adverse events. This should encourage further investigation of this hypothesis with additional results from other laboratories.

In summary, a useful model to predict the risk of NSAIDs in causing GI toxicity from *in vitro* parameters is the inhibition of NSAIDs on COX-1 activity. However, the ratio of COX-2/COX-1 should be assessed further. Thus, further analyses of *in vitro* data from other sources are encouraged to validate the usefulness of this model in prediciting the GI side-effects of each particular NSAID.

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