Characterization of Cox-1 inhibitors effect on platelets by in vitro platelet aggregation assays

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1 INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in inflammatory diseases. Therapeutic and side effects of NSAIDs are dependent on cyclooxygenase (COX) inhibition. COX isoforms have been named constitutive: COX-1, and inducible: COX-2 as it is induced by various factors. In platelets, COX-1 transforms Arachidonic acid (AA) previously synthetized from membrane phospholipids to Thromboxane A2, a potent platelet aggregating agent (Fig.1). The AA pathway shows considerable differences between and within species with this pathway being prominent in human and monkey platelets.

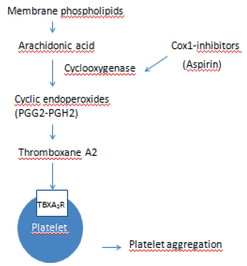

Fig 1: Outline of Arachidonic acid metabolism leading to the formation of Thromboxane A2 and platelet aggregation.

NSAIDs are classified in 4 categories, among these are the selective Cox-1 inhibitors such as aspirin, and non-selective COX inhibitors which exhibit Cox1/Cox2 inhibition such as Ibuprofen and Naproxen (Ref 1). Only the COX inhibitors that inhibit COX-1 can inhibit synthesis of TxA2. Selective COX-2 inhibitors do not inhibit Thromboxane A2 and have no effect on platelets.

Inhibitor	Example of compound	Inhibition of PLT aggregation
Selective Cox 1-inhibitors	Acetylsalicylic acid (aspirin)	Marked
Non Selective COX inhibitors	Ibuprofen	Mild to Moderate
	Naproxen	Mild to Moderate
Relatively selective COX-2 inhibitors	Meloxicam	Not expected
Highly selective COX-2 inhibitors	Refecoxib	no

Table 1: Different types of COX inhibitors and their effects on platelets (modified from Ref 1).

There is currently no specific guidance on platelet testing in the context of investigational new drug testing. If platelet function is impaired, hemorrhages are encountered even if the platelet numbers are within historical range. Cox -1 inhibition effects on platelet function may be characterized in vivo on human or cynomolgus monkey platelets but can be easily determined by in vitro methodology.

2 PLATELET AGGREGATION METHOD

The Chrono-Log ® Aggregometer is used for performing optical aggregation tests. The platelets of a citrated whole blood sample are isolated yielding the platelet rich plasma (PRP) sample. A reference sample, platelet poor plasma (PPP), is also made. The PRP sample is diluted using PPP to obtain a final platelet concentration between 200 x 10^3 cells/µL to 300 x 10^3 cells/µL.
Light transmission through both samples is recorded simultaneously by the aggregometer, the PPP sample being the 100% light transmission reference while the PRP is set at 0% light transmission. Upon agonist addition in the PRP sample, the platelets undergo shape changes and the aggregation event begins. The software generates an aggregation curve and calculates the maximal amplitude, in percent, of the aggregation curve.

3 EVALUATION OF COX-1 INHIBITION

First step:

For the characterization of the inhibitory effect of a compound on COX- 1, arachidonic acid is used as an agonist. Serial concentrations of arachidonic acid are tested on PRP samples from different monkey and human donors in order to determine the optimal concentration for which 50% of the platelets are aggregated (IC50). Similarly the IC90 can be determined (90% inhibition of platelet aggregation).

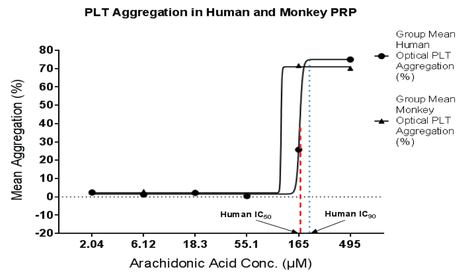

Fig 2: Determination of the optimal concentration of Arachidonic acid for platelet aggregation in human and monkey PRP. In cynomolgus monkeys a concentration of 105 uM of AA was sufficient to inhibit maximal platelet aggregation by 50%. In humans a concentration of 165 uM of AA was needed to inhibit maximal platelet aggregation by 50%.

Second step:

The compound is tested on PRP and its activity is compared to Aspirin (acetylsalicylic acid (solubilized in dimethyl sulfoxide (DMSO)) and saline .

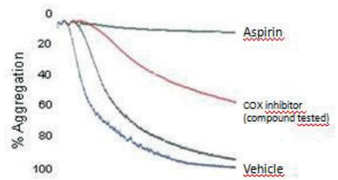

Fig 3 : Amplitude curve of platelet aggregation in human PRP with Arachidonic acid agonist (1uM) on Chronolog® aggregometer.

Serial concentrations of the compound are tested on PRP samples from different monkey or human donors in order to determine the optimal concentration for which 50% of the platelets are aggregated (IC50). The IC50 obtained is then compared to the expected maximal concentration of the compound (Cmax) in order to predict its potential effects on platelets. If the IC50 obtained in higher than the Cmax, it is likely that the compound will not show an effect on platelets in vivo.

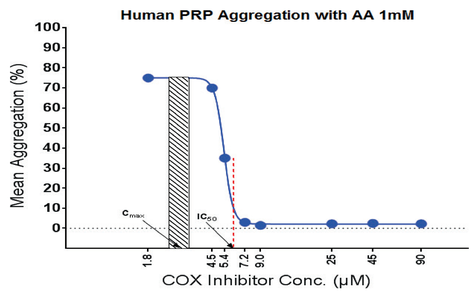

Fig 4: Inhibition of COX-1 activity on human PRP aggregation. Serial concentrations of the compounds are tested to determine the IC50 for which 50% of aggregation reactions are inhibited.

Third step:

Effects of compounds on platelets are also tested with other agonists such as low concentrations of ADP or collagen. These agonists induce, at low concentrtaion, platelet aggregation through the production of thromboxane A2 and their activity on platelets is blocked by COX-1 inhibitors. Platelet aggregation induced by high concentrations of collagen and thrombin does not involve the production of thromboxane A2. Platelet aggregation induced by non Cox-1 dependent activity is usually not altered by COX-1 inhibitors.

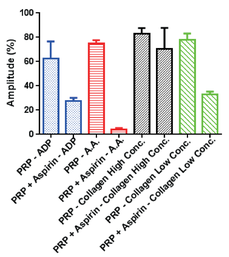

Fig 5: Effect of Aspirin (2mM) on human PRP aggregation induced with ADP 10 uM, Arachidonic acid 1 mM and Collagen 0.5 ug/mL (low dose) and 2 ug/mL (high dose). In the presence of aspirin, platelet aggregation was inhibited totally with Arachidonic Acid, partially with ADP and a low concentration of collagen, and not inhibited with a high concentration of collagen.

Fourth step:

Evaluation of drug-drug interactions on platelets can be assessed by combining a COX-1 inhibitor with another compound and evaluating the potential combined effect on platelet aggregation with one or more agonists depending on the nature of the compounds tested.
An example of such interaction is Naproxen which interferes with the inhibitory effect of aspirin on platelet COX-1 activity and function, and the drugs interaction might undermine the sustained inhibition of platelet COX-1 that is necessary for aspirin cardio protective effects (Ref 2).

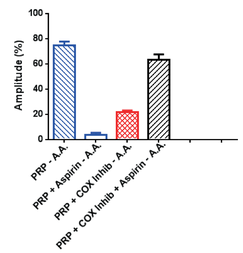

Fig 6: Platelet aggregation with Arachidonic acid was totally inhibited with aspirin, partially inhibited by a second COX-inhibitor, and was not inhibited by the combination of aspirin and candidate Cox-inhibitor, showing drug interaction on platelet aggregation.

4 CONCLUSION

The evaluation of the effect of COX-1 inhibitors on platelets can easily be assessed by in vitro testing and several approaches are available. The choice of the agonist and its concentration are essential in order to keep a good sensitivity of the assay. It is also possible to evaluate two drug combinations as these may synergize or inhibit platelet effects.

References:
1-Suleyman H. and al. Anti-inflammatory and side effects of cyclooxygenase inhibitors. Pharmacological reports. 2007, 59, 247-258.
2-Capone M.L. and al. Pharmacodynamic interaction of naproxen with low-dose aspirin in healthy subjects. Journal of the Am Coll of Cardiol. 2005, Vol. 45.