PHARMACOLOGICAL & TOXICOLOGICAL SCREENING METHODS-I (PTSM-I)

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Unit-III

Preclinical screening of new substances for the pharmacological activity using in vivo, in vitro, and other possible animal alternative models.

- Respiratory Pharmacology: anti-asthmatics, drugs for COPD and anti allergics.
- Reproductive Pharmacology: Aphrodisiacs and antifertility agents
- Analgesics, antiinflammatory and antipyretic agents. Gastrointestinal drugs: anti ulcer, anti-emetic, antidiarrheal and laxatives.
SCREENING OF ANTI-INFLAMMATORY AGENTS
Topics to discuss

- Introduction
- General considerations
- \textit{In-vitro} methods
- \textit{In-vivo} methods
- Side effects of anti-inflammatory agents
Introduction

- **Inflammation** is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants.
  - Protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

- **Non-steroidal anti-inflammatory drugs (NSAIDs)** are drugs with analgesic, antipyretic (lowering an elevated body temperature and relieving pain without impairing consciousness) and, in higher doses, with anti-inflammatory effects (reducing inflammation).
  - Act primarily on peripheral; and in CNS to raise the pain threshold.
## Comparison between acute and chronic inflammation

<table>
<thead>
<tr>
<th></th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Causative agent</strong></td>
<td>Pathogens, injured tissues</td>
<td>Persistent acute inflammation due to non-degradable pathogens, persistent foreign bodies, or autoimmune reactions</td>
</tr>
<tr>
<td><strong>Major cells involved</strong></td>
<td>Neutrophils, mononuclear cells (monocytes, macrophages)</td>
<td>Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts</td>
</tr>
<tr>
<td><strong>Primary mediators</strong></td>
<td>Vasoactive amines, <strong>eicosanoids</strong></td>
<td>IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes</td>
</tr>
<tr>
<td><strong>Onset</strong></td>
<td>Immediate</td>
<td>Delayed</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td>Few days</td>
<td>Up to many months, or years</td>
</tr>
<tr>
<td><strong>Outcomes</strong></td>
<td>Resolution, abscess formation, chronic inflammation</td>
<td>Tissue destruction, fibrosis</td>
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**Note:**
- **Screening of antiinflammatory agents**
General considerations

- Inflammation has different phases:
  - the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space,
  - the second one by infiltration of leukocytes from the blood into the tissues and
  - the third one by granuloma formation.
- anti-inflammatory tests - acute inflammation, subacute inflammation and chronic repair processes.
- In some cases, the screening is directed to test compounds for local application.
- Predominantly, however, studies are aimed to find new drugs against polyarthritis and other rheumatic diseases.
Biosynthesis of eicosanoids

Diacylglycerol or phospholipid

Phospholipase C

Phospholipase A₂

Arachidonic acid

Lipooxygenase (FLAP, Alox5)

HPETE (hydroperoxy-eicosatetraenoic acid)

PGH₂ synthase (cox-1 or -2 and peroxidase)

PGD₂ synthase

PGD₂

PGE synthase

PGE₂

Prostaglandin H₂ (PGH₂)

6-keto-PGF₁α

Endothelium

Prostacyclin (PGI₂)

Thromboxane (TXA₂)

Platelets

PGE₂ synthase

Prostaglandin E₂ (PGE₂)

Prostacyclin synthase

Prostacyclin (PGI₂)

Thromboxane synthase

Thromboxane (TXA₂)

Leukotriene A₄

H₂O

LTB₄

Glutathione

Glutathione-S-transferase

Leukotriene C₄

Glutamic acid

Leukotriene D₄

Leukotriene E₄

Leukotriene A₄
FIGURE 1. Algorithm of the biochemical pathway shows that the formation of prostaglandins occurs via both cyclooxygenase enzymes (COX-1 and COX-2).
Screening of anti-inflammatory methods

**In-vitro methods**
- $^3$H-Bradykinin receptor binding
- $^3$H-Substance P receptor binding
- Assay of polymorphonuclear leukocyte chemotaxis *in vitro*
- Constitutive and inducible cellular arachidonic acid
- Formation of leukotriene B4 in human white blood cells *in vitro*
- Formation of lipoxygenase products from 14C-arachidonic acid in human polymorphonuclear neutrophils (PMN) *in vitro*
- COX-1 and COX-2 inhibition
- Flow cytometric analysis of intracellular cytokines
- Screening for interleukin-1 antagonists

**In-vivo methods**
- Ultraviolet erythema in guinea pigs
- Inhibition of leukocyte adhesion to rat mesenteric venules *in vivo*
- Oxazolone-induced ear edema in mice
- Croton-oil ear edema in rats and mice
- Paw edema
- Granuloma pouch technique
- Urate-induced synovitis
- Cotton wool granuloma
- Pleurisy test
- Glass rod granuloma
- Methods for testing the proliferative phase (granuloma formation)
**In vitro methods for anti-inflammatory activity**

- **Autacoids**, are involved in the process of inflammation and repair.
- Histamine, serotonin, bradykinin, substance P, and the group of **eicosanoids** (prostaglandins, thromboxanes and leucotrienes), the platelet-activating factor (PAF) as well as **cytokines** and **lymphokines**.
- Their discovery makes the use of **in vitro studies** possible.
- The influence of **non-steroidal anti-inflammatory agents** on the **eicosanoid pathway** gave rise to numerous studies.
In-vitro methods
3H-Bradykinin receptor binding

Purpose and rationale

- Bradykinin produces pain by stimulating A and C fibers in the peripheral nerves, participates in the inflammatory reaction and lowers blood pressure by vasodilatation.
- The 3H-bradykinin receptor binding is used to detect compounds that inhibit binding of 3H-bradykinin in membrane preparations obtained from guinea-pig ileum.
- Two types of bradykinin receptors (BK1 and BK2 receptors) are known.
  - Pulmonary BK3 receptors
  - Three subtypes of B2 receptors, B2a, B2b, and B2c
3H-Bradykinin receptor binding

Procedure

- Guinea pig – ileum (2 cm length)
- Homogenized for 30 s in ice-cold TES buffer, pH 6.8, containing 1 mM 1,10-phenanthroline, in a Potter homogenizer.
- Homogenates are filtered through 3 layers of gauze and centrifuged twice at 50 000 g for 10 min with an intermediate re-homogenization in buffer.
- In the competition experiment,
  - 50 μl 3H-bradykinin (one constant concentration of 0.5–2 × 10–9 M),
  - 50 μl test compound (6 concentrations, 10–5–10–10 M) and
  - 150 μl membrane suspension from guinea pig ileum (approx. 6.6 mg wet weight/ml) per sample are incubated in a shaker bath at 25 °C for 90 min.
3H-Bradykinin receptor binding

- **Total binding** is determined in the presence of incubation buffer, non-specific binding is determined in the presence of non-labeled bradykinin (10–6 M).
- The reaction is stopped by rapid vacuum filtration through glass fibre filters.
- Thereby the membrane bound radioactivity is separated from the free one.
- The retained membrane-bound radioactivity on the filter is measured after addition of 3 ml liquid scintillation cocktail per sample in a liquid scintillation counter.
3H-Bradykinin receptor binding

Evaluation

- The following parameters are calculated:
  - total binding of 3H-bradykinin
  - non-specific binding in the presence of 10 μM bradykinin
  - specific binding = total binding – non-specific binding
  - % inhibition: 100 – specific binding as percentage of control value
- Compounds are first tested at a single high concentration (10 000 nM) in triplicate. For those showing more than 50% inhibition a displacement curve is constructed using 7 different concentrations of test compound.
- Binding potency of compounds is expressed either as a relative binding affinity (RBA) with respect to the standard compound (bradykinin) which is tested in parallel or as an IC50.

\[
RBA = \left(\frac{IC_{50\text{ standard compound}}}{IC_{50\text{ compound}}}\right) \times 100\%
\]
Polymorphonuclear leukocytes aggregation induced by FMLP

Purpose and rationale

- Aggregation of polymorphonuclear leukocytes (PMNs) can be induced by FMLP (formyl-L-methionyl-Leucyl- L-phenylalanine).
- The aggregation can be inhibited by xanthine derivatives.
Polymorphonuclear leukocytes aggregation induced by FMLP

Procedure

- PMNs cell suspensions are prepared from peritoneal exudates obtained 17 h after intraperitoneal injection of 10 ml 6% sodium caseinate into Sprague Dawley rats.
- The cells are washed twice in Geys-balanced-salt solution (Gibco GBSS) and resuspended to a final concentration of $15 \times 10^6$ cells/ml.
- The test compounds and the standard (pentoxiphylline) are dissolved in GBSS.
- FMLP is dissolved in DMSO. The further dilutions are made up to a final concentration of $10^{-7}$ mol FMLP in GBSS.
- Before addition of FMLP, the cell suspensions are pre-incubated for 10 min with the drugs.
- PMNs aggregation is carried out in a Born aggregometer.
Polymorphonuclear leukocytes aggregation induced by FMLP

Evaluation

- The results are expressed as change in transmittance, measured in mm on the recorder. The mean peak of the untreated cells is set 100%.
TNF-alpha antagonism

Purpose and rationale

- There are two distinct types of tumor necrosis factors, TNF-alpha (cachectin) and TNF-beta (lymphotoxin) with biological activities going beyond the necrosis of tumor cells.
- The complex sequence of hemodynamic and metabolic collapse which leads to shock and death during lethal endotoxinemia appear to represent the response of the infected host to the acute, systemic release of TNF-alpha.
- Thus, drugs that antagonize the activity of this mediator could be of clinical value in combating its fatal effects.
**TNF-alpha antagonism**

**Procedure**

- Twenty hours before the initiation of the experiments, L 929 cells are harvested from stock cultures and are plated in 96 well culture plates (2 × 10⁴ cells/well) and incubated at 37 °C and 5% CO2 in air.

- For each group 6 wells are set up. The cells are then pre-incubated for 30 min with test substances or solvent before TNF alpha is added (between 1 and 10 IU/well).

- After an additional incubation time of 20 h, the culture plates are flicked out and the remaining living cells are lysed by the addition of bidistilled water (100 μl).

- After 30 min incubation at room temperature, 100 μl of LDH reagent are given to each culture well. After 15 min, the enzyme activity is determined photometrically at 490 nm.
TNF-alpha antagonism

Evaluation

- The percent inhibition is calculated according to the formula:

\[
% \text{ inhibition} = 100\% - \frac{\text{ext. test group} - \text{ext. spontaneous lysis}}{\text{ext. positive control} - \text{ext. spontaneous lysis}}
\]

- The positive control is the group which receives vehicle and TNF-alpha. The spontaneous lysis is based on cultures which receive vehicle without TNF-alpha.
**In vivo methods for anti-inflammatory activity**

- The **inflammatory process** involves a series of events that can be elicited by numerous stimuli, e.g., infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury.
- The response is accompanied by the clinical signs of erythema, edema, hyperalgesia and pain.
- Inflammatory responses occur **in three distinct phases**, each apparently mediated by different mechanisms:
  1. *an acute, transient phase*, characterized by local vasodilatation and increased capillary permeability,
  2. *a subacute phase*, characterized by infiltration of leukocytes and phagocytic cells,
  3. *and a chronic proliferative phase*, in which tissue degeneration and fibrosis occur.
Methods for testing acute and subacute inflammation are:

- UV-erythema in guinea pigs
- Vascular permeability
- Oxazolone-induced ear edema in mice
- Croton-oil ear edema in rats and mice
- Paw edema in rats (various modifications and various irritants)
- Pleurisy tests
- Granuloma pouch technique (various modifications and various irritants)

The proliferative phase is measured by methods for testing granuloma formation, such as:

- Cotton wool granuloma
- Glass rod granuloma
- PVC sponge granuloma.

Furthermore, methods for testing immunological factors have been developed, such as:

- Adjuvant arthritis in rats (various modifications)
- Experimental allergic encephalomyelitis
- Schultz-Dale-reaction
- Passive cutaneous anaphylaxis
- Arthus type immediate hypersensitivity
- Delayed type hypersensitivity
Paw edema

Purpose and rationale

- One of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent.

- Many phlogistic agents (irritants) have been used, such as brewer’s yeast, formaldehyde, dextran, egg albumin, kaolin, sulfated polysaccharides like carrageenin or naphthoylehparamine.

- The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls.

- Some irritants induce only a short lasting inflammation whereas other irritants cause the paw edema to continue over more than 24 h.
Paw edema

Procedure

- Male or female Sprague-Dawley rats with a body weight between 100 and 150 g are used.
- The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume.
- Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw.
- The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark.
- The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge.
Paw edema

Evaluation

- The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal.
- Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated.
- The difference at the various time intervals give some hints for the duration of the anti-inflammatory effect.
- A dose- response curve is run for active drugs and ED50 values can be determined.
Oxazolone-induced ear edema in mice

Purpose and rationale

- The oxazolone-induced ear edema model as first described by Evans (1971) in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration.
Oxazolone-induced ear edema in mice

**Procedure**

- **Mice** of either sex with a weight of 25 g are used.
- Before each use a fresh 2% solution of oxazolone (4- ethoxymethylene-2-phenyl-2-oxazolin-5-one) in acetone is prepared.
- The mice are sensitized by application of 0.1 ml on the shaved abdominal skin or 0.01 ml on the inside of both ears under halothane anesthesia.
- The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2% oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is dissolved.
- Groups of 10 to 15 animals are treated with the irritant alone or with the solution of the test compound. The left ear remains untreated.
- The maximum of inflammation occurs 24 h later. A disc of 8 mm diameter is punched from both sides. And weighed immediately.
- The weight difference is an indicator of the inflammatory edema.
Oxazolone-induced ear edema in mice

Evaluation
- Average values of the increase of weight are calculated for each treated group and compared statistically with the control group.
- A 0.003% solution of hydrocortisone and a 1% solution of indomethacin were found to be active.

Critical assessment of the method
- The method is suitable for both steroidal and non-steroidal compounds as well as for the evaluation of various topical formulations.
Granuloma pouch technique

Purpose and rationale

- The method originally invented by Selye has been developed for screening by Robert and Nezamis (1957) using **croton oil as irritant**.
- An aseptic inflammation resulting in large volumes of hemorrhage exudate is elicited which resembles the **subacute type of inflammation**. Instead of croton oil carrageenan can be used as irritant.
Granuloma pouch technique

Procedure

- Male or female Sprague-Dawley rats (n=10) of 150 and 200 g
- The back of the animals is shaved and disinfected.
- With a very thin needle, a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anesthesia.
- Into the resulting oval airpouch, 0.5 ml of a 1% solution of Croton oil in sesame oil is injected.
- 48 hours later, the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken.
- Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard.
- For testing local activity, the test compound is injected directly into the air sac at the same time as the irritant.
- On the 4th or the 5th day, the animals are sacrificed under anesthesia. The pouch is opened, and the exudate is collected in glass cylinders.
- Controls have an exudate volume between 6 and 12 ml, which is reduced dose dependent in the treated animals.
Granuloma pouch technique

Evaluation

- The average value of the exudate of the controls and the test groups is calculated.

- A clear dose response curve could be found by s.c. injection of 0.5, 1.0 and 2.0 mg hydrocortisone acetate/rat. Also doses of 1.5 mg/kg indomethacin were found to be active.

Critical assessment of the method

- The method has been very useful to estimate the potency of anti-inflammatory corticosteroids both after local and after systemic application.

- By injection of a depot-preparation and induction of the granuloma pouch after various time intervals up to 4 weeks the duration of action can also be determined (Vogel 1963, 1965).
Cotton wool granuloma

Purpose and rationale

- The method has been described first by Meier et al. (1950) who showed that foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton.
- After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration.
- The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal.
- More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan.
Cotton wool granuloma

Procedure

- Male Wistar rats with 200 g are anaesthetized with ether.
- The back skin is shaved and disinfected with 70% ethanol. An incision is made in the lumbar region.
- By a blunted forceps subcutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region.
- The pellets are standardized weighing 20 mg or pellets formed from raw cotton which produce a more pronounced inflammation than bleached cotton.
- The animals are treated for 7 days subcutaneously or orally. Than, the animals are sacrificed, the pellets prepared and dried until the weight remains constant.
- The net dry weight, i.e. after subtracting the weight of the cotton pellet is determined.
Cotton wool granuloma

Evaluation

- The average weight of the pellets of the control group as well as of the test group is calculated.
- The percent change of granuloma weight relative to vehicle control group is determined.

Critical assessment of the method

- The method has been useful for evaluation of steroidal and nonsteroidal anti-inflammatory drugs.
- For testing corticosteroids, the test can be performed in adrenalectomized rats.
Side effects of anti-inflammatory compounds

- **Gastrointestinal side effects** are among the most frequent of the untoward or adverse reactions associated with orally ingested anti-inflammatory or anti-arthritic agents.

- The risk of **gastrointestinal ulceration, bleeding and even perforation** with non-steroidal anti-inflammatory drug therapy is well known.

- The mechanisms by which these drugs cause **gastro-intestinal irritation are complex** (Rainsford 1989).

- Deleterious effects may result from local actions, which cause injuries to the submucosal capillaries with subsequent necrosis and bleeding, or from inhibition of the formation of protective prostaglandins.
Side effects of anti-inflammatory compounds

- Ulcerogenic effect in rats
- Measurement of gastric mucosal damage by intragastric inulin
- Determination of blood loss