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Research paper

In vitro based index of topical anti-inflammatory activity to compare a series of NSAIDs

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Abstract

The aim of the present work was to generate an index to predict topical efficiency of a series of nonsteroidal anti-inflammatory drugs (NSAIDs): indomethacin, diclofenac, ketoprofen, piroxicam, tenoxicam and ketorolac. This index took into account both biopharmaceutic and pharmacodynamic aspects. The biopharmaceutic aspect, based on the maximal flux (J_m), was determined experimentally from transdermal studies carried out with human skin in previous work. The pharmacodynamic aspect, based on the ability to inhibit cyclooxygenase-2 (COX-2) in vitro, was determined by incubating human dermal fibroblasts in culture, pre-treated with phobol-12-myristate-13-acetate (PMA) for 6 h, with 25 μM [^{14}C]-arachidonic acid (AA) in the presence of several drug concentrations. The most potent inhibitor of COX-2 activity in induced fibroblasts was diclofenac while indomethacin, ketoprofen and ketorolac were approximately equipotent. Piroxicam and tenoxicam were inhibitors at higher concentrations. Based on the proposed index of the topical anti-inflammatory activity (ITAA) diclofenac, ketorolac, ketoprofen and indomethacin exhibited acceptable efficiency for external use. However, piroxicam and tenoxicam showed the lowest topical anti-inflammatory activity of the series assayed. In conclusion, indomethacin ketorolac, ketoprofen and diclofenac have shown good intrinsic feasibility for formulation into topical pharmaceutical forms. However, for dermatological formulations of oxicams, use of penetration enhancers may be unavoidable.

Keywords: Topical NSAID; Cyclooxygenase; Indomethacin; Diclofenac; Ketoprofen; Piroxicam; Tenoxicam; Ketorolac

1. Introduction

When a topically applied drug is ineffective in clinical trials, we cannot determine whether it is inherently inactive or whether its delivery was insufficient [1]. Not only biopharmaceutical but also pharmacological factors should be considered to formulate an efficacious topical formulation. The evaluation of this topical formulation would be based on both the facility to reach the target into the skin (biopharmaceutics) and the demonstration of the local therapeutic effect (pharmacology). Therefore, an index of the topical anti-inflammatory activity (ITAA) was proposed for comparing the intrinsic efficacy of a series of NSAIDs after their application to the skin.

The inhibition of the enzyme cyclooxygenase (COX), also called prostaglandin endoperoxide H synthetase, is the basis for the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs) [2]. Two forms of COX are identified: a constitutively expressed COX-1 and an inducible isoform COX-2. COX-1 is important in circumstances where prostaglandins have a protective effect such as gastric mucus production and renal blood flow maintenance, whereas the inflammatory prostaglandins seem to be derived from COX-2 at the site of inflammation. As non-selective NSAIDs inhibit both COX-1 and COX-2, the current hypothesis is that their therapeutic activity is due to COX-2 inhibition, while the side effects associated with NSAID therapy are due to inhibition of COX-1 in normal tissues [3–8]. Therefore, a selective inhibition of COX-2 may eliminate unwanted side effects leading to a significant improvement over currently available NSAIDs.

To develop new topical anti-inflammatory agents, a series of in vivo inflammation models are applicable [9]. However, none of the existing models fully reflect the whole set of symptoms and mechanisms normally encountered in inflammatory conditions. In general in vitro models are easier to carry out, less expensive, less time-consuming and are designed to serve as a general screening tool. Although extrapolation of data obtained in vitro to human topical activity is always speculative, the rank order of potency of

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NSAIDs as inhibitors of PG synthesis in vitro tends to reflect their anti-inflammatory potency in vivo [10]. Masferrer et al. [4] suggested that macrophages and fibroblasts are the source of COX-2-derived PGs present at the site of inflammation. Therefore, the COX-2 inhibitory activity (IC_{50}) of NSAIDs was assessed in vitro in human fibroblasts culture.

The skin is a heterogeneous multilayer tissue consisting of stratum corneum, viable epidermis and dermis [11]. As a dermatological drug needs to be delivered to the skin layers where the dermal disorder lies, dermis was assumed to be the viable skin tissue where the NSAID should be targeted.

2. Theoretical development

The ITAA index was defined by the ratio of the drug concentration at the target site (C_d) to the concentration that yields a relevant anti-inflammatory activity (C_a). This ratio should be higher than one unit for likely candidates for topical formulation.

2.1. Estimation of C_d

Firstly two statements should be introduced. On one hand, for diffusants across the skin with very large partition coefficients (like the NSAIDs assayed), the rate-determining step can be the diffusion through the aqueous layer. Following the method of Zwolinski et al. [12] we can plot the reciprocals of the experimental permeability coefficients ($1/P_T$) as a function of the membrane-water partition coefficients ($1/PC$). When diffusion in the aqueous layer is controlling the process, a horizontal line results. Wenkers and Lippold [13] stated that the hidrophilic layer (dermis or epidermis), not the stratum corneum, is the rate-limiting barrier for the transport of NSAIDs across the skin. On the other hand, in percutaneous absorption the concentration gradients develop over many strata in a multilayered barrier. Each layer contributes to the total diffusional resistance ($R_T = \sigma R_i$) which is proportional to the layer thickness (h_i) and is indirectly proportional to the product of the layer diffusivity (D_i) and the partition coefficient (PC_i) with respect to the external phases (we assume that are of the same composition). For the ith layer

$$R_i = \frac{h_i}{D_i \times PC_i} = \frac{1}{P_i} \tag{1}$$

If one segment has a much greater resistance than the other segments (i.e. that of the dermis compared with that of the stratum corneum), then the single high-resistance phase determines the composite barrier property.

Therefore, under dermal diffusion flux control the overall permeability can be given by

$$P_T = \frac{D_d \times PC_{d/v}}{h_d} \tag{2}$$

being D_d, $PC_{d/v}$ and h_d the coefficient of diffusion, partition coefficient dermis-vehicle and thickness of the dermis, respectively. Wenkers and Lippold [13] also proposed this expression when the viable tissue is decisive in permeation. The maximum flux (J_m) of a drug results from the multiplication of the skin permeability (P_T) with the vehicle solubility (C_v) (Eq. 3)

$$J_m = P_T \times C_v \tag{3}$$

As diffusion layer control is approached, under simple zero-order flux case and steady state conditions, the dermal concentration at epidermis/dermis interface becomes

$$C_d = PC_{d/v} \times C_v \tag{4}$$

The average drug concentration in dermis is half of the concentration at the first layer of the dermis because of the essentially zero concentration prevailing in the receptor compartment. If we make use of the Eqs. (3) and (4), we can rewrite Eq. (2) as

$$C_d = \frac{J_m \times h_d}{2D_d} \tag{5}$$

where D_d is the drug diffusion coefficient in dermis and h_d is the thickness of the dermis from epidermis/dermis junction to the bottom layer of the skin.

2.2. Estimation of C_a

The relationship between concentration and effect can be described by the Hill equation [14]. Expressing drug effect in terms of inhibition (I), the inhibitory concentration of an anti-inflammatory agent can be expressed as

$$C_a = \mathrm{IC}_{50} \times \left(\frac{I}{I_{\max} - I}\right)^{\frac{1}{n_H}} \tag{6}$$

where I is the inhibitory activity of an agent applied at a concentration C_a; $I_{\max}$ is the maximal inhibition; n_H is the Hill coefficient; and IC_{50} is the drug concentration that produces 50% inhibition of COX-2.

2.3. Estimation of ITAA

The NSAID concentration in the target tissue can be estimated from Eq. (5). The drug concentration responsible of the therapeutic effect was calculated by means of Eq. (6). Therefore, the proposed index of topical anti-inflammatory activity (*ITAA*) for each NSAID can be given by the following expression

$$ITAA = \frac{C_d}{C_a} = \frac{\frac{J_m \times h_d}{2D_d}}{\mathrm{IC}_{50} \times \left(\frac{I}{I_{\max} - I}\right)^{\frac{1}{n_H}}} \tag{7}$$

For simplicity in the *ITAA* estimation, two assumptions can be made for a particular NSAID: (1) A COX-2 inhibition of 50% ($I = 50$) is sufficient to achieve therapeutic relevance; and (2) 100% COX-2 inhibition ($I_{\max} = 100$) can be

achieved by all the drugs assayed. Therefore, from Eq. (6) it can be deduced that $C_a = IC_{50}$ and consequently Equation (7) can be simplified as follows

$$ITAA = \frac{J_m \times h_d}{IC_{50} \times 2D_d} \quad (8)$$

To estimate the *ITAA* of a NSAID, J_m and IC_{50} should be determined in vitro. The former was directly obtained from in vitro transdermal studies carried out with human skin from saturated aqueous solutions of the drug. The latter was calculated experimentally from a pharmacological in vitro model with human fibroblasts, previously induced to express COX-2.

3. Materials and methods

3.1. Drugs

Indomethacin, ketoprofen, diclofenac, piroxicam, tenoxicam and ketorolac were supplied by Acofarma, Sigma Química, Roig-Pharma, Insalquisa, Ferrer Int. and Chemo Ibérica, respectively. In all cases the free acid form of the drug was used. Stock solutions of the drugs were dissolved in dimethyl sulfoxide (DMSO) and further dilutions were done with Dulbecco's modified Eagles' medium (DMEM). Drug vehicle, at the concentrations employed, did not affect enzyme activities.

3.2. Materials

Culture plates of six 35 mm wells were purchased from Techno Plastic-Products, Switzerland. DMEM, fetal bovine serum (FBS), glutamine, pyruvate, and penicillin/streptomycin solutions were purchased from Biological Industries, Kibbutz Beit Haemek, Israel. Collagenase (50 000 U mg^{-1} purity >98%) was obtained from Boehringer–Mannheim SA, Barcelona, Spain. Phorbol 12-myristate 13-acetate (PMA) came from Sigma, St. Louis, MO. The electrophoresis reagents were from Bio-Rad Laboratories (Spain). Rabbit polyclonal antiserums against COX-1 (PG 20) and COX-2 (PG 27) were from Oxford Biomedical Research, Inc. (USA). COX-1 (ram seminal vesicles) and COX-2 (sheep placenta) electrophoresis standards were from Cayman Chemical Company (USA). Polyvinylidene difluoride transference membrane Immobilon-P was supplied by Millipore Ibérica, Barcelona, Spain. [^{14}C]-AA (50–53 mCi/mmol) and the ECL chemiluminescence kit were purchased from Amersham Ibérica (Madrid, Spain). The scintillation cocktail was Ready flow III, Beckman, San Ramón, CA. All HPLC solvents were supplied by Scharlau SA, Barcelona, Spain.

3.3. Fibroblast culture and COX-2 induction

Human dermal fibroblasts were isolated from skin samples of breast and abdomen taken from women who had undergone plastic surgery, and were cultured as described previously [15]. Experiments were performed with cells between passages 4 and 6. Cells in confluent state were seeded into 6 well plates at a density of 10^5 cells per well. After 72 h cells were made quiescent in medium containing 1% fetal bovine serum (FBS) for 24 h. Quiescent cells were treated with 10 nM of phorbol 12-myristate 13-acetate (PMA) for the indicated period of time.

3.4. Determination of COX activity

The culture medium was then removed, cells were washed and incubated at 37°C for 10 min. with 0.5 ml of DMEM containing 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 25 μM [^{14}C]-AA. The reactions were then stopped by adding 1 N HCl to yield pH 3 followed by a volume of cold methanol. Samples were kept at −80°C until analysis. HPLC analysis of eicosanoids was performed as previously described [16]. COX activity was evaluated as the substrate transformed into the sum of all COX-derived eicosanoids by 10^5 cells in 10 min.

3.5. Western blotting of COX-1 and COX-2

Western blot analysis of COX-1 and COX-2, in fibroblasts treated with 10 nM of PMA for the indicated periods of time, was performed as described previously [17]. Immunoreaction and detection were performed with an ECL chemiluminescence western blotting kit (rabbit) following the manufacturer's instructions.

3.6. Evaluation of the IC_{50} of NSAIDs on COX-2

Cells incubated in the presence of 10 nM of PMA for 6 h were washed and then treated with 0.5 ml of DMEM containing the indicated drug concentrations dissolved in DMSO for 5 min (final DMSO concentration 0.1% v/v). Control samples were performed incubating cells in the presence of vehicle instead of drug. [^{14}C]-AA was then added to a final concentration of 25 μM and the reactions were allowed to stand for another 10 min. Reactions were then stopped and the samples analyzed as described above.

As suggested by the international recommendations on quantitative pharmacology [14], the IC_{50} values were estimated by fitting the Hill equation to the experimental results (% of inhibition referred to the control as a function of concentration). They were calculated by a non-linear least squares regression method using InPlot™ version 4.03 (1992) from GraphPad Software Inc. on a PC computer.

4. Results

4.1. Metabolism of AA and characterization of COX isoforms present in PMA-activated fibroblasts

A typical chromatogram of eicosanoids formed by human dermal fibroblasts in culture is shown in Fig. 1. As expected,

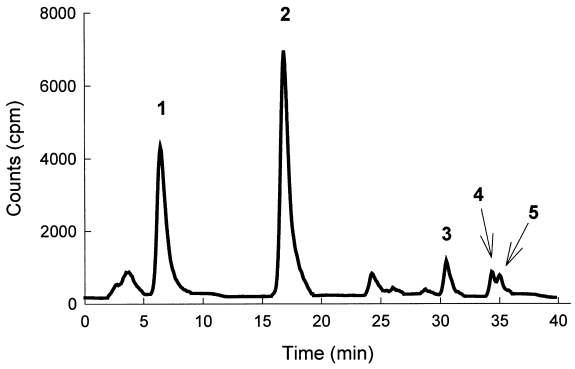

Fig. 1. Representative chromatogram of the eicosanoid analysis from samples of PMA-treated fibroblasts. Cells were treated with 10 nM PMA for 6 h and incubated with 25 μM [^{14}C]-AA for 10 min. Samples were analysed by HPLC. Peak identity was assessed by coelution with authentic standards: 1, 6-keto-$PGF_{1\alpha}$; 2, PGE_2; 3, HHT; and 4 and 5, unresolved 11-HETE and 15-HETE.

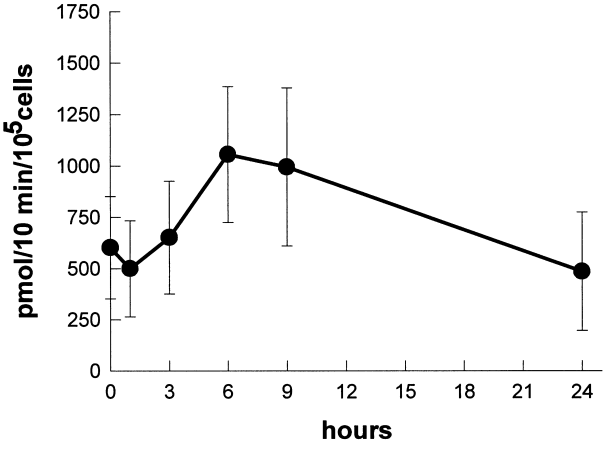

Fig. 2. Time-course response of the COX activity in fibroblasts treated with PMA. Cells were incubated for 0, 1, 3, 6, 9 and 24 h in the presence of 10 nM PMA. COX activity is expressed as pmol of [^{14}C]-AA transformed through COX pathway by 10^6 cells after incubation with 25 μM substrate for 10 min. Data shown are the means ± SD, $n = 4$.

fibroblasts formed PGE_2 and PGI_2 (determined as 6-keto-$PGF_{1\alpha}$) as the major compounds derived from AA, and lesser amounts of 12-hydroxyheptadecatrienoic acid (HHT), 15-hydroxy-eicosatetraenoic acid (15-HETE) and 11-HETE were also present.

Treatment of fibroblasts with PMA resulted in a higher production of the aforementioned eicosanoids than in untreated cells. PGE_2 was the most synthesized prostanoid after PMA-stimulation. 11- and 15-HETE were quantified together, since they resolved poorly with the chromatographic technique used. Biosynthesis of all eicosanoids was increased in a time-dependent fashion after exposure of fibroblasts to PMA. Indomethacin and the other compounds tested inhibited the formation of all eicosanoids in a concentration-dependent manner (not shown), which indicated a COX origin for all of them. Therefore, COX activity, expressed as [^{14}C]-AA transformed through COX pathway, was evaluated as the sum of all the aforementioned compounds. COX activity was evaluated as a function of time exposure to PMA. Fig. 2 shows that COX activity reached a maximum 6–9 h after PMA addition, and decreased later on.

The western blot analysis of the COX isoforms from dermal fibroblasts is shown in Fig. 3. The time-course of COX-1 and COX-2 protein expression was measured by incubating fibroblasts in the presence of 10 nM PMA for various time intervals. No cross-reactivity was observed either between COX-1 antiserum and COX-2 protein isolated from sheep placenta or between COX-2 antiserum and COX-1 protein isolated from ram seminal vesicles at the concentrations used in this assay. The antibody against COX-1 recognized a band corresponding to the migration of purified COX-1 and the antibody against COX-2 peptide recognized a major band corresponding to the migration of purified COX-2. Expression of COX-1 protein was scarce in our experimental conditions and no variation of this protein as a function of time of treatment with PMA was observed. In contrast, after PMA treatment there was a significant

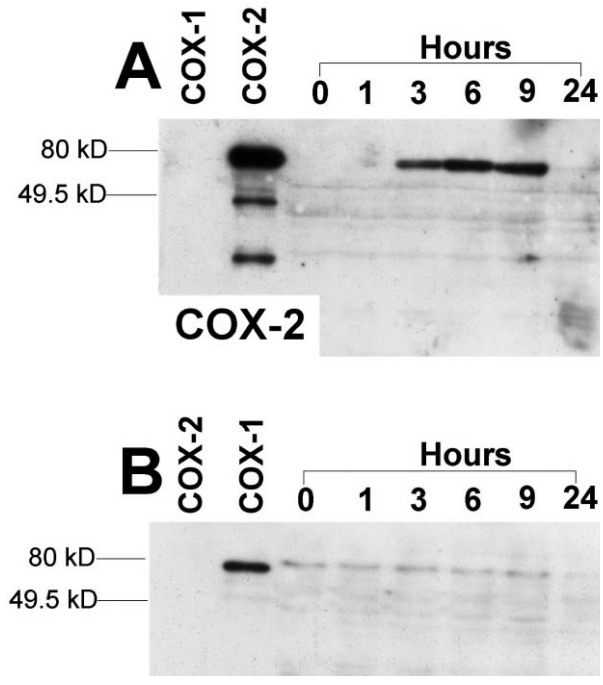

Fig. 3. Time-dependent induction of COX-2 by PMA in dermal fibroblasts. Quiescent confluent fibroblasts were incubated with 10 nM PMA for 0, 1, 3, 6, 9 and 24 h. For each condition, equal amounts of proteins (50 μg) were resolved by SDS-PAGE on 10% acrylamide gels, transferred to nitrocellulose membranes, and immunoblotted either with anti-COX-2 (PG 27) (panel A) or anti-COX-1 (PG 20) (panel B) antiserums, followed by enhanced chemiluminescence detection. Electrophoresis standards of COX-1 (from ram seminal vesicles, 0.3 μg) and COX-2 (from sheep placenta, 0.3 μg) were used as a reference. Similar results were obtained with cell extracts from two different batches of cells. Location of the standards of molecular weight in kDa is indicated.

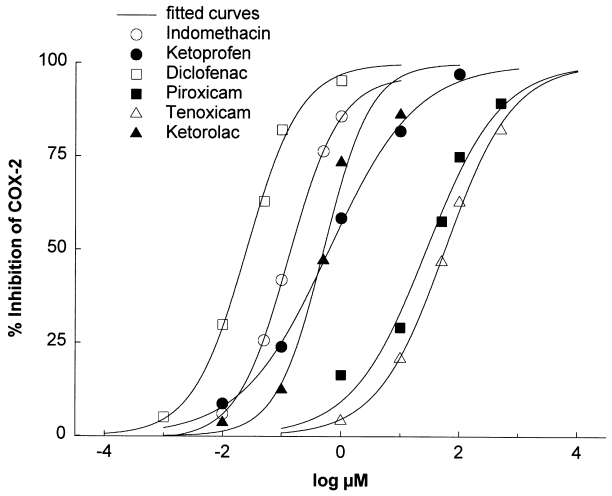

Fig. 4. Dose-response curves for inhibition of COX-2 activity by NSAIDs. Human fibroblasts induced over 7 h with PMA to express predominantly COX-2, were then preincubated with an NSAID at 37°C for 5 min. Then [^{14}C]-AA was added to initiate the COX reaction and incubation was continued for a further 10 min at 37°C. The % of inhibition were calculated as described in Section 3 and the theoretical Hill equation (Eq. (6)) was fitted to the experimental data (%inhibition vs. concentration). The curves fitted to mean data are represented above.

increase of COX-2 protein expression in a time-dependent manner with a maximum between 3 and 9 h of exposure to PMA, followed by a decrease at 24 h.

4.2. COX-2 inhibition

The COX-2 inhibition curves for the six NSAIDs assayed were similar in shape, as shown in Fig. 4; the IC_{50} values are shown in Table 1. As can be seen in Fig. 4, diclofenac was the most potent inhibitor of COX-2 and the maximum effect was attained at 1 μM, whereas oxicams (piroxicam and tenoxicam) were inhibitors at higher concentrations, with their maximum effects reached at more than 500 μM. Indomethacin, ketoprofen and ketorolac were approximately equipotent inhibitors of COX-2.

4.3. Calculation of the ITAA values

The values of J_m were previously estimated to be 0.7, 16, 1.4, 0.08, 0.7 and 13 μg h^{-1} cm^{-2} for indomethacin, ketoprofen, diclofenac, piroxicam, tenoxicam and ketorolac, respectively [18]. The method of Zwolinski [12] plotting $1/P_T$ and $1/PC$ (both at 0% of ionization) shows an horizontal line (slope $= 3 \times 10^{-7}$) indicating that the permeation through the skin is controlled by diffusion across living tissues, not across the stratum corneum. To apply Equation (8) the value of h_d can be considered constant since all drugs were assayed under the same experimental conditions. The D_d value was also considered constant due to the similar drug molecular weights of the series (from 254.3 to 357.8). A value of 200 μm was chosen for h_d as the thickness of the aqueous diffusion layer from the dermal/epidermal junction until the internal surface of dermatomed skin. The D_d value of living skin tissues is typically in the range of 10^{-6} to 10^{-5} cm^2 s^{-1} [19–21]. For the NSAIDs assayed a value of 10^{-5} cm^2 s^{-1} was estimated as an approximation for comparative purposes. This value was calculated from the experimental results of the transdermal lag time (T_l) [18], taking into account that the diffusion coefficient of the living skin tissues is approximately 1000 times greater than that of stratum corneum [11,22].

The calculated *ITAA* values for the NSAIDs series from Eq. (7) are shown in Table 1. These results indicated that the highest topical anti-inflammatory activity is caused by diclofenac, followed by ketorolac and ketoprofen. Indomethacin exhibited substantially lower *ITAA* than these NSAIDs. Oxicams (piroxicam and tenoxicam) showed the lowest *ITAA* of the series. Furthermore, the *ITAA* values were calculated considering that therapeutic relevance was reached after 75 or 90% inhibition of COX-2 (Table 1). Slight qualitative differences from the above results were observed in the cases of diclofenac and ketorolac. Nevertheless, as percentage inhibition increased, differences between ketoprofen and indomethacin decreased.

The *ITAA* of two reference drugs (indomethacin and diclofenac) were also calculated from other published data using Eq. (8). The J_m was obtained from Singh & Roberts

Table 1
The estimated parameters (mean ± SEM.) of COX-2 inhibition curves from four independent experiments and the *ITAA* at 50, 75 and 90% of COX-2 inhibition

Drug	n_H[a]	IC_{50} (μM)	MW (g × mol^{-1})	J_m[b] (μg × h^{-1} × cm^{-2})	*ITAA*[c] (*I* = 50%)	*ITAA*[c] (*I* = 75%)	*ITAA*[c] (*I* = 90%)
Indomethacin	1.47 ± 0.33	0.16 ± 0.04	357.8	0.7	3.4	1.6	0.8
Ketoprofen	0.63 ± 0.11	0.74 ± 0.37	254.3	16	23.6	4.1	0.7
Diclofenac	1.30 ± 0.39	0.03 ± 0.01	296.2	1.4	43.8	18.8	8.1
Piroxicam	0.97 ± 0.19	34.90 ± 7.64	331.4	0.08	0.0019	0.0006	0.0002
Tenoxicam	0.99 ± 0.01	55.26 ± 21.82	337.4	0.7	0.0104	0.0034	0.0011
Ketorolac	1.63 ± 0.08	0.38 ± 0.04	255.3	13	37.2	19.0	9.7

[a] Hill coefficient.
[b] In vitro permeation flux across human skin (see Ref. [18]).
[c] Index of topical anti-inflammatory activity calculated from equation (7).

Table 2
The index of topical anti-inflammatory activity calculated from bibliographic data using Eq. (8)

Drug	J ($\mu g \times h - 1 \times cm^{-2}$)	IC_{50} (μM)	In vitro model[a]	*ITAA* ($I = 50\%$)
Indomethacin	0.025[b]	1.68[c]	1	0.012–0.036
Diclofenac	0.048[b]	1.18[c]		
Indomethacin	0.025[b]	1.4[d]	2	0.01–0.42
Diclofenac	0.048[b]	0.1[d]		
Indomethacin	0.025[b]	0.026[e]	3	0.75–32
Diclofenac	0.048[b]	0.001[e]		
Indomethacin	0.025[b]	0.022[f]	4	0.09–25
Diclofenac	0.048[b]	0.002[f]		
Indomethacin	0.025[b]	0.044[g]	5	0.44–43
Diclofenac	0.048[b]	0.001[g]		

[a] 1, Endotoxin-activated J774.2 macrophages; 2, Microsomal preparations from COS-7 cells with human recombitant COX-2; 3, CHO cells stably transfected with human COX-2; 4, LPS-induced COX-2 activity of human peripheral blood cells; 5, Interleukin 1-β-stimulated human synovial cell COX-2.
[b] From Ref. [23].
[c] From Ref. [3].
[d] From Ref. [25].
[e] From Ref. [5].
[f] From Ref. [7].
[g] From Ref. [8].

[23] because of the differences from authors data in order to know the ITAA robustness. As very different IC_{50} values have been reported depending on the pharmacological model and the experimental variables used, only those studies where both diclofenac and indomethacin were assayed under the same experimental conditions were selected (see Table 2). Despite the fact that *ITAA* values varied greatly, due to the variability of IC_{50}, in all cases diclofenac was more active than indomethacin (3–100-fold higher) in terms of *ITAA*.

5. Discussion

Results of western blotting showed that the phorbol ester mainly induced COX-2 in the cultured human fibroblasts used, which was consistent with data reported using Swiss 3T3 cells [24]. These results indicate that PMA-stimulated human fibroblasts are suitable for measuring the inhibitory effect of NSAIDs on human COX-2. The value of IC_{50} of each NSAID for COX-2 activity in induced fibroblasts gives a comparative indication of their intrinsic anti-inflammatory potencies. The variability of the COX-2 inhibitory potencies (IC_{50}) of the drugs investigated spans a range of 10^3 which is similar to that stated by other authors working with a series of NSAIDs [3,25]. For comparative purposes, care must be taken when results come from different pharmacological models and different experimental conditions. Certain factors, such as the type of cells used for the in vitro test (purified enzyme, microsomes, broken or intact cells, etc.), the preincubation time before the addition of the subtract (AA) or the analytical method used, are the main sources of disagreement. For example, the IC_{50} values that we obtained in COX-2 inhibition for diclofenac, indomethacin and piroxicam follow the same ranking order (Table 1), but are approximately one order of magnitude lower than those previously reported by O'Neill et al. [25] (IC_{50}: 0.10, 1.4 and >300 μM, respectively). These authors used human COX-2 obtained from microsomal preparations of COS-7 cells. Although non-human cells were used (J774.2 macrophages), Mitchell et al. [3] found that diclofenac (IC_{50}: 1.2 μM) and indomethacin (IC_{50}: 1.7 μM) were equipotent. Engelhardt et al. [26] worked with a different pharmacological model (guinea-pig peritoneal macrophages in vitro) but the ranking order of potencies was the same as we found: diclofenac > indomethacin > piroxicam > tenoxicam. Bradshaw et al. [27], using COX from sheep seminal vesicles, stated that tenoxicam and piroxicam were equipotent (110 and 120 μM, respectively) and that both drugs were about 100-fold less active than indomethacin (IC_{50}: 1.4 μM) in this test. Differences of the same order were observed in the present study. We should emphasize that the most suitable in vitro models for investigating the pharmacology of inflammatory responses in the skin are those based on cultured dermal cells where the expression of COX-2 is predominant.

In addition to PGE_2 (65%) and 6-keto-$PGF_{1\alpha}$ (20%), other COX-2 derived compounds such as 15-HETE (4%) were also detected. Meade et al. [28] suggested that 15-HETE may have an important role in the prevention of colon cancer. The complete inhibition of 15-HETE synthesis was reached at 500 μM for tenoxicam and piroxicam, at 10 μM of ketoprofen and ketorolac, at 1 μM of indomethacin and around 0.1 μM for diclofenac.

The comparatively high values of *ITAA* obtained for diclofenac, ketorolac and ketoprofen suggest that they are the best candidates of the series assayed to be efficient in topical formulations. Indomethacin and oxicams exhibited lower topical effectiveness than the foregoing NSAIDs, in terms of *ITAA*. Considering diclofenac and indomethacin as

representative of the two categories (high and low topical efficacy, respectively), the *ITAA* values calculated from other reported data showed that diclofenac is a better candidate for topical formulation than indomethacin (Table 2). This is consistent with the effectiveness of the commercial formulation of diclofenac [29]. In the case of ketorolac, which seems one of the most dermally active products of the series studied, no topical preparations have been launched.

The *ITAA* is useful for biopharmaceutical studies since high *ITAA* values of a particular drug indicate that it can very feasibly be formulated as a topical pharmaceutical preparation. Drugs with scarce local activity (low *ITAA*) may be formulated topically but additional studies with skin penetrants would be indicated. Of the *ITAA* values calculated with 90% inhibition of COX-2, only in two cases (diclofenac and ketorolac) was this index higher than one (Table 1). Drugs with high *ITAA* values and low systemic absorption after application to the skin are the candidates with the best biopharmaceutical profile. Therefore, if 90% enzyme inhibition were required in order to achieve therapeutic relevance on the skin, only diclofenac and ketorolac would be good candidates for topical formulations. Nevertheless, extrapolation of *ITAA* values to therapeutic efficacy should be cautiously done unless conclusive proofs of clinical efficacy were provided.

Recently, clinical efficacy, safety and percutaneous absorption of topical NSAIDs have been reviewed [30]. More than twenty clinical studies of topical NSAIDs were included after a reference search covering the last 5 years. Overall evaluation of these studies indicates that piroxicam gel seems to show less efficacy than other alternatives [31], while diclofenac seems to account for higher efficacy than placebo [32,33] or a gel of felbinac [34]. These conclusions support our results, though care must be taken when extrapolating from results obtained in vitro to humans. In general, there is a good relationship between the therapeutic plasma levels of this drugs at clinically active doses [18] and the in vitro IC_{50} values obtained in present manuscript ($r = 0.908$, $n = 6$). This fact justifies that 50% inhibition could be considered therapeutically relevant in the estimation of *ITAA*. In addition, diclofenac is one of the best candidates of the series assayed for a topical formulation, which can also be concluded from a clinical trial with 1575 patients treated for acute soft-tissue injury [31]. These authors concluded that treatment with ketoprofen and diclofenac gels was significantly more effective than with piroxicam gel. No significant differences were observed between ketoprofen and diclofenac in terms of efficacy, which is also consistent with the present results. Sengupta et al. [35] also demonstrated that diclofenac gel was more efficient than piroxicam gel in healthy volunteers. The greater efficacy of ketorolac than placebo was demonstrated in a clinical trial with 36 patients for the treatment of ankle sprain [36]. This in vivo assay confirms the other conclusion drawn from the *ITAA* values, i.e. ketorolac has good intrinsic characteristics for formulation in an efficacious anti-inflammatory topical application.

6. Conclusion

An in vitro-based index to compare the topical anti-inflammatory activity of a series of six NSAIDs was calculated. For piroxicam and tenoxicam, transdermal studies with penetration enhancers may be required to increase their intrinsic topical efficiency. Diclofenac, ketorolac, ketoprofen and indomethacin appear as acceptable candidates for formulation as topical pharmaceutical forms.

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