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INFLUENCE OF THE ANTI-INFLAMMATORY COMPOUND FLOSULIDE ON GRANULOCYTE FUNCTION

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Abstract—Polymorphonuclear leukocytes (PMN) are involved in inflammatory reactions. It is thought that oxygen-derived free radicals released from activated PMN may participate in tissue damage during inflammation. We have shown that flosulide (6-(2,4-difluorophenoxy)-5-methylsulfonylamino-1-indanone), a novel highly potent anti-inflammatory compound, inhibits superoxide production induced by *N*-formyl-Met-Leu-Phe (FMLP), C5a and PMA without impairing bacterial killing or chemotaxis. Flosulide (10^{-5}–10^{-7} M) was more potent in inhibiting the FMLP-induced respiratory burst of PMN than the structurally related compound nimesulide. FMLP-induced superoxide generation was also inhibited by two human flosulide metabolites. A good correlation between this *in vitro* effect and *in vivo* anti-inflammatory potency in rat adjuvant arthritis was found for flosulide and its metabolites. Indomethacin, piroxicam and ibuprofen did not inhibit the respiratory burst at 10^{-5} M. FMLP receptor number was decreased by 36% in the presence of 10^{-5} M flosulide. However, a 250-fold molar excess of flosulide could not displace labeled FMLP from the receptor. Inhibition of degranulation of primary and secondary granules was a common effect of all anti-inflammatory compounds tested. At a concentration of 10^{-5} M, all drugs inhibited degranulation to about the same degree, independent of their *in vivo* anti-inflammatory activity.

Inflammation is characterized by the accumulation of polymorphonuclear leukocytes (PMN‖) at extravascular sites [1]. Inflammatory tissue destruction is at least partially due to activated PMN which produce oxygen radicals [2] and release lysosomal enzymes, such as elastase and collagenase [3]. It is therefore conceivable that inhibitors of PMN-functions could be potent anti-inflammatory drugs. However, such an anti-inflammatory mechanism is potentially harmful, since extravascular PMN are indispensable for the elimination of microorganisms at the site of inflammation.

Flosulide (6-(2,4-difluorophenoxy)-5-methylsulfonylamino-1-indanone) is a new highly potent non-steroidal anti-inflammatory drug (NSAID) [4]. Unlike established NSAID, flosulide is nearly inactive in inhibiting cyclooxygenase, although prostaglandin production is suppressed in various cells and tissues [4]. It is still not clear if inhibition of prostaglandin synthesis is the only mechanism responsible for the high anti-inflammatory potency of flosulide. We therefore studied the effect of flosulide on superoxide (O_2^-) production and PMN degranulation, i.e. two PMN functions which are supposed to be involved in inflammatory tissue destruction [2, 3, 5–7]. In order to exclude a harmful effect on host defense [5, 8], we also tested the influence on chemotaxis and phagocytic-bactericidal activity of PMN.

The effect of flosulide on PMN functions was compared to that of established NSAID (indomethacin, piroxicam, ibuprofen) and to nimesulide, which is structurally related to flosulide. Two major flosulide metabolites identified in human plasma were also tested.

MATERIALS AND METHODS

Reagents. Reagents used and their sources were: bovine serum albumin, cytochalasin b, cytochrome *c* (type IV), Hepes, DMSO, phenolphthalein glucuronic acid solution, phenolphthalein, phenolphthalein standard solution, Triton X-100, formylpeptide (FMLP) and phorbol myristate acetate (Sigma Chemical Co., St Louis, MO, U.S.A.); dextran T-500 and Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); ^{57}Co-B12 and FML[^{3}H]P (New England Nuclear, Boston, MA, U.S.A.); EDTA and activated charcoal (Merck AG, Zürich, Switzerland); and Harleco Diff-Quik (Merz & Dade AG, Düdingen, Switzerland). Purified C5a was kindly provided by Dr C. Dahinden (Bern, Switzerland). Hepes Hanks' balanced salt solution with (HBSS) or without calcium and magnesium (mHBSS) were prepared according to standard methods [9].

Drug preparation. Flosulide and the metabolites M I(2-OH) and M II (3-OH), piroxicam, ibuprofen, indomethacin and nimesulide were kindly provided by Ciba-Geigy Ltd as pure substances. Flosulide, its metabolites and indomethacin were dissolved in 80% DMSO/NaCl (0.9%) to a stock

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‖ Abbreviations: PMN, polymorphonuclear leukocyte(s); DMSO, dimethyl sulfoxide; flosulide, 6-(2,4-difluorophenoxy) - 5 - methylsulfonylamino - 1 - indanone; FMLP, *N*-formyl-Met-Leu-Phe; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; HBSS, Hepes Hanks' balanced salt solution; NSAID, non-steroidal anti-inflammatory drug(s).

solution of 10^{-2} M. Piroxicam was dissolved in 100% DMSO to a stock solution of 10^{-2} M. Ibuprofen was dissolved in 0.1 N NaOH at a concentration of 10^{-1} M and further diluted with distilled water to a stock solution of 10^{-2} M. Nimesulide was dissolved in 0.1 N NaOH to a stock solution of 10^{-2} M. All stock solutions were kept at −20° and further diluted with mHBSS to the appropriate working solution just prior use.

Isolation of PMN. Human blood PMN were purified from EDTA-anticoagulated (5 mM final concentration) blood, drawn from healthy volunteers. Purification included dextran T-500 (3% in 0.9% saline) sedimentation and subsequent centrifugation (350 g × 30 min at 4°) on a 53%/67% Percoll gradient as described previously [10]. PMN accumulating at the interphase of the gradient were washed twice in 0.9% NaCl. Hypotonic lysis was performed, if necessary, with distilled water and correction of the osmolarity with 1.8% NaCl after 15–30 sec. The final cell preparation contained >97% viable PMNs.

Chemotaxis assay. PMN migration was quantitated using a micropore filter technique in a 48-well microchamber (Neuro Probe, Inc., Cabin John, MD, U.S.A.), as previously described by Harvath *et al.* [11]. A 10 μm thick polycarbonate filter sheet with 3-μm pores (Nucleopore Corp., Pleasanton, CA, U.S.A.) was placed between the lower compartments containing chemoattractants and the upper compartments containing PMN (2.5×10^{-6}/mL). PMN were tested in the presence of 0.08% DMSO, and 10^{-5} M flosulide in the same microchamber. The assembly was incubated for 30 min at 37° in humidified air with 5% CO_2. After incubation, the filter was removed, air-dried and stained with Diff-Quik. PMN on both sides of the filter were counted using a graduated ocular (×8) and a ×63 objective on a Zeiss microscope. For each stimulus and cell type six different fields were chosen at random for counting. Results are reported as per cent PMN migration through the filter.

Phagocytic bactericidal capacity of PMN. This assay has been described in detail previously [12]. We used *Staphylococcus aureus* Wood 46 as the test strain. This strain depends mainly on complement for its opsonization. The test incubation contained 0.1 mL of the washed and appropriately diluted bacterial suspension, 0.1 mL of serum from a normal pool, and 0.8 mL HBSS containing 5×10^6 PMN. This test mixture was incubated either with 0.08% DMSO (control), or with 10^{-5} M of the anti-inflammatory drug. The tubes were incubated at 37° for 30 min on an overhead rotator. The bacterial killing by PMN was calculated from bacterial counts at time 0 and 30 min. Each test contained two control tubes, namely pooled serum without PMN to exclude an antibacterial activity of serum, and PMN with heat-inactivated serum to verify opsonic requirements.

Superoxide production. O_2^- production was determined at 37° spectrophotometrically (550 nm, millimolar extinction coefficient of 21,000 $M^{-1}cm^{-1}$) by continuously monitoring the O_2^- dismutase (300 units/mL)-inhibitable reduction of cytochrome c (120 μg/mL) in a double-beam spectrophotometer (model 35, Beckman Instruments, Fullerton, CA, U.S.A.) with a temperature regulator set [13]. The effect of anti-inflammatory drugs was tested by adding the substance at the appropriate concentration in the cuvette 5 min before PMN stimulation. Control PMN were incubated in the presence of 0.08% DMSO.

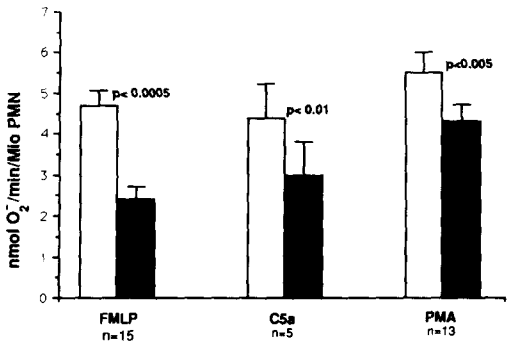

Fig. 1. Effect of flosulide on superoxide (O_2^-) production induced by different stimuli. O_2^- production was measured in the presence of DMSO (0.08%, □) or flosulide (10^{-5} M, ■), respectively, after stimulation with cytochalasin b/FMLP (10^{-6} M), cytochalasin b/C5a (10^{-7} M) or PMA (100 ng/mL), respectively. Results from the indicated number of experiments were given as mean ± SEM. The P values are calculated with the paired t-test.

Degranulation assay. PMN (5×10^6) were incubated in a shaking waterbath at 37°. At time zero 5 μg/mL cytochalasin b was added. Five minutes later 10^{-5} M test drug and 10^{-6} M FMLP (final concentration) were added. After another 15 min the reaction was stopped on ice, the tubes centrifuged (8000 g × 3 min), the supernatant replaced with NaCl/Triton X-100 (0.1%) and after 5 min at 0° centrifuged again. Primary granules (β-glucuronidase) and secondary granules (β_{12}-binding protein), respectively, were determined in the supernatant and in the Triton X-100 (0.1%) lysed pellet with standard methods [9]. Spontaneous degranulation was determined in control tubes without cytochalasin b and FMLP. Results are given as per cent of total cellular content as follows: 100% − (remaining granules in PMN after assay/total granules in unstimulated PMN) × 100.

FML[^{3}H]P binding assay. The incubation medium for the binding assay was mHBSS with 0.2% bovine serum albumin and flosulide (10^{-5} M) or DMSO (0.08%), respectively. FML[^{3}H]P binding to PMN was carried out at 4° for 20 min using a silicon–oil technique as described previously [14]. For Scatchard analyses final concentrations of FML[^{3}H]P in binding assays varied from 3 to 200 nM. Non-specific binding was determined with a parallel series of samples by adding a 250-fold excess of unlabeled FMLP. Scatchard plots of the experimental data (specific binding) were fitted by linear regression. The dissociation constant (K_d) and receptor number (B_{max}) were estimated from the fitted slopes and x-intercepts [14].

Adjuvant arthritis [15]. Adjuvant arthritis was induced in male Lewis rats (LEW/MOL, 145–170 g

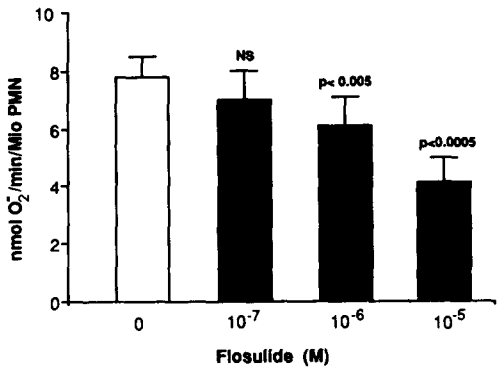

Fig. 2. FMLP-induced superoxide (O_2^-) production in PMN treated with different concentrations of flosulide. After 5 min of preincubation with either DMSO (0.08%, □) or increasing concentrations of flosulide (■), PMN were stimulated with cytochalasin b/FMLP (10^{-6} M). Results are expressed as mean ± SEM of 5 different experiments. The P values are calculated with the paired *t*-test.

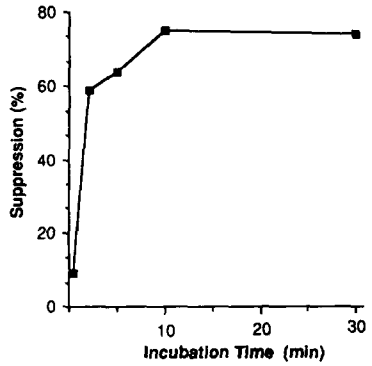

Fig. 3. Time course of the suppression of superoxide (O_2^-) production by flosulide (10^{-5} M). PMN were stimulated with cytochalasin b/FMLP (10^{-6} M) after the indicated time of preincubation. Results are reported as suppression in comparison to PMN incubated in 0.08% DMSO. Two other experiments gave similar results.

body wt, five animals per group) by intraplantar injection of 0.2 mg *Mycobacterium butyricum* (Difco) in 0.05 mL paraffin oil into the left hind paw. The drugs (suspended in 0.75% methylcellulose) were given p.o. once daily from day 11 to day 14 after induction of arthritis. The oedema of the injected paw was measured plethysmometrically on days 11 and 15. The anti-inflammatory effect is expressed as per cent inhibition of the oedema of the arthritic controls. ED_{40} values were calculated by graphical interpolation.

RESULTS

Influence on stimulated O_2^- generation

Figure 1 shows the effect of flosulide on the stimulated O_2^- production in human PMN induced by FMLP, C5a or PMA. Regardless which stimulus was used, flosulide (10^{-5} M) caused a significant inhibition of the respiratory burst.

The inhibition of FMLP-induced O_2^- production by flosulide was further investigated. Concentrations from 10^{-5} to 10^{-7} M inhibited this response dose-dependently (Fig. 2). Figure 3 shows a typical experiment analysing the time-dependency of the flosulide effect. Only 10 min after the drug was added to FMLP-stimulated PMN, maximal suppression of the respiratory burst was reached.

For comparison, the reference NSAID indomethacin, piroxicam, ibuprofen and nimesulide were tested for their effect on FMLP-induced O_2^- production under identical conditions as for flosulide (Fig. 4). At a concentration of 10^{-5} M, flosulide and nimesulide, which are structurally related compounds, were the only drugs significantly inhibiting the O_2^- production. Flosulide was more potent than nimesulide ($P < 0.005$).

Inhibition of PMN degranulation

Flosulide as well as all reference NSAID tested inhibited FMLP-induced degranulation of

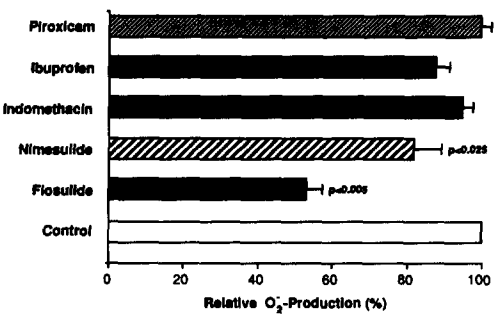

Fig. 4. Effect of different anti-inflammatory compounds on FMLP-induced superoxide (O_2^-) production. PMN were incubated with DMSO (0.08%), or the indicated drug (10^{-5} M), respectively. After a preincubation period of 5 min, PMN were stimulated with cytochalasin b/FMLP (10^{-6} M). Results are means ± SEM from at least 5 different experiments. The P values were calculated with the paired *t*-test, comparing the absolute values of drug-incubated and control PMN. Bars without P values are not significantly different from controls.

β-glucuronidase and B_{12}-binding protein at a concentration of 10^{-5} M (Fig. 5).

Influence on bactericidal activity and chemotaxis

PMN produce O_2^- in excess. Therefore, a partial reduction of the stimulated oxidative burst does not necessarily result in an impaired bacterial killing. Staphylococcal killing was indeed not influenced in the presence of 10^{-5} M flosulide (83 ± 6%), indomethacin (88 ± 3%), piroxicam (85 ± 2%), ibuprofen (83 ± 4%) and nimesulide (86 ± 3%) when compared to control PMN (83 ± 7%). All compounds were tested in at least three experiments.

Similarly, chemotaxis of PMN induced by FMLP or activated serum was also not inhibited in the presence of 10^{-5} M flosulide (Fig. 6), nor by indomethacin, piroxicam, ibuprofen and nimesulide, respectively (data not shown).

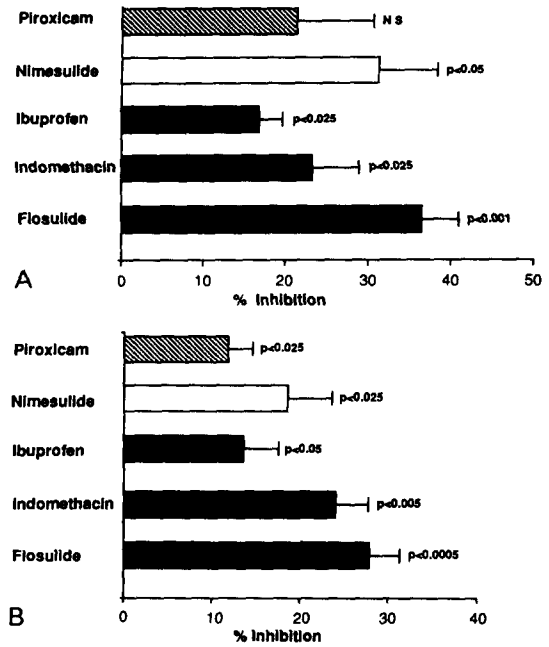

Fig. 5. Inhibition of FMLP-induced degranulation of B_{12}-binding protein (panel A) and β-glucuronidase (panel B), respectively. Degranulation was measured after stimulation with cytochalasin b/FMLP (10^{-6} M) in the presence of the indicated drug (10^{-5} M). Results are expressed as % inhibition in comparison to DMSO (0.08%)-incubated control PMN. The P values indicate the significance of the difference between drug-incubated and control PMN (paired *t*-test).

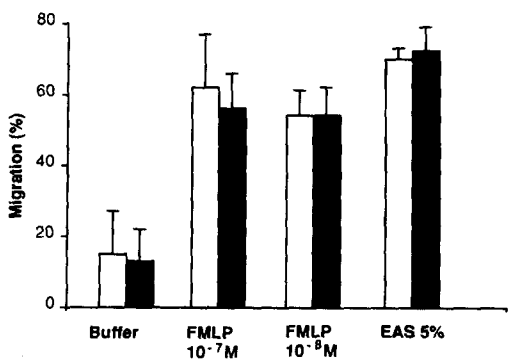

Fig. 6. Influence of flosulide on chemotaxis. Migration was assessed in a 48-well microchamber. PMN were simultaneously incubated in the identical chamber, either in the presence of DMSO (0.08%, □) or flosulide (10^{-5} M, ■). Results are reported as % PMN which migrated through the 10-μm filter within 30 min. Data are the means ± SEM from 5 different experiments. No difference was significant.

Effect of flosulide on FML[^{3}H]P-binding

To further investigate the inhibitory mechanism of flosulide on FMLP-induced O_2^- production and degranulation, we have tested its influence on

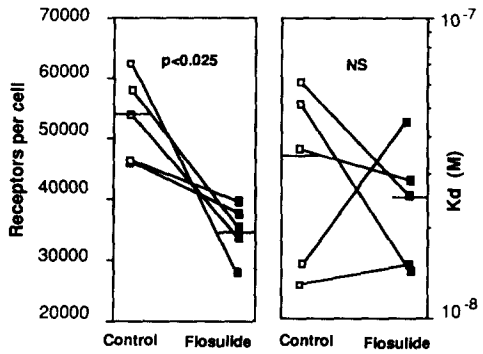

Fig. 7. Effect of flosulide (10^{-5} M) on FML[^{3}H]P binding. Each point is calculated from a complete Scatchard analysis. The differences between control PMN (0.08% DMSO) and flosulide PMN were calculated with the paired *t*-test.

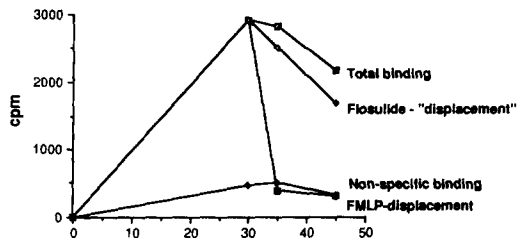

Fig. 8. Displaceable FML[^{3}H]P binding. PMN were incubated with 50 nm labeled FML[^{3}H]P in the presence (non-specific binding) or absence (total binding) of a 1000-fold excess of unlabeled FMLP. After a 30-min incubation at 4° a 250-fold molar excess of FMLP (FMLP-displacement) or flosulide (flosulide-"displacement") was added. FML[^{3}H]P binding was determined again 5 and 15 min after displacement.

FML[^{3}H]P binding. In five out of five Scatchard analyses the presence of 10^{-5} M flosulide decreased specific FML[^{3}H]P binding by about 35% (Fig. 7). The receptor affinity was not influenced by flosulide.

Figure 8 shows that the FML[^{3}H]P binding, which was perfectly displaceable by a 250-fold excess of cold FMLP, could not be displaced by a 250-fold molar excess of flosulide. These results may indicate an indirect effect of flosulide on the FMLP-receptor.

Effect of flosulide metabolites on PMN functions

Two major metabolites, M I(2-OH) and M II(3-OH), have been identified in human plasma after single and repeated oral administration of flosulide. Their effects on PMN functions were investigated in comparison to flosulide (Table 1). Like flosulide, M I caused significant inhibition of O_2^- generation at 10^{-5} M, but to a lower degree than flosulide. M II was nearly inactive at 10^{-5} M.

On the other hand, no differences in activity were found between flosulide and its metabolites in inhibiting PMN degranulation (Table 1).

Correlation between in vitro *inhibition of PMN functions and* in vivo *anti-inflammatory activity of flosulide and its metabolites*

We hypothesized that the inhibition of PMN

Table 1. Inhibition of PMN function *in vitro*

Drug	O_2^- generation Inhibition (%)		Degranulation Inhibition by 10^{-5} M (%)	
	10^{-5} M	10^{-6} M	Primary	Secondary
Flosulide	41 ± 14	19 ± 8	21 ± 7	43 ± 24
Metabolite I	24 ± 11*	13 ± 14	21 ± 4	35 ± 16
Metabolite II	9 ± 9‡	0 ± 0†	16 ± 6	32 ± 17

Results of *in vitro* tests are given as mean ± SD.
Flosulide and its metabolites were tested simultaneously in triplicate at least 5 times.
* $P < 0.025$, † $P < 0.005$, ‡ $P < 0.0005$ (compared to flosulide, paired *t*-test).

Table 2. Anti-inflammatory activity of flosulide metabolites in comparison to flosulide in rat adjuvant arthritis

Drug	Number of animals (N)	Dose (mg/kg)	Oedema inhibition (%) Expt1	2	3	ED_{40} (mg/kg)
Flosulide	15	0.03	27	8	15	
	15	0.1	48	36	49	
	15	0.3	58	45	66	
	15	1	59	54	71	
	5	3			73	~0.05
Metabolite I	5	0.1	22			
(M I)	10	0.3	42	36		
	5	1	58			
	10	3	68	61		
	5	30		57		~0.3
Metabolite II	10	0.3	13	8		
(M II)	5	1		26		
	10	3	32	37		
	5	10		56		
	10	30	48	59		~4

functions by flosulide might be a mechanism contributing to its potent anti-inflammatory activity. We therefore compared the *in vitro* effects on PMN functions (Table 1) and the *in vivo* anti-inflammatory potency of flosulide and its two metabolites (Table 2). Compounds were tested in rat adjuvant arthritis, a chronic model for rheumatoid arthritis and related diseases. The arthritic animals were treated during the early stage of the disease, which is characterized by rapid oedema development and massive PMN infiltration in the inflamed joints. Both metabolites of flosulide (M I and M II) were distinctly less active than flosulide in rat adjuvant arthritis (Table 2). On the basis of ED_{40} values, M I and M II were about 6 and 80 times less potent than flosulide. The anti-inflammatory potency of flosulide and its metabolites was therefore parallel to their inhibition of the respiratory burst, but was unrelated to their inhibitory effects on PMN degranulation.

DISCUSSION

Inhibition of prostaglandin synthesis is thought to be the major mechanism responsible for the anti-inflammatory activity of many NSAID [4, 16]. Recently, the suppression of PMN activation has been proposed as an alternative or additional mechanism of action of NSAID [16–20]. Several observations are in favour of this concept: (i) PMN accumulation is involved in acute and chronic inflammatory tissue destruction [7]; (ii) the anti-inflammatory potency and inhibition of prostaglandin synthesis are not always strictly correlated [4]; (iii) several NSAID act on PMN activation independently on inhibition of prostaglandin synthesis [18, 21, 22].

The development of new NSAID should consider this concept, since suppression of prostaglandin synthesis accounts for many side-effects of these agents such as gastro-intestinal bleeding, impairment of platelet function, decrease of renal function and bronchoconstriction [16, 23]. Flosulide is a novel NSAID with potent anti-inflammatory action and improved gastro-intestinal tolerance in preclinical studies [4]. We found that flosulide was the most potent inhibitor of FMLP-induced O_2^- production when compared to piroxicam, ibuprofen, indomethacin and nimesulide. The previously described inhibition of the respiratory burst by nimesulide could be confirmed [18]. However, flosulide was distinctly more potent than nimesulide (Fig. 4). In

contrast, we did not find the inhibitory effect of piroxicam described by Altman [16] and by Abramson *et al.* [17]. The reason for this may be the 5-fold lower molar concentration of piroxicam which we used in our experiments.

The inhibition of O_2^- production by flosulide was significant at concentrations of 10^{-6} M. This suppression is thought to be of clinical relevance, since plasma peak concentrations of 3×10^{-6} M are reached after a single oral dose of 25 mg flosulide (unpublished data).

The comparison of the anti-inflammatory potency and suppression of the oxidative burst by flosulide, its two metabolites and the structurally related nimesulide revealed a parallel hierarchy. This observation supports the hypothesis that suppression of O_2^- generation may be, at least in part, responsible for the anti-inflammatory activity of flosulide and nimesulide. However, since indomethacin, piroxicam and ibuprofen, independent of their anti-inflammatory potency, did not inhibit O_2^- production at 10^{-5} M, this mechanism is not a therapeutical principle for these NSAID.

On the other hand, inhibition of degranulation of primary and secondary granules was a common effect shown for all compounds tested. There was no correlation to the anti-inflammatory activity. Therefore, suppression of the respiratory burst seems to be more important for the inhibition of inflammation than the blockade of granule release.

Phenylbutazone and sulfinpyrazone have previously been shown to inhibit competitively FMLP-induced enzyme release, O_2^- production, hexose monophosphate activity and adhesiveness [21]. Both drugs interfered with specific FML[^{3}H]P binding to PMNs indicating receptor-antagonistic properties [21]. We have therefore tested the influence of flosulide on FML[^{3}H]P binding on PMN.

The presence of 10^{-5} M flosulide reduced FML[^{3}H]P binding sites by about 35% without significant modification of the receptor affinity. However, the FMLP binding could not be displaced by a 250-fold molar excess of flosulide. Therefore, the inhibition of FML[^{3}H]P binding is either non-specific or, alternatively, flosulide binding has a K_d which is more than 250-fold higher than that of FMLP. The antagonism of FMLP binding cannot be the only mechanism by which flosulide inhibits the respiratory burst, since C5a- and PMA-induced O_2^- production was also depressed, although to a lower degree. Another mechanism of action could be the blockade of PMN functions via cyclooxygenase inhibition. However, since indomethacin had no effect on the respiratory burst, this mechanism seems to be unlikely. Furthermore, cyclooxygenase products can by themselves inhibit PMN functions [24].

The effect of flosulide on PMN functions was completely reversible after a single washing step (data not shown). This observation indicates that flosulide did not irreversibly damage PMN within the concentration range used.

The described inhibition of the respiratory burst by flosulide and nimesulide could be potentially harmful, since it could interfere with host defense. Our data, however, indicate that this is not the case for these compounds. Chemotaxis as well as staphylococcal killing were not impaired in the presence of 10^{-5} M flosulide, nimesulide and reference NSAID.

In conclusion, flosulide and to a lesser degree nimesulide inhibit O_2^- production in human PMN. This property differentiates these compounds against indomethacin, piroxicam and ibuprofen and may be a potential anti-inflammatory mechanism for these compounds. It was not associated with compromised host defense mechanisms.

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