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Effects of PAF-antagonists in mouse ear oedema induced by several inflammatory agents

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1 Several platelet activating factor (PAF)-antagonists of different chemical structures were tested in the arachidonic acid-, tetradecanoylphorbol acetate-, dithranol-, and benzoic acid-induced mouse ear oedema models.

2 Topical application of UR-10324, UR-11353, CV-6209 and WEB-2086 markedly inhibited ear oedema induced by the four irritants tested, mimicking the profile obtained with dexamethasone. YM-461 was highly effective only in the dithranol-induced ear oedema, while BN-52021 failed to inhibit ear oedema in all models tested.

3 Leukocyte recruitment into the inflamed ears was prevented by PAF-antagonists, as measured by myeloperoxidase activity in the supernatants of ear homogenates.

4 A relationship between PAF-antagonist and anti-inflammatory activities was found in some cases, but other mechanisms cannot be excluded to explain the topical anti-inflammatory effect of these compounds.

5 Our results suggest that topical formulations containing PAF-antagonists could be useful in the treatment of some inflammatory skin diseases and provide evidence on the involvement of PAF in these inflammatory processes.

Keywords: PAF-antagonists; mouse ear oedema; arachidonic acid; phorbol esters; dithranol; benzoic acid; myeloperoxidase

Introduction

Inflammation of the skin is characterized by increased vascular permeability, plasma protein exudation and migration of inflammatory cells. All the features of the inflammatory response are closely mimicked by platelet-activating factor (PAF) (Camussi & Brentjens, 1987). Thus, intradermal injection of PAF causes an acute wheal and flare response followed by delayed erythema in man (Pirotski *et al.*, 1985). Moreover, PAF and PAF-like materials have been isolated from psoriatic scales (Mallet & Cunningham, 1985) and from the blood of patients with primary acquired cold urticaria (Grandel *et al.*, 1985). All these observations support the role of PAF in the pathophysiology of the inflammatory skin responses, suggesting that PAF-antagonists could be useful in the treatment of some skin diseases.

The most commonly used animal model in the search for novel therapeutic agents useful in the topical treatment of inflammatory skin conditions is rodent ear oedema induced by application of an irritant agent (Young & De Young, 1989). In this work we have chosen some of the currently used primary irritant agents, such as arachidonic acid (AA), 12-O-tetradecanoylphorbol 13-acetate (TPA), dithranol (D), and benzoic acid (BA). Arachidonic acid produces an acute transient inflammatory reaction with immediate erythema, oedema and an elevated tissue concentration of prostaglandins and leukotrienes (Opas *et al.*, 1985). Although not very selective, this test has been used to evaluate 5-lipoxygenase inhibitors *in vivo* (Pignat *et al.*, 1986). TPA has been isolated from croton oil and identified as the active constituent. This substance has been reported as a tumour promoter and an inducer of epidermal hyperplasia (Young *et al.*, 1984). On the other hand, dithranol is one of the most important drugs used for the local treatment of psoriasis. The usefulness of dithranol is highly limited by its irritant effect on the surrounding skin (Keméry *et al.*, 1989). Topical administration of dithranol in the mouse ear produces a delayed irritant effect, which may be partly due to the generation of free radicals (Finnen *et al.*, 1984). Benzoic acid exposure provokes contact urticaria both in man and in guinea-pigs (Lahti & Maibach, 1984). We have therefore selected benzoic acid for testing in the mouse ear oedema model in this present study.

We have prepared a new series of ionic compounds with PAF-antagonist activity (Bartrolí *et al.*, 1990). Two of these compounds, together with standard PAF-antagonists of similar or different structure, were selected for testing in the above mentioned models of topical inflammation in the mouse ear. In addition to anti-inflammatory activity, we have evaluated PAF antagonist activity of the selected compounds, in order to verify a possible correlation between the two pharmacological activities. Standard anti-inflammatory agents were also tested, i.e. indomethacin (cyclo-oxygenase inhibitor), phenidone (lipoxygenase inhibitor) and dexamethasone (corticoid).

Methods

Dermatitis

Groups of 8 to 10 Swiss male mice, 6–8 weeks old, were used in the experiments. Animals had free access to food and water. Irritant dermatitis was elicited by painting both faces of the left ear of the mouse with 20 μl (10 μl each face) of the inflammatory agents. For screening purposes, concentrations (w/v) of the irritants were 10% arachidonic acid, 0.1% dithranol, 0.01% TPA, each dissolved in acetone, and 10% benzoic acid dissolved in ethanol. Total applied doses were 2, 0.02, 0.002, and 2 mg/ear, respectively. Treatment was effected by application of a total of 20 μl of the test compound dissolved in acetone to both faces of the left ear of the mouse 30 min before the application of the irritant agent. Control animals received only the vehicle. At 45 min (benzoic acid), 1 h (arachidonic acid), 6 h (TPA) and 24 h (dithranol) after administration of the irritant, animals were killed by cervical dislocation. An 8 mm diameter punch biopsy was performed on each ear, the

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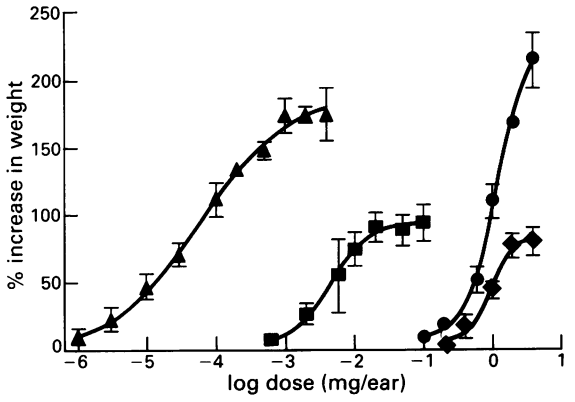

Figure 1 Inflammation caused by topical administration of various doses of 12-O-tetradecanoylphorbol 13-acetate (TPA) (▲); dithranol (■); arachidonic acid (●); and benzoic acid (◆) to both faces of the mouse ear, expressed as the percentage increase of the 8 mm-biopsy weight, compared to the biopsy-weight of the contralateral ear receiving the solvent alone. The application of the solvent alone caused no increase in ear weight. Values are means with s.d. shown by vertical bars (n = 8–10).

right (untreated) ear being the control for each animal. The swelling induced by the irritant was quantified as the percentage increase in the weight of left ear biopsy over that of the right ear biopsy. Percentage inhibition of oedema was calculated according to the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{wt. of inflamed-treated ear} - \text{wt. of control ear}}{\text{wt. of inflamed ear} - \text{wt. of control ear}}\right) \times 100$$

Activity of the inhibitors was expressed as the ID_{50} value, i.e. the dose required to inhibit the increase in weight by 50%, with respect to control animals.

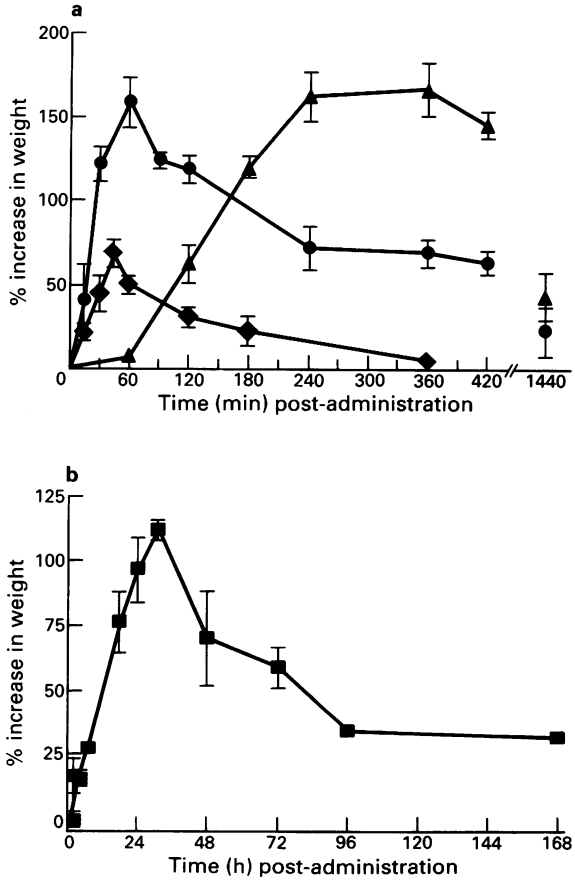

Figure 2 Time course of the ear oedema induced by (a) 12-O-tetradecanoylphorbol 13-acetate (TPA) 2 μg/ear (▲); arachidonic acid 2 mg/ear (●); and benzoic acid 2 mg/ear (◆); (b) dithranol 20 μg/ear (■). Results are expressed as the percentage increase of the 8 mm-biopsy weight compared to the biopsy weight of the contralateral ear receiving the solvent alone. The application of the solvent alone caused no increase in ear weight. Values are means with s.d. shown by vertical bars (n = 8–10).

Myeloperoxidase activity

Tissue biopsy specimens were homogenized in 0.5% hexadecyltrimethylammonium bromide with a Polytron apparatus. The entire process was performed in an ice bath to avoid warming of the sample. The homogenate was centrifuged at 6000 *g* for 30 min at 4°C. The supernatant was assayed for myeloperoxidase activity according to the method of Suzuki *et al.* (1983) with 3,3',5,5'-tetramethylbenzidine used as the substrate.

Platelet aggregation test

Blood was collected from male New Zealand rabbits (2–2.5 kg body weight) in 3.16% sodium citrate (1 volume to 9 volumes of blood) by cardiac puncture. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 250 *g* for 10 min at 4°C. The PRP was diluted with platelet-poor plasma, obtained by further centrifuging at 3000 *g* for 10 min. The platelet count was adjusted to 3.5×10^8 cells ml^{-1}. Platelet aggregation was induced by C_{18}-PAF (15 nM) and measured with a dual-channel aggregometer Chrono-log 500. Activity of the inhibitors was expressed as the IC_{50} value, i.e. the concentration required to inhibit platelet aggregation by 50%.

Hypotension test

Male Sprague-Dawley rats, weighing 180–220 g, were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Blood pressure was recorded from the left carotid artery with a Beckman pressure transducer coupled to a Beckman R611 polygraph. Right and left femoral veins were catheterized for injection of C_{18}-PAF (0.5 μg kg^{-1}) or the test compound. Test compounds were administered intravenously (1 ml kg^{-1}, dissolved in saline), 3 min before PAF injection. Control animals received only the vehicle. Blood pressure was monitored and percentage inhibition of PAF-induced hypotension with respect to controls was calculated. The results are expressed as ID_{50} values, i.e. the dose of the test compound required to inhibit PAF-induced hypotension by 50%.

Statistics

Results of percentage increase in weight are expressed as mean ± s.d. Regression analysis of not less than five data points was used to calculated ID_{50} and IC_{50} values, each point being the mean of the percentage inhibition at a given dose/concentration obtained from two or more independent experiments; 95% confidence limits were calculated with a standard calculation program (Tallarida & Murray, 1981).

Materials

Dexamethasone, dithranol, hexadecyltrimethylammonium bromide, phenidone, 3,3',5,5'-tetramethylbenzidine and TPA were obtained from Sigma (St. Louis, MO, U.S.A.) Arachidonic acid and benzoic acid were from Fluka (Buchs, Switzerland. Indomethacin was provided by Urquima (Sant

Table 1 Effect of topically applied PAF-antagonists on the different models of mouse ear oedema in comparison with their *in vitro* and *in vivo* PAF-antagonist activity

	PAF-induced platelet aggreg. IC_{50} (μM)	*PAF-induced hypotension* (ID_{50}, mg kg^{-1}, i.v.)	*Ear oedema induced by*			
			AA	*TPA*	*D*	*BA*
			ID_{50} (mg/ear)			
UR-10324	0.012 (0.009–0.017)	0.018 (0.016–0.020)	0.058 (0.040–0.083)	0.025 (0.019–0.040)	0.61 (0.47–0.75)	0.28 (0.19–0.45)
UR-11353	0.010 (0.008–0.014)	0.025 (0.022–0.029)	0.075 (0.038–0.14)	0.94 (0.69–1.3)	0.085 (0.052–0.14)	0.43 (0.25–0.76)
CV-6209	0.012 (0.010–0.014)	0.008 (0.006–0.011)	0.059 (0.037–0.088)	0.056 (0.036–0.086)	0.057 (0.042–0.076)	0.17 (0.12–0.24)
WEB-2086	0.091 (0.071–0.117)	0.17 (0.12–0.27)	0.76 (0.38–1.5)	0.39 (0.24–0.61)	0.29 (0.17–0.50)	0.47 (0.27–0.82)
YM-461	0.88 (0.78–0.98)	0.035 (0.023–0.053)	>2	1.52 (1.13–2.1)	0.12 (0.05–0.29)	>2
BN-52021	0.55 (0.38–0.77)	>5	>1	>1	>1	>1
Indomethacin	NT	NT	1.25 (0.61–2.6)	0.015 (0.007–0.029)	0.086 (0.053–0.14)	0.34 (0.21–0.57)
Phenidone	NT	NT	0.013 (0.005–0.034)	0.32 (0.22–0.46)	>2	0.57 (0.34–0.95)
Dexamethasone	NT	NT	0.12 (0.061–0.24)	0.023 (0.016–0.034)	0.14 (0.10–0.19)	0.33 (0.18–0.59)

The results are expressed as IC_{50} and ID_{50} values with their 95% confidence limits given below in parentheses. NT: not tested. AA: arachidonic acid; TPA: 12-O-tetradecanoylphorbol 13-acetate; D: dithranol; BA: benzoic acid.

Fost, Spain). C_{18}-PAF, UR-10324 (2-[[N-acetyl-N-[[2-[2-[heptadecylcarbamoyloxy] ethylthio]ethoxy] carbonyl]-amino] methyl]]-1-ethylpyridinium chloride), UR-11353 (2-[[N - acetyl - N - [[[2 - octadecyloxy]] - 4 - tetrahydrofuranyl] methoxy] carbonyl] amino]methyl] - 1 - ethylpyridinium chloride), CV-6209 (2-[N-acetyl-N-[[[2-methoxy-3-[(octadecylcarbamoyl)oxyl] propoxy] carbonyl] amino] methyl] - 1-ethylpyridinium chloride) (Takeda, Japan), and YM-461 (1-(3-phenylpropyl) - 4 - [2 - (3 - pyridyl) - thiazolidin - 4 - ylcarbonyl] - pipeazine fumarate) (Yamanouchi, Japan) were synthesized in our laboratories following published procedures. WEB-2086 (3 - (4 - (2 - chlorophenyl) - 9 - methyl - 6H - thieno - (3,2 - f)(1,2,4) - triazolo - (4,3 - a)(1,4) - diazepine - 2yl) - 1 - (4 - morpholinyl) - 1 - propanone) and BN- 52021 (3-[1,1-dimethylethyl] hexahydro-1,4,7b - trihydroxy - 8 - methyl - 9H - 1,7a (epoxymethanol) - 1H, 6aH - cyclopenta[c]furo[2,3 - 6] furo[3',2':3,4] cyclopenta-[1,2-d]furan- 5,9,12[4H]-trione) were generous gifts from Dr Heuer (Boehringer Ingelheim, Germany) and Dr Braquet (Henri Beaufour, France), respectively. All other chemicals used were of reagent grade or of the purest commercially available grade.

Results

The four irritants tested produced a marked and dose-dependent inflammatory reaction in the mouse ear (Figure 1). In the arachidonic acid-induced ear oedema, a dose of 2 mg/ear of arachidonic acid was selected for studying the activity of inhibitors for practical reasons, even though a maximal response was not achieved. In contrast, a dose of 20 μg/ear of dithranol was used, since higher doses did not induce a greater inflammatory response. TPA was the most potent irritant tested, showing an inflammatory response in the range of 0.01 to 4 μg/ear. Benzoic acid produced only mild oedema at doses above 1 mg/ear. The time-course of the oedema at the selected doses was also studied. A sharp rise in inflammatory response was observed in the arachidonic acid-induced ear oedema model, reaching a maximum at 1 h, after which the response declined slowly (Figure 2a). A similar time-profile was obtained with benzoic acid, with a maximal effect at about 45 min, returning to basal values at 6 h post-application (Figure 2a). In contrast, the maximal response in the TPA-induced oedema was reached at about 6 h after application of the irritant (Figure 2a). Dithranol-induced ear oedema showed a characteristic delayed-type inflammation, with a maximal effect at about 24–30 h after application of the irritant (Figure 2b). A certain degree of inflammation persisted even one week after dithranol administration.

The potency of PAF-antagonists in the *in vitro* PAF-induced platelet aggregation test and the *in vivo* PAF-induced hypotension test in normotensive rats is given in Table 1. UR-compounds showed a similar potency in the platelet aggregation test, with IC_{50} values of the order of 10^{-8} M, an activity

Table 2 Inhibition of leukocyte accumulation into the inflamed ear, measured as myeloperoxidase activity, in relation to the reduction of arachidonic acid-induced ear oedema

Product	*Dose* (mg/ear)	*% inhibition of AA-induced ear oedema*	*% inhibition of myeloperoxidase activity*
UR-10324	0.1	70.4	60.2
UR-11353	0.1	47.6	46.7
CV-6209	0.1	58.5	67.3
WEB-2086	0.3	37.7	37.8
Dexamethasone	0.1	48.8	35.8

Groups of 10 animals were used for the calculation of percentage inhibition of arachidonic acid (AA)-induced ear oedema. Data of myeloperoxidase activity are mean percentages of inhibition of two or more experiments in which sets of five ears were homogenized and processed as indicated in Methods.

comparable with that of CV-6209 and superior to that of WEB-2086, YM-461 and BN-52021. Potency of URs in the hypotension test was slightly lower than that of CV-6209, but higher than that of YM-461, WEB-2086 and BN-52021.

Topical administration of various PAF-antagonists produced an inhibition of ear oedema in mice. Inhibition was dose-related, and reached values greater than 70% when active compounds were considered, thus allowing calculation of ID_{50} values (Table 1). URs and CV-6209 showed a comparable and marked inhibition of arachidonic acid-induced ear oedema, whereas YM-461 and BN-52021 were practically ineffective and WEB-2086 showed an intermediate activity. Differences of activity between URs were more pronounced (ID_{50} values in the range from 0.025 to 0.94 mg/ear) when dithranol, TPA and benzoic acid were used as the inducers. CV-6209 exhibited a high degree of activity in the three models. WEB 2086 and YM-461 showed variable potency depending on the test, and BN-52021 was without significant activity. Among other reference compounds with no PAF-antagonist activity, only dexamethasone was highly effective in all models of oedema. Indomethacin was only slightly effective in the arachidonic acid-induced ear oedema, while it was very useful in suppressing TPA-, dithranol- and benzoic acid-induced ear oedema. Phenidone was particularly effective in the arachidonic acid-induced ear oedema test, but failed to inhibit dithranol-induced oedema. Inhibition of oedema was accompanied by a concomitant decrease of myeloperoxidase activity in the arachidonic acid-inflamed ears, as shown in Table 2. This result signifies that leukocyte recruitment into the damaged tissue can also be prevented by PAF-antagonists.

The PAF-antagonist and anti-inflammatory activities of structural analogues of UR-10324 are given in Table 3. The deacetylated compound and the non-ethylated neutral molecule showed a considerable reduction of both activities as compared with UR-10324. These results suggest a possible relationship between PAF-antagonist and anti-inflammatory activities.

Topical application of PAF-antagonists at pharmacologically effective doses did not provoke macroscopic changes in treated ears. Only at the highest dose tested, i.e. 3 mg, a mild irritative effect of the ionic compounds was observed, with a peak at about 45–60 min postadministration and a maximum increase of ear weight of about 10%.

Discussion

This study shows that PAF-antagonists of different chemical structures are capable of preventing the inflammatory response provoked by several primary irritants in the mouse ear. It is well known that arachidonic acid-induced ear oedema is accompanied by a rapid production of PGE_2 and leukotriene C_4 (LTC_4)/LTD_4 (Arner *et al.*, 1985), but there are no reports on the participation of PAF in this model of ear oedema. We have found that ionic and non-ionic PAF-antagonists, such as UR-10324, UR-11353, CV-6209 and WEB-2086 are able to inhibit arachidonic acid-induced ear oedema. Moreover, PAF-antagonists can also prevent leukocyte recruitment into the inflamed tissue, as shown by their ability to decrease myeloperoxidase activity in the supernatants of ear homogenates.

It has been well established that the inflammation observed in the TPA model is related to the activation of phospholipase A_2 (Furstenberger *et al.*, 1981) and is primarily mediated by prostaglandin E_2 (PGE_2) (Ashendel & Boutwell, 1979). In consequence, cyclo-oxygenase inhibitors (e.g. indomethacin) are very effective in this test. On the other hand, a relationship between TPA-stimulatory effects and PAF production has been found in some cases. Thus, TPA enhances PAF biosynthesis in human polymorphonuclear leukocytes (Sánchez-Crespo & Nieto, 1989), and CV-3988, a PAF-antagonist with PAF-like structure, suppresses pleurisy induced by TPA in rats (Ohishi *et al.*, 1986).

Kémery *et al.* (1989) demonstrated that dithranol-induced oedema was dose-dependently inhibited by intraperitoneal administration of BN-52021, but also showed the inhibitory activity of indomethacin, clemastine (H_1-antihistamine) and superoxide dismutase. These findings suggest a possible involvement of PAF, prostaglandins, histamine and reactive oxygen radicals in dithranol-induced dermatitis in mice. Nevertheless, in our studies, a unique topical treatment with a PAF-antagonist has managed to produce a near-total suppression of oedema (e.g., CV-6209 induced 87% inhibition at a dose of 0.2 mg/ear). Surprisingly, topical administration of BN-52021 was ineffective in preventing oedema in our conditions. The suppression of dithranol-induced dermatitis in the mouse by local administration of PAF-antagonists could be useful in developing antipsoriatic dithranol-formulations with improved local tolerance and an enhanced therapeutic profile.

No information is available with respect to the mediators involved in the benzoic acid-induced ear oedema. The inhibition caused by treatment with indomethacin and PAF-antagonists suggests a contribution by prostaglandins and PAF in this model.

PAF-antagonist activity may account, at least in part, for the topical anti-inflammatory activity. Indirect evidence in favour of PAF participation in these models of topical inflammation includes the following: (a) structurally different PAF-antagonists (i.e., ionic and non-ionic compounds) are able to suppress oedema and higher anti-inflammatory potency of ionic compounds correlates with their superior PAF-antagonist activity; (b) structural analogues of UR-10324, the deacetylated and the non-ethylated neutral molecule, which are much less potent PAF-antagonists than the parent compound, showed a markedly lower anti-inflammatory activity (Table 3). Nevertheless, we have also found that the PAF-antagonist YM-461 lacks effect in arachidonic acid- and benzoic acid-induced ear oedema, while this compound is highly effective in dithranol-induced oedema; UR compounds show differences in anti-inflammatory potency in spite of their similar PAF-antagonist activity, and we have reported that CV-6209 inhibits not only the rat paw oedema induced by PAF, but also the oedema induced by histamine, 5-hydroxytryptamine, compound 48/80 and carrageenin (Merlos *et al.*, 1990). All these data suggest that other mechanisms

Table 3 PAF-antagonist and anti-inflammatory activities of UR-10324 and its deacetylated and non-ethylated analogues

	PAF-induced platelet aggreg. IC_{50} (μM)	*PAF-induced hypotension* ID_{50} (mg kg^{-1}, i.v.)	*AA*	*Ear oedema induced by TPA* ID_{50} (mg/ear)	*D*	*BA*
UR-10324	0.012 (0.009–0.017)	0.018 (0.016–0.020)	0.058 (0.040–0.083)	0.025 (0.019–0.040)	0.61 (0.47–0.75)	0.28 (0.19–0.45)
Deacetylated analogue	6.1 (4.1–9.1)	>5	>1	>1	>1	>1
Non-ethylated analogue	6.4 (6.3–6.5)	>5	0.46 (0.41–0.52)	>1	>1	>1

The results are expressed as IC_{50} and ID_{50} values with their 95% confidence limits given below in parentheses. Abbreviations as in Table 1.

such as prostaglandin and/or leukotriene biosynthesis inhibition and/or phospholipase inhibition, may be involved in the action of PAF antagonists and explain their anti-inflammatory activity. In fact, preliminary studies have shown that both UR compounds inhibit phospholipase A_2 from snake venom with IC_{50} values of about 10 μM. This additional activity could explain, in part, the anti-inflammatory activity of UR compounds, but to date there is no well-founded explanation for the discrepant activities of UR-10324 and UR-11353 on ear oedema models. Studies to assess the relative contribution of other mechanisms to the overall anti-inflammatory effects of these compounds are now in progress.

In conclusion, the improvement observed in several models of irritant dermatitis in mice after local administration of PAF-antagonists provides evidence of the involvement of PAF in these topical inflammatory processes, and supports the use of PAF-antagonists in the treatment of some inflammatory skin diseases.

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