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Reduction of inflammation and pyrexia in the rat by oral administration of SDZ 224-015, an inhibitor of the interleukin-1β converting enzyme

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1 The aim of this study was to determine whether a synthetic inhibitor of the interleukin-1β converting enzyme (ICE) displays oral activity in models of inflammation.

2 To this end, the ICE inhibitor, SDZ 224-015, was examined in rat paw oedema, pyrexia and nociception tests.

3 SDZ 224-015 (0.3-300 $\mu g\ kg^{-1}$) potently reduced carrageenin-induced paw oedema, with an oral ED_{50} of approximately 25 $\mu g\ kg^{-1}$. This effect was independent of endogenous glucocorticoid, as shown by retention of activity upon adrenalectomy.

4 Pyrexia induced by lipopolysaccharide (0.1 mg kg^{-1} s.c.) or by interleukin-1β (100 ng i.v.) was also reduced, over a similar dose-range to oedema (oral ED_{50}s 11 $\mu g\ kg^{-1}$ and 4 $\mu g\ kg^{-1}$ respectively).

5 SDZ 224-015 (0.2-5 mg kg^{-1}, p.o.) displayed analgesic activity in the Randall-Selitto yeast-inflamed paw pressure test, significant at a dose of 1 mg kg^{-1}, p.o.

6 Thus, SDZ 224-015 has potent oral activity in several acute models for inflammation, suggesting that ICE inhibitors may constitute a novel type of anti-inflammatory agent.

Keywords: Fever; inflammation; interleukin-1; interleukin-1 converting enzyme; oedema

Introduction

Interleukin-1 (IL-1) appears to be a central mediator of the pathogenesis of acute and chronic inflammation (reviewed by Dinarello, 1991; 1993). Administration of the cytokine has been demonstrated to produce cardinal signs of inflammation such as leukocyte influx, pain and tissue breakdown (Ferreira *et al.*, 1988; Henderson & Pettipher, 1988; Wankowicz *et al.*, 1988). Furthermore, elevated levels can be demonstrated in inflammatory effusions (Bochner *et al.*, 1990; Sim *et al.*, 1994). The suitability of IL-1 as a target for anti-inflammatory therapy has been confirmed by the efficacy in a variety of animal models of the naturally-occurring IL-1 receptor antagonist (IL-1ra), soluble IL-1 receptors or with neutralising antibodies to IL-1. (Jacobs *et al.*, 1991; Thompson *et al.*, 1992; van den Berg *et al.*, 1994).

Of the two IL-1 proteins that exist, α and β, it is the latter that is predominantly released from activated human monocytes (Bailly *et al.*, 1994). IL-1β is synthesized as a cytosolic inactive precursor of 33K, being released only upon cleavage to the mature 17.5K form. The protease (termed interleukin-1β converting enzyme, ICE) responsible for this processing has recently been cloned (Thronberry *et al.*, 1992) and the crystal structure elucidated (Walker *et al.*, 1994; Wilson *et al.*, 1994). ICE is a homodimeric (consisting of two heterodimer subunits p10 and p20) cysteine protease with unique substrate specificity, cleaving proIL-1β between Asp 116 and Ala 117. Since IL-1β release is dependent upon the action of ICE (Young *et al.*, 1988), it follows that inhibition of this enzyme provides a rational therapeutic target for the development of anti-inflammatory agents. Indeed, an example from nature already exists in that cowpox virus produces an inhibitor of ICE, the *crmA* protein, as a means of blocking the inflammatory response; mutants lacking this protein are less pathogenic (Marrack & Kappler, 1994).

Based on the work of Smith *et al.* (1988) who produced acyloxymethyl ketones as selective inactivators of cathepsin B, we have designed irreversible substrate-based inhibitors of ICE. Here we demonstrate specific inhibition of IL-1β *in vitro* and use models of acute inflammation, pyrexia and nociception, in order to show for the first time *in vivo* activity of a synthetic inhibitor of ICE, SDZ 224-015.

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Methods

Animals

For all studies, male Sprague-Dawley rats weighing 140-170 g were used, being supplied by Interfauna AG, Tuttlingen, Germany. They were fed *ad libitum* with a standard diet (Nafag Ecosan AG, Gossau, Switzerland) and allowed at least 4 days acclimatisation before an experiment.

Release of cytokines from THP-1 cells

THP-1 cells 500,000 were cultured for 3 h in 1 ml RPMI with 5% heat-inactivated foetal bovine serum containing 100 u interferon gamma, in 24-well multiwell plates. The acid of SDZ 224-015 (or vehicle) was then added to duplicate wells to achieve the required final concentration, together with 5 μg lipopolysaccharide. After a further 40 h, IL-1β, interleukin-6 (IL-6) or tumour necrosis factor-α (TNF-α) in the cell-free media were determined by ELISA and the amount measured related to total DNA. The latter was determined by the fluorometric method of Kapuscinski & Skoczylas (1977). Lactate dehydrogenase (LDH) was determined in the same media (without freezing which destroys LDH) as described by Schnyder *et al.* (1990).

Carrageenin-induced paw oedema

Rats were divided into groups of 4 and dosed orally by gavage either with the vehicle (Tween 80/1% BSA in 10 mM phosphate buffer pH 6), diclofenac as reference substance, or with various amounts of SDZ 224-015. A zero reading of right paw volume was then taken with an antiphlogmeter according to

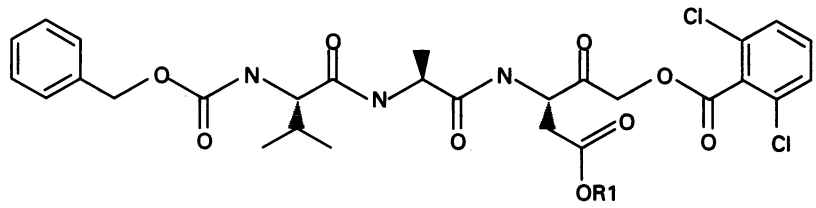

Figure 1 Structure of SDZ 224-015. R1 = Et = SDZ 224-015. R1 = H = active principle.

Kemper & Ameln (1959). This is an electrical method for measuring volumes whereby the paw of the conscious rat is inserted into a tube where a constant high frequency electric field exists; the presence of the paw alters the frequency of the field in proportion to its volume. One hour later, 0.1 ml of a w/v 1% suspension of carrageenin in saline at 37°C was given (without anaesthesia) by subplantar injection into the right paw. Animals were returned to the cage and 3 h later paw oedema measured with the antiphlogmeter. After subtraction of the zero values, the means of the compound-treated groups were compared to the vehicle-treated group to give the percentage inhibition of paw swelling.

Adrenalectomy

Under isoflurane narcosis, a 1 cm lateral incision was made in the dorsal flank and the adrenals exposed. For sham-operated rats the incision was then merely closed, whilst for actual adrenalectomy both adrenals were completely removed with eye forceps. Immediately following surgery all animals received 1 mg 11-deoxycorticosterone acetate i.m. and thereafter were maintained with drinking water containing 0.9% NaCl. Five days after adrenalectomy the standard carrageenin-induced paw oedema test was carried-out as described above.

Lipopolysaccharide (LPS)-induced pyrexia

Rats were placed in single cages and fasted overnight and then divided into groups of three. LPS (0.1 mg kg^{-1}) was administered s.c. and 2 h later, after the initial hypothermic response had occurred, the baseline temperature was measured with a rectal thermocouple probe. Four hours after LPS, compound (or vehicle alone) was given by oral gavage and at 6 h the end temperature recorded. This dose of LPS produces a temperature rise of 2–2.5°C. The difference between the basal and final temperature was calculated and the mean values of the compound-treated groups compared to that of the vehicle-treated in order to determine the percentage inhibition of fever. For pyrexia experiments DuP 697, an inhibitor of prostaglandin E_2 synthesis (Gans *et al.*, 1990), was used as reference compound.

IL-1β-induced pyrexia

Rats were placed in individual cages and fasted overnight and then divided into groups of four. Basal rectal temperature was measured and animals immediately dosed orally with compound (or vehicle alone); 30 min later 100 ng recombinant human IL-1β was administered i.v. via the tail vein. Four hours later the fever was determined (this amount of cytokine induces a temperature rise of 1.5–2°C) and the difference between the basal and final values calculated. Mean values of the compound-treated groups were compared to that of the vehicle-treated to give percentage inhibition of fever.

Inflamed paw pressure test

This was performed according to the method of Randall & Selitto (1957). Rats were divided into groups of 5 and 0.1 ml of a 20% w/v solution of baker's yeast in water at 37°C given by subplantar injection into the hind paw; 2 h later compound (or vehicle alone) was administered by oral gavage. After a further hour, nociception was measured by applying an increasing weight upon the paw with a pressure meter (Ugo Basile, Varese, Italy) until vocalization. The weight applied to each rat was recorded and comparison made between values for the compound-treated and vehicle-treated rats. Diclofenac was used as reference compound.

Statistical analysis

Comparison between vehicle-treated and compound-treated groups was carried-out by analysis of variance and unpaired *t* test, or Dunnett's multiple comparison test (parametric data) or the Mann-Whitney (nonparametric) test.

Materials

THP-1 cells (human monocytic leukaemia) were from the European Collection of Animal Cell Cultures (Porton Down, UK). Bovine serum albumin (fraction V), lipopolysaccharide (*Salmonella abortus equi*) and Tween 80 were obtained from Sigma (St. Louis, MO, U.S.A.). Human recombinant interferon γ was from Boehringer Mannheim (Mannheim, Germany). ELISA kit for human IL-1β was purchased from Cayman chemicals (Paris, France), those for IL-6 and TNF-α were from Innogenetics (Zwijndrecht, Belgium). Carrageenin (satiagum standard) was from Sugro AG (Basel, Switzerland). Baker's yeast was from Fleischmann (Bern, Switzerland). Isoflurane 'Forene' was purchased from Abbot (Cham, Switzerland). 11-Deoxy-corticosterone acetate was from Fluka (Buchs, Switzerland). Corticosterone RIA kit was from ICN Biochemicals (Costa Mesa, CA, U.S.A.). Recombinant human interleukin-1β was supplied by Dr P. Ramage, Biotechnology Department of Sandoz Ltd. (Basel, Switzerland). SDZ 224-015 (Z-valyl-alanyl-3(S)-3-amino-4-oxo-5-(2,6-dichlorobenzoyl-oxypentanoic acid) ethyl ester) was ground to a particle size of 99% < 88 μm diameter (Milling Department, Sandoz, Basel, Switzerland). The structure of SDZ 224-015 and its acid is shown in Figure 1.

Results

The free acid which is the active principle of SDZ 224-015, dose-dependently inhibited the release of IL-1β from the human monocytic cell line THP-1, with an IC_{50} of 0.24 μM (Figure 2a), without affecting release of IL-6, TNFα or lactate dehydrogenase (Figure 2b-d, respectively). This demonstrates that the action of the compound is specific for IL-1β and also that it is not due to cellular toxicity.

SDZ 224-015 was then tested in carrageenin-induced paw oedema, a model for acute inflammation. The results of a series of dose-response experiments are presented in Table 1. It can be seen that the compound is very potent, with significant reduction in paw swelling always apparent with an oral dose of 3 μg kg^{-1}. When these data are combined, a 50% inhibition of total swelling is obtained with a dose of 25 μg kg^{-1} p.o. In-

terestingly, the effect of SDZ 224-105 reaches a plateau at around 60% inhibition, with increasing doses failing to reduce swelling further. Thus, a component of carrageenin-oedema exists that is refractory to this compound class.

Since glucocorticoids potently reduce inflammation, it was important to exclude the possibility that SDZ 224-015 might act indirectly, via induction of endogenous corticosteroid production. Therefore, the carrageenin oedema test was performed on adrenalectomized rats. As shown in Table 2, adrenalectomy did not influence the action of the compound, indicating that its effect was independent of the adreno-pituitary axis. This was subsequently confirmed by radioimmunoassay of circulating corticosterone in intact rats ($n=4$) that had been dosed 2 h earlier with SDZ 224-015 10 mg kg^{-1}, p.o. These animals had a mean (s.e.mean) plasma corticosterone of 25.4 (8.0) ng ml^{-1} compared to 15.1 (1.6) ng ml^{-1} for those dosed with vehicle (values below 50 ng ml^{-1} being normal).

SDZ 224-015 was also examined in LPS-pyrexia, since IL-1 is an endogenous pyrogen induced by LPS. The dose-response curve is presented in Figure 3, the ED_{50} being 11 $\mu g\ kg^{-1}$. Table 3 shows that SDZ 224-015 also reduced pyrexia fol-

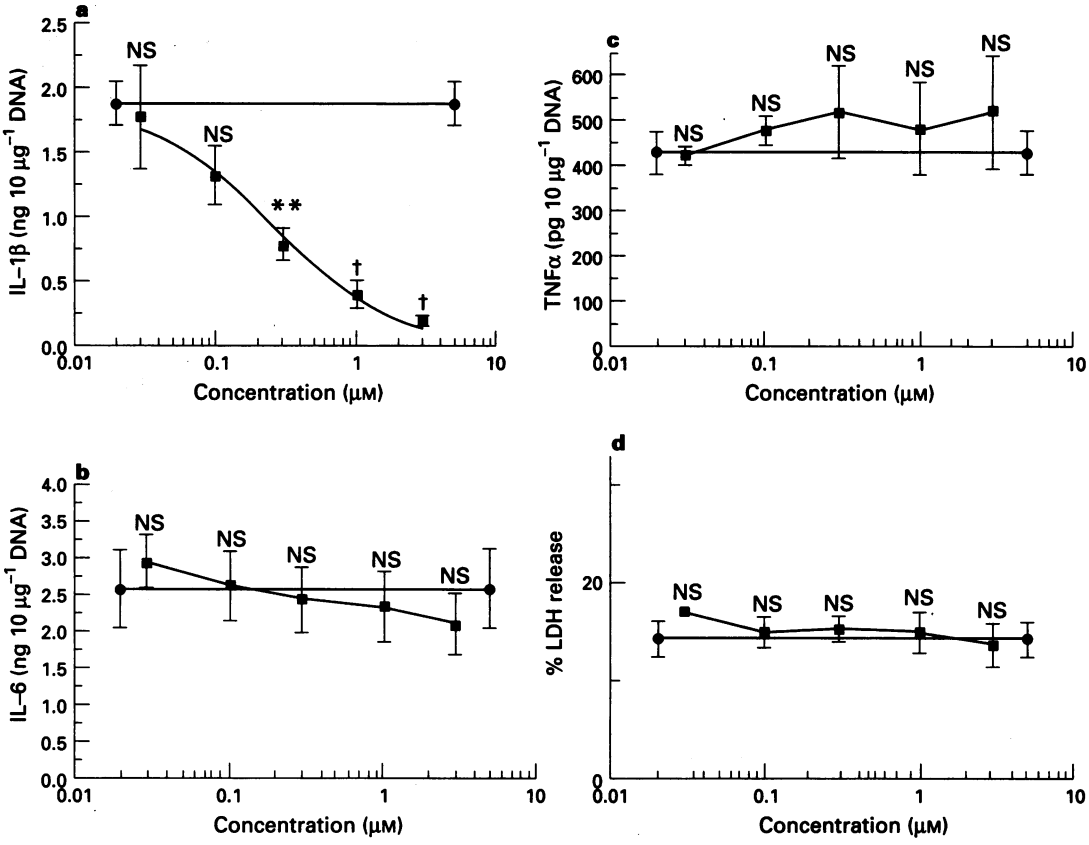

Figure 2 Dose-response for the inhibition of cytokine release from THP-1 cells by the free acid of SDZ 224-015. Following a 43 h culture in the presence of various concentrations of compound (or vehicle), together with interferon γ and LPS as stimulant, cytokines in the media were measured by specific ELISA. The compound inhibited IL-1β release with an IC_{50} of 0.24 μM (a) without causing a change in levels of interleukin 6 (IL-6) (b) and tumour necrosis factor α (TNF-α) (c) or of lactate dehydrogenase (LDH) (d) in the media. Results are the mean ± s.e.mean of 3 experiments. (●) = control in (a), (b), (c) and (d). NS = not significant; $^{**}P<0.01$; $\dagger P<0.001$.

Table 1 Effect of SDZ 224-015 in carrageenin paw oedema

Dose of SDZ 224-015 ($\mu g\ kg^{-1}$ p.o.)	*% inhibition of paw swelling compared to vehicle-treated group*			
	Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*
0.3	33 (2)**	22 (2)*	36 (6)**	30 (5)NS
3	50 (6)**	30 (6)*	38 (3)†	43 (2)*
30	59 (3)†	42 (2)**	59 (7)†	56 (5)*
300	59 (4)†	52 (6)**	59 (2)†	63 (3)**
3000	61 (4)†	ND	ND	ND
Diclofenac 3 mg kg^{-1} p.o.	73 (3)†	64 (7)**	73 (4)†	70 (4)**

Rats were dosed by gavage with SDZ 224-015, or diclofenac as reference and 1 h later paw inflammation induced by injection of carrageenin (see methods); 3 h thereafter paw swelling was measured and compared to that of the vehicle-treated group to ascertain the effect of the substance. The table shows means (s.e.mean) of 4 experiments; ND = not done; NS = not significant; $^{*}P<0.05$; $^{**}P<0.01$; $\dagger P<0.001$.

lowing intravenous administration of IL-1β itself, with ED_{50} of 4 $\mu g\ kg^{-1}$. In this case, it may be that the ICE inhibitor acts by blocking an amplification cascade, since IL-1β is known to stimulate its own production in some *in vitro* systems.

In further studies we tested whether the compound possessed antinociceptive activity, using a model for inflammatory pain, the Randall-Selitto inflamed paw pressure test. As shown in Figure 4, SDZ 224-015 significantly increased the paw pressure threshold required for a nociception response, indicating a probable analgesic action. However, the dose required to produce a significant effect was several orders of magnitude greater than that for oedema, indicating that the mechanism is probably distinct from the reduction of paw swelling induced by lower doses of SDZ 224-015.

Table 2 Effect of adrenalectomy upon the inhibition of carrageenin paw oedema by SDZ 224-015

	% inhibition of paw swelling compared to vehicle-treated control group	
	Sham-operated	*Adrenalectomized*
SDZ 224-015 (50 $\mu g\ kg^{-1}$ p.o.)	48 (4)†	48 (4)†
Diclofenac (3 $mg\ kg^{-1}$ p.o.)	62 (4)†	64 (5)†

Rats were adrenalectomized or merely sham-operated as described in methods; 5 days later the standard carrageenin paw oedema test was performed. Results are means (s.e.mean) using 5 rats per group; 2 such experiments were performed with the same outcome.
†$P<0.001$.

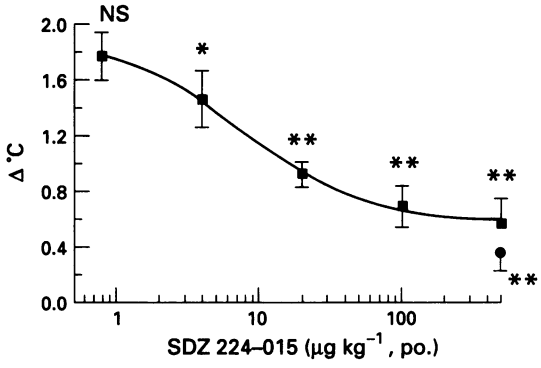

Figure 3 Dose-response to SDZ 224-015 in LPS-pyrexia in the rat. Animals were injected s.c. with 0.1 $mg\ kg^{-1}$ LPS and 2 h later the basal temperature measured. After a further 2 h, substance was administered orally. Six hours after LPS, the final temperature was recorded and values compared to that of the vehicle-treated group. The results are the mean and s.e.mean of 3 experiments. (■) SDZ 224-015; (●) Du697. NS = not significant; *$P<0.05$; **$P<0.01$.

Table 3 Effect of SDZ 224-015 upon pyrexia induced by interleukin-1β (IL-1β)

Dose of SDZ 224-015 ($\mu g\ kg^{-1}$ p.o.)	*% inhibition of fever*
0.1	15 (11) NS
1	46 (14)*
10	54 (15)*
100	77 (13)**
DuP 697 0.5 $mg\ kg^{-1}$ p.o.	62 (10)**

Basal temperature was determined and rats dosed orally with compound or vehicle in groups of 4. 30 minutes later fever was induced by intravenous administration of 100ng IL-1 beta. 4 hours after IL-1 beta, final temperature was recorded and compared to the vehicle treated group. Results are means (s.e.means) from 1 of 3 experiments producing similar results.
NS = not significant; *$P<0.05$; **$P<0.01$.

Discussion

In this study it has been shown that a synthetic peptide inhibitor of ICE reduces parameters of inflammation, consistent with an inhibition of IL-1β production. Although a peptide aldehyde ICE inhibitor has also recently been reported to inhibit specifically IL-1β release from human peripheral blood monocytes *in vitro* (Miller *et al.*, 1993), this to our knowledge is the first report demonstrating therapeutic efficacy of an ICE inhibitor *in vivo*. Although such peptide ICE inhibitors also inhibit cathepsin B, they demonstrate a marked preference for ICE, the acid of SDZ 224-015 displaying 180-fold selectivity for the latter (Dolle *et al.*, 1994). SDZ 224-015 inhibits isolated cathepsin B with an IC_{50} of 0.2 μM; however, other compounds with much lower potency for the latter are also effective in the oedema and pyrexia models. For instance, exchanging the dichlorobenzoic acid moiety for diphenyl acetate removes most activity against cathepsin B (10 μM producing only 7% inhibition), whereas inhibition of IL-1 release from THP-1 cells (IC_{50} 0.4 μM) and of oedema (ED_{50} 20 $\mu g\ kg^{-1}$, p.o.) is retained. The potency of SDZ 224-015 is remarkable, with oral ED_{50}s for inhibition of oedema and fever lower than that of the powerful cyclo-oxygenase inhibitor, diclofenac (which in our laboratory has an oral ED_{50} of 100 $\mu g\ kg^{-1}$ in both tests). ICE is thought to be derived from an autocatalytic precursor, being cleaved at sites with Asp in the P1 position, to form the active subunits p10 and p20 (Wilson *et al.*, 1994). Thus, the remarkable potency of SDZ 224-015 could be due to an amplifying action, through the ability not only to inhibit conversion

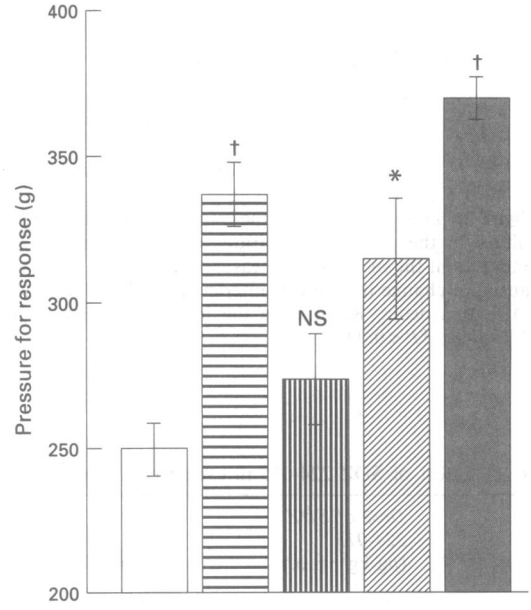

Figure 4 Effect of SDZ 224-015 in the inflammed paw pressure test for antinociception. Two hours after subplantar injection of yeast, compound was given orally and 1 h thereafter the paw pressure required to induce vocalization determined. Data are the mean ± s.e.mean, of 5 rats and are taken from 1 of 2 experiments, the results of which were very similar. NS = not significant; *$P<0.05$; †$P<0.001$. Open column, vehicle; vertically lined column, diclofenac (3 $mg\ kg^{-1}$, p.o.); horizontally lined column, SDZ 224-015 (0.2 $mg\ kg^{-1}$, p.o.); hatched column, SDZ 224-015 (1 $mg\ kg^{-1}$, p.o.); stippled column, SDZ 224-015 (5 $mg\ kg^{-1}$, p.o.).

of proIL-1β but also that of ICE itself, a property of ICE inhibitors also alluded to by others (Ayala *et al.*, 1994).

Part of the paw swelling in the carrageenin oedema model is refractory to SDZ 224-015, dose-response curves reproducibly displaying a plateau of around 60%. It is well established that a variety of mediators are involved in oedema formation (Henson & Murphy, 1989) and therefore it is perhaps not surprising that blockade of a single cytokine does not yield complete inhibition. This might have implications for the efficacy of ICE inhibitors in inflammatory disorders, where elimination only of IL-1β may be counteracted by continued presence of other inflammatory mediators. On the other hand, IL-1 appears to be a crucial player, triggering a cascade of other cytokines, mediators, proteases etc. (Dinarello, 1991); this is corroborated by the efficacy of the soluble IL-1 receptor, receptor antagonist or neutralising antibodies in experimental inflammation models.

Our results extend previous studies using the IL-1 receptor antagonist, which has been reported to be effective in carrageenin-induced pleurisy (Meyers *et al.*, 1993) and IL-1β induced pyrexia (Coceani *et al.*, 1992). In the latter regard, the effectiveness of SDZ 224-015 in IL-1β pyrexia may be related to inhibition of autocrine IL-1β release induced by the cytokine (Sakai *et al.*, 1987).

Endogenous prostaglandins and tachykinins appear to mediate some effects of IL-1, such as hyperalgesic action (Ferreira *et al.*, 1988; Perretti *et al.*, 1993). This may explain the analgesic action of SDZ 224-015, although it is unclear why it appears only at significantly higher doses than those in oedema or pyrexia unless the IL-1 involved in the induction of hyperalgesia is produced in the CNS in which case rather poor penetration of the blood-brain barrier might explain the weaker potency.

Chronic inflammatory disease, such as arthritis, is probably the most obvious disorder where IL-1β blockade could be of benefit. However, it is possible to speculate that therapeutic applications of an ICE inhibitor may be far broader. For example, treatment with the IL-1 receptor antagonist reduces osteoporotic bone loss (Kimble *et al.*, 1994) and ischaemic neuronal damage in rats (Relton & Rothwell, 1992). The viral ICE inhibitor, *crmA*, protects transfected ganglion neurones from apoptosis induced by nerve growth factor depletion (Gagliardini *et al.*, 1994); thus programmed cell death observed in degenerative neuronal diseases may be influenced by inhibitors of ICE.

In conclusion, it has been shown that the synthetic ICE inhibitor SDZ 224-015 potently reduces both acute inflammation and pyrexia. Although it has not actually been proven that this is due to inhibition of IL-1β release, specific blockade of IL-1β by the compound *in vitro*, and the known efficacy of proteinaceous IL-1 inhibitors in inflammatory models, makes this interpretation plausible. ICE inhibitors may constitute a novel type of therapeutic agent, the possible applications of which may well extend beyond inflammatory disease.

Note added in proof

Since submission of the paper, it has been confirmed by Ramage *et al.* (*J. Biol. Chem.*, **270**, 9378–9383) that processing of precursor ICE is autocatalytic and that autocatalysis is inhibited by the free acid of SDZ 224-015.

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