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Xanthine derivatives: comparison between suppression of tumour necrosis factor-α production and inhibition of cAMP phosphodiesterase activity

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SUMMARY

Several *in vitro* and *in vivo* studies have demonstrated suppression of tumour necrosis factor-α (TNF-α) synthesis by pentoxifylline. In the present study we compared the effect of pentoxifylline with that of five other xanthine derivatives. We addressed two questions. First, what is the relative potency of those chemically related compounds in suppressing the lipopolysaccharide (LPS)-induced production of TNF-α in human mononuclear cells? Second, does suppression of TNF-α production by these xanthine derivatives correlate with their capacity to inhibit 3′,5′-cAMP phosphodiesterase (PDE) activity? The experimental drug A 80 2715 [1-(5-hydroxy-5-methylhexyl)-3-methyl-7-propylxanthine] was identified as the most potent agent with an IC_{50} (concentration exerting 50% suppression of LPS-induced TNF-α production) of 41 μM (mean of 13 individuals). The IC_{50} values of the other substances ranged between 106 μM for HWA 138 and 419 μM for theophylline. The LPS-induced interleukin-1β (IL-1β) production was not influenced by all substances tested at comparable concentrations. Inhibition of PDE activity was determined in a cell-free system using PDE isolated from bovine heart. All xanthine derivatives dose-dependently inhibited PDE activity. Furthermore, with the exception of theophylline, there was a high degree of correlation between the potency to suppress TNF-α production in the cell culture system and the potency to inhibit PDE activity in the cell-free enzymatic assay. This argues for a crucial role of PDE inhibition in the suppression of TNF-α synthesis by xanthine derivatives.

INTRODUCTION

Suppression of tumour necrosis factor-α (TNF-α) by pentoxifylline is a pharmacologically interesting effect investigated in a number of studies[1–6] with possible therapeutic implications. We compared a series of xanthine derivatives—clinically used substances (theophylline, pentoxifylline) and experimental compounds (A 80 2715, HWA 138, HWA 448, BL 194)—in their relative potency of TNF-α suppression. The chemical names of these compounds are given in Table 1. A possible protective role of HWA 138 and A 80 2715 in animal models of septic shock syndrome has been described by some investigators.[7–9] We addressed the possible mechanism responsible for the marked differences in the efficacy of these substances, comparing for each individual compound its potency to suppress TNF-α production in a cellular system with its capacity to inhibit 3′,5′-cAMP phosphodiesterase (PDE) activity in a cell-free

Abbreviations: cAMP, cyclic 3′,5′-adenosine monophosphate; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MNC, mononuclear cells; PDE, 3′,5′-cAMP phosphodiesterase (3′,5′-cyclic-nucleotide-nucleotidohydrolase).

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Table 1. Nomenclature and molecular composition of the six xanthine derivatives tested

Generic name	Chemical name
Theophylline	1,3 Di-MX*
Pentoxifylline	1-(5-Oxohexyl)-3,7-di-MX
BL 194	1-(5-Hydroxyhexyl)-3,7-di-MX
HWA 138	1-(5-Hydroxy-5-methylhexyl)-3-MX
HWA 448	7-Ethoxymethyl-1-(5-hydroxy-5-methylhexyl)-3-MX
A 80 2715	7-Propyl-1-(5-hydroxy-5-methylhexyl)-3-MX

* MX, methylxanthine.

biochemical system. This allows quantitation of the specific effect of the compounds tested independently of the numerous other factors that might affect their activity in a cell culture system.

MATERIALS AND METHODS

Cellular system for determination of TNF-α and interleukin-1β (IL-1β) production

Preparation of mononuclear cells. Blood was drawn from healthy fasting volunteers, who had been without any medication for at least 2 weeks. The mononuclear cell (MNC) fraction was obtained by gradient centrifugation (400 **g**, 45 min) of heparinized blood, diluted with pyrogen-free physiological saline (1 part blood, three parts saline) over Ficoll–Hypaque (Biochrom, Berlin, Germany). MNC were washed twice in saline. This protocol provides a population of MNC consisting of approximately 20% monocytes and 80% lymphocytes, as determined by light microscopy. RPMI-1640 culture medium (Serva, München, Germany) was supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin and was ultrafiltered (hollow fibre filter U 2000; Gambro, Hechingen, Germany) in order to remove endotoxins, as described previously.[10] The cells were adjusted to $2{\cdot}0\times10^6$/ml in this medium, supplemented with 1% heat-inactivated sterile human serum (from donor with AB blood group) and were kept at room temperature until stimulation for a maximum of 1 hr.

Preparation of the test compounds. BL 194 (penthydroxifylline) and A 80 2715 (both from Hoechst AG, Werk Kalle-Albert, Wiesbaden, Germany)—supplied in powder form—were dissolved in the ultrafiltered RPMI medium by vigorous vortexing. Pentoxifylline, HWA 448, HWA 138 (same source) and theophylline (Klinge, München, Germany), each provided in pyrogen-free saline, were directly diluted in the ultrafiltered RPMI medium. One hundred microlitres of the inhibitors were aliquoted at the required dilutions into 12 × 75 mm capped polypropylene tubes (Falcon, München, Germany).

Preparation of the stimulus. Lipopolysaccharide (LPS; *Escherichia coli* 055:B5; Sigma, München, Germany) was freshly diluted from a frozen aliquot with the supplemented RPMI medium containing 10 mg/ml endotoxin-free human albumin (Behringwerke, Marburg, Germany) and was aliquoted at 100 μl into the test tubes to a final concentration of 10 ng/ml.

Mononuclear cell stimulation. Finally, 900 μl of the MNC suspension were added to each test tube. Thus, the cells were exposed to the LPS stimulus and the test compound simultaneously. The final concentration of MNC was $1{\cdot}6\times10^6$/ml. A 20-hr incubation period at 37° in fully humidified air containing 5% CO_2 followed. Incubation was terminated by shock-freezing the tubes at −70°, thus obtaining combined cell lysate plus supernatant.

Measurement of TNF-α and IL-1β. Cell lysis was completed by two more freeze–thaw cycles. For radioimmunoassay (RIA) the samples were diluted in 0·01 M phosphate-buffered saline (PBS) containing 0·25% bovine serum albumin and 0·05% sodium azide. TNF-α and IL-1β were determined by specific RIA, as described previously.[11,12] ^{125}I Bolton Hunter labelled cytokines from NEN Dupont (Bad Homburg, Germany) were used as tracers. Cytokines for calibration of the standard curves were kindly provided by the National Institute for Biological Standards and Control (Potters Bar, U.K.).

Cell-free system for phosphodiesterase assay

The PDE assay followed the protocol used in the Kontroll-Labor Biochemie, edn 2, 32766, Jan. 1986 (Boehringer Mannheim, Mannheim, Germany).

The test volume of 3·0 ml contained the final concentrations of the following reagents: glycylglycine buffer (pH 7·5) at 73 mM, $MgSO_4$ at 1·37 mM, cAMP solution at 90 μM, adenosine deaminase from calf intestine at 6·7 μg/ml and alkaline phosphatase from calf intestine at 3·3 μg/ml (all reagents obtained from Boehringer Mannheim). The inhibitors (xanthine derivatives) were dissolved in 40% ethanol at neutral pH and were added at concentrations ranging from 100 nM to 1 mM. The reaction was started at 25° by the addition of PDE from bovine heart (EC 3.1.4.17; Boehringer Mannheim) at 8·3 μg/ml. The final ethanol concentration was 0·67% v/v and the final pH 7·5. Change of absorption over time at 256 nm was read in the linear range. PDE activity was calculated as mU/ml based on an absorption coefficient of $\varepsilon_{265}=8{\cdot}1/(\text{mmol}\times\text{cm})$.

Statistical analysis

The paired two-tailed Student's *t*-test was performed for statistical analysis, using MICROSOFT EXCEL 3·0 for Windows. Differences were considered significant for $P\leqslant0{\cdot}05$. Values are indicated as means ± SEM.

RESULTS

Dose-dependent suppression of TNF-α production by different xanthine derivatives

Stimulation of human MNC by 10 ng/ml of LPS over 20 hr in the absence of inhibitors induced TNF-α at a mean of 7·2 ± 1·5 ng/ml. This was set at 100%. All substances tested exhibited a dose-dependent suppression of TNF-α production (Fig. 1). Maximal inhibition induced by 1000 μM ranged from 78% for theophylline to 92% for A 80 2715 (down to 8% of LPS-stimulated controls). The clinically used xanthines, pentoxifylline and theophylline, were remarkably weaker suppressors than the experimental compound A 80 2715. This new substance influenced TNF-α production even at 4 μM, the lowest concentration tested, in contrast to theophylline for example, which only achieved an effect from a concentration of 63 μM.

Comparison of IC_{50} values in suppression of TNF-α production

The series of xanthine derivatives tested can be divided into three groups (Fig. 2). First, A 80 2715 with an IC_{50} value of 41 ± 3 μM (mean ± SEM, $n=13$), that proved to be the strongest inhibitor. Secondly HWA 138, pentoxifylline and BL 194 with almost identical IC_{50} values: HWA 138 at 106 ± 10 μM, pentoxifylline at 113 ± 10 μM and BL 194 at 120 ± 11 μM. And lastly, the least potent substances in suppressing *in vitro* TNF-α production, i.e. HWA 448 (IC_{50} value of 241 ± 15 μM) and, the even less potent theophylline (419 ± 65 μM). The two latter substances showed an incomplete suppression even at concentrations of 1000 μM (Fig. 1). There remained a basal TNF-α production of approximately 20% of control values.

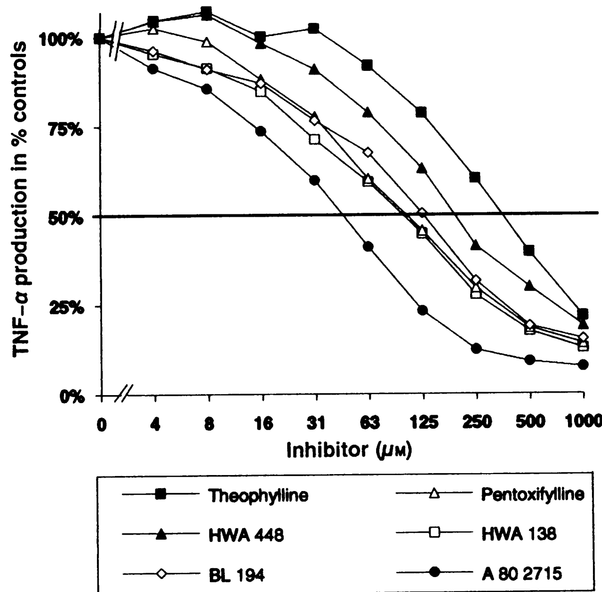

Figure 1. Xanthine derivatives-dose dependently suppress LPS-induced TNF-α production by human MNC. $1{\cdot}6 \times 10^6$ MNC/ml were stimulated with 10 ng/ml LPS for 20 hr in the presence of inhibitor concentrations ranging from 4 to 1000 μM. Concentrations of total TNF-α (i.e. intracellular and extracellular) were measured by RIA. Values are indicated as per cent of controls, i.e. MNC incubated with LPS stimulus in the absence of inhibitors. The data represent the means of eight to 11 experiments. Statistically significant TNF-α suppression at the 0·05 level was obtained for (in order of potency): A 80 2715 above 8 μM; HWA 138, pentoxifylline and BL 194 above 16 μM; HWA 448 above 31 μM and theophylline above 125 μM.

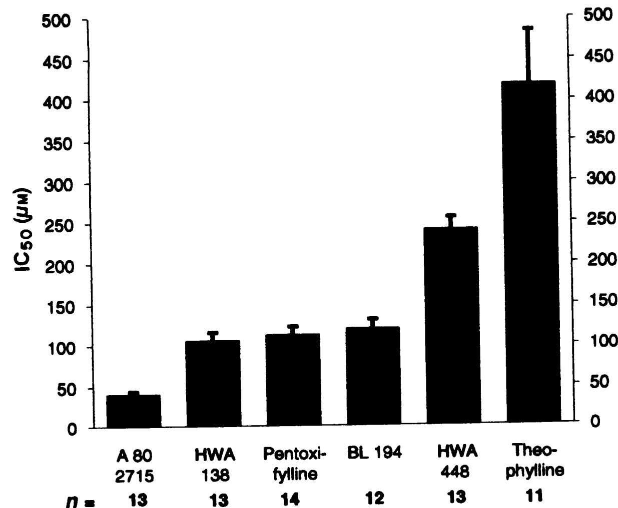

Figure 2. The series of xanthine derivatives was compared in terms of their suppressive power on LPS-induced TNF-α production by human mononuclear cells. The potency of each compound is given by its IC_{50}, i.e. the dose inhibiting TNF-α production to 50% of controls. The means of 11–14 experiments are indicated. The data of three additional experiments are included, that did not use the same dosage intervals as Fig. 1. Error bars indicate SEM. A 80 2715 proved a more powerful inhibitor than the clinically used pentoxifylline and theophylline. HWA 138, also an experimental compound, is comparable to pentoxifylline in its TNF-α suppressive capacity.

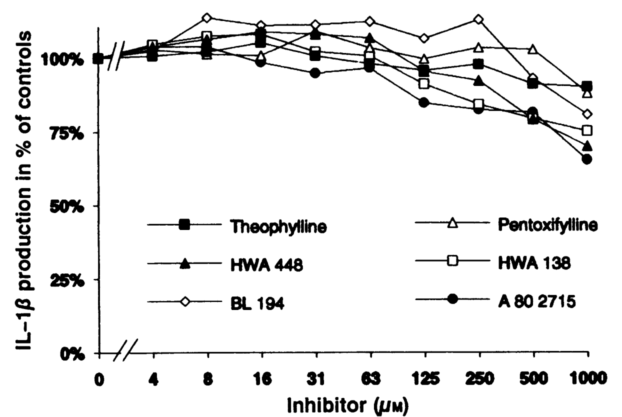

Figure 3. The tested xanthine derivatives do not suppress the IL-1β synthesis in human MNC. For methods see legend to Fig. 1. IL-1β concentration was determined in the same samples as used for TNF-α measurements. Pentoxifylline and theophylline showed no significant reduction of IL-1β production. The slight decrease of IL-1β synthesis was significant for HWA 138 only at concentrations above 250 μM, for HWA 448 above 500 μM, for BL 194 and for A 80 2715 at the maximum concentration of 1000 μM.

Specificity of the TNF-α suppressive effect by xanthine derivatives

In contrast to TNF-α production, LPS-induced IL-1β synthesis was only slightly influenced (Fig. 3). Stimulation of MNC by 10 ng/ml of LPS in the absence of the inhibitors induced IL-1β at a mean of $6{\cdot}2 \pm 1{\cdot}2$ ng/ml. This was set at 100%. Even at concentrations as high as 500 μM IL-1β synthesis still reached 79% or more for all substances. Concentrations that decreased TNF-α production by 50% did not cause a significant change in IL-1β synthesis in any case.

Inhibition of PDE activity by xanthine derivatives

In the cell-free test system all substances showed a dose-dependent inhibition of the PDE activity (Fig. 4). The PDE activity in the control samples, measured in the absence of inhibitors, was set at 100%. Their mean ± SD activity (regularly determined at the beginning of each test series) amounted to $211{\cdot}5 \pm 11{\cdot}5$ mU/ml ($n = 24$); the unit U is the amount of PDE required to split 1 μM cAMP/min at 22°. Almost complete suppression was reached by inhibitor concentrations in the range of 400 μM (8% resting PDE activity for HWA 448, 0% for A 80 2715). Again, the strongest inhibition of PDE activity at remarkably low concentrations was caused by the experimental compound A 80 2715, whereas HWA 448 was the weakest PDE inhibitor of the six substances tested.

Comparison of TNF-α suppression and PDE inhibition

Regarding the different biological activities—suppression of TNF-α production and inhibition of PDE activity—the rank order in the potency of the substances, indicated by the respective IC_{50} values, corresponded well (Table 2). Only theophylline appears to form an exception, achieving strong inhibition of PDE activity accompanied by comparatively little suppression of TNF-α production. Although the suppression of TNF-α production and inhibition of PDE activity were meas-

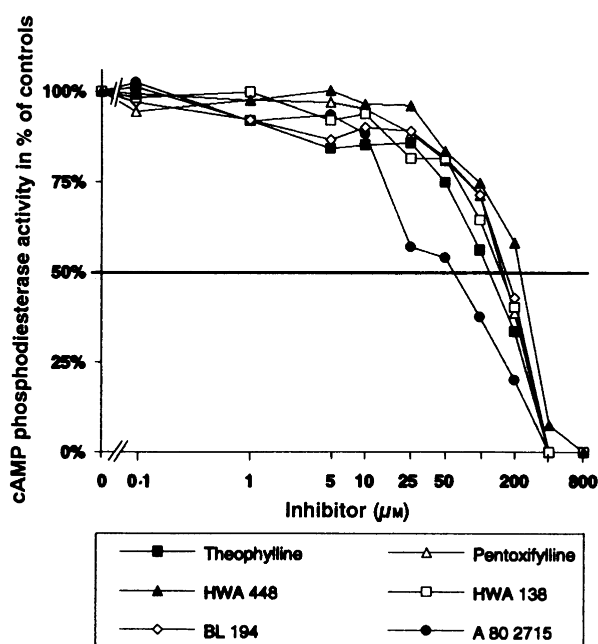

Figure 4. All xanthine derivatives tested suppressed 3′,5′-cAMP phosphodiesterase (PDE) activity dose dependently in a cell-free biochemical system. PDE activity isolated from bovine heart was measured as described in Materials and Methods. Values are given as means of triplicates. For each xanthine, PDE activity was calculated as a per cent of the control (activity without addition of inhibitor). The strongest inhibition of PDE activity at comparatively low concentrations was achieved by the experimental compound A 80 2715.

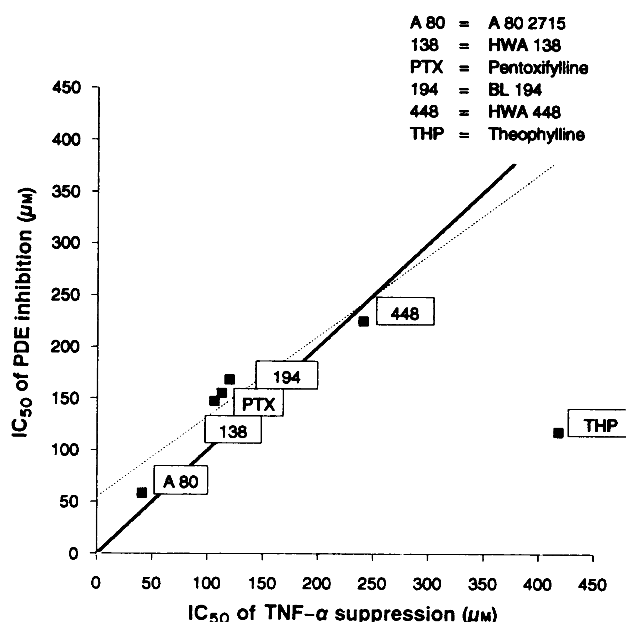

Figure 5. Inhibition of PDE correlates with the TNF-α suppressive action of the xanthine compounds tested. Data are extracted from the dose–response curves illustrated in Figs 1 and 4. With the exception of theophylline, the observed IC_{50} values for the individual substances showed a high degree of correlation between the two biological activities: for these five compounds the correlation coefficient of the experimental regression line (scattered) is 0·94. Furthermore, the absolute values were almost identical, as indicated by the position of the symbols close to the arithmetic diagonal (continuous line).

Table 2. Comparison between TNF-α suppressive activity of different xanthines and their capacity to inhibit 3′,5′-cAMP phosphodiesterase (PDE) activity. The potency of the substances is indicated by their IC_{50} values, i.e. the dose necessary to suppress LPS-induced TNF-α production in human mononuclear cells or the activity of isolated PDE, respectively, to 50% of control. The values were calculated as means of 11–14 experiments for TNF-α and as means of triplicate measurements for PDE activity

Inhibitor	TNF-α IC_{50} (μM)	cAMP PDE IC_{50} (μM)
A 80 2715	41	58
HWA 138	106	147
Pentoxifylline	113	155
BL 194	120	168
HWA 448	241	225
Theophylline	419	118

ured in completely different assay systems, the observed IC_{50} values for the individual compounds (theophylline excluded) showed a high degree of correlation. The correlation coefficient for these five xanthine derivatives with a long side chain in N1 position was $r=0{\cdot}94$, significant at $P\leqslant 0{\cdot}05$. Furthermore the absolute values were almost identical, as indicated by the position of the symbols close to the diagonal in Fig. 5. The calculated slope of the regression line (slope m in $y=b+mx$) was 0·78.

DISCUSSION

In these experiments we investigated the inhibitory effect of six chemically related compounds containing a xanthine moiety with different side chains on TNF-α production by human mononuclear cells and on the cAMP PDE activity. The experimental drug A 80 2715 was identified as the most potent agent by its suppression of TNF-α production, with an IC_{50} value at 41 μM, remarkably stronger than the clinically used substances pentoxifylline and theophylline. In a cell-free biochemical assay all xanthine derivatives dose dependently inhibited PDE activity. With the exception of theophylline the potency of the compounds to suppress TNF-α production in the cell culture system correlated significantly with the potency to inhibit PDE activity in the cell-free enzymatic assay. Thus PDE inhibition is a central mechanism in the TNF-α suppressive activity of xanthine derivatives on mononuclear cells. In other cell types additional mechanisms may contribute, such as the induction of prostacyclin by xanthine derivatives observed in endothelial cells.[13]

TNF-α and IL-1β are peptides synthesized *de novo* by MNC upon LPS stimulation. Thus, the unaffected level of IL-1β production argues for a specific action of xanthine derivatives on TNF-α production and against non-specific cytotoxicity. Even several-fold higher concentrations of the xanthine compounds than those reqired for half-maximal inhibition of TNF-α production did not substantially affect production of IL-1β in

the same system. The slight reduction of IL-1β depicted in Fig. 3 may indeed be explained by the strong decrease of TNF-α production under the experimental conditions employed. Endogenous TNF-α is a stimulus for IL-1β production, in addition to the direct stimulation by LPS. The specifity of xanthine derivatives for suppression of TNF-α is probably based on decreased gene transcription, as has been demonstrated for pentoxifylline.[14] This contrasts with the action of steroids that block cytokine production at the translational level.

The differences in the capacity of xanthine derivatives to suppress TNF-α production raise the question of a structure–function relationship. Remarkably, the five compounds with substitution of a methyl group in N1 position by a side chain with six carbon atoms exhibited distinctly lower IC_{50} values than theophylline, which lacks such a substitution. Within the five compounds containing the six carbon atom side chain there is no apparent connection between specific substituents and their suppressive efficacy.

The comparison between cytokine production in cell culture and PDE activity in a cell-free system allows the determination of the impact of the xanthine derivatives tested on the enzyme level directly, independently of numerous other factors, like membrane permeability, receptor binding and metabolism. This is of particular importance because a role of adenosine receptors in the transmission of the effects of xanthine compounds has been discussed. On the one hand, xanthine derivatives can block adenosine receptors;[15] on the other hand selective TNF-α suppression by adenosine analogues has been described.[16] The close relation between PDE inhibition and TNF-α suppression observed in this study, however, argues for a direct action of xanthines on the PDE without additional transmission through cellular structures. This conclusion corresponds with our recent finding of the strong inhibitory effect of rolipram, a specific cAMP PDE type IV inhibitor with phenyl-pyrrolidinone structure, on TNF-α production of MNC.[17] The pivotal role of cAMP in suppression of TNF-α production is also emphasized by studies that demonstrated TNF-α inhibition by cAMP and its analogues.[18,19]

Theophylline, characterized by strong inhibition of PDE and relatively weak suppression of TNF-α production, seems to form an exception to the other compounds studied. This phenomenon may have several different explanations. Theophylline may be less membrane permeable than the other xanthines containing long side chain substituents. Furthermore, possible differences of the PDE involved in the studied cell culture system and the PDE studied in cell-free assay should be taken into account. The bovine heart PDE used in the cell-free assay is an isoenzyme type III, in contrast to the human monocyte PDE, which belongs predominantly to type IV.[20,21] An important role of species or isoenzyme differences, however, is not likely because theophylline is definitely described as a non-specific PDE inhibitor.[22]

Concerning therapeutic consequences, this study reveals A 80 2715 and HWA 138 as substances with high pharmacological potential. However, from the animal studies published so far, no information is available about the relative toxicities of these compounds compared to xanthines, which are clinically used at present. Both substances have effects at low concentrations may be taken orally as well as parenterally, and may have beneficial effects both in chronic, moderate and acute, severe diseases mediated by excessive TNF-α production.

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Part of this study has been presented at the III International Workshop on Cytokines, Stresa, Italy, November 1991, and has been published in abstract form (*Cytokine* 1991 **3**, 5). The experimental data of this study (except Fig. 4) are part of the dissertation of J. Semmler: 'Phosphodiesterasehemmer als Inhibitioren der TNF-Synthese' (Medizinische Fakultät der Ludwig-Maximilians-Universität München, in preparation).

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