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SYNTHESIS of IL-1β and TNFα by human monocytes-macrophages was significantly inhibited by eleven bisbenzylisoquinolines and one half-molecule (benzylisoquinoline), with IC_{50} values in the μM range. The results indicate that these compounds may have value in the therapy of human diseases where these inflammatory cytokines have a central role in pathogenesis.

Key words: Bis-benzylisoquinolines, Cytokines, IL-1β, TNF-α

Inhibitory effects of bisbenzylisoquinolines on synthesis of the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha

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Introduction

The pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF) are potent molecular mediators with wide-ranging effects on a large array of cells, tissues and organs.[1,2] They are produced by a wide variety of cell types, but monocytes-macrophages are the major source of these polypeptides which share structural similarities and have overlapping activities.[3,4] They have been implicated in the pathogenesis of chronic inflammatory (e.g. rheumatoid arthritis[5]), autoimmune (e.g. insulin-dependent diabetes mellitus[6]), allergic (e.g. asthma[7]), infectious (e.g. leprosy[8]) and malignant (e.g. ovarian carcinoma[9]) diseases.

There is therefore a great interest in drugs that can control their generation and/or action because of the potential for therapeutic application. It has recently been shown that tetrandrine, a novel bisbenzylisoquinoline alkaloid, has potent inhibitory effects on the production of IL-1β and TNFα by human monocyte-macrophage cultures.[10–12] In addition, it has been shown to have therapeutic efficacy in a number of animal models of human disease, such as relapsing experimental allergic encephalitis in rats (multiple sclerosis),[13] spontaneous diabetes in BB rats (insulin-dependent diabetes mellitus),[14] air-pouch inflammation in rats (rheumatoid arthritis)[15] and airways microvascular leakage in guinea-pigs (asthma).[16] In an attempt to gain some mechanistic insight into the relationship between the bisbenzylisoquinoline structure and the extent of inhibition of the synthesis of TNF-α and IL-1β, the inhibitory potencies of tetrandrine and a series of ten other bisbenzylisoquinolines and one benzylisoquinoline (half-molecule) are now compared. The bisbenzylisoquinolines vary in the type of substituent, location of their ether bridges and the stereochemistry at a chiral centre. The sole benzylisoquinoline tested is a monomeric component of the dimeric benzylisoquinoline, tetrandrine,

and serves to identify whether the dimeric bisbenzylisoquinolines are active as a result of their inherent macrocyclic structure or because of their constituent functional groups which are also present in their smaller monomeric fragments.

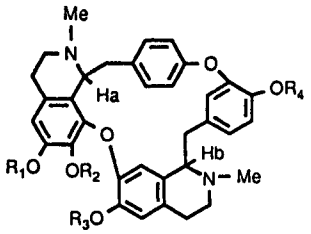
Materials and Methods

Compounds: Four types of compounds were used in these experiments (Fig. 1). Three of these were macrocyclic ether compounds of isoquinolines bridged by alpha-benzyloxy substituents. These compounds differ in the position of the linkages between the two monomeric benzylisoquinoline components, and the nature and number of substituents on the aromatic rings. The fourth type of compound was represented by *N*-methylcoclaurine, a monomeric benzylisoquinoline.

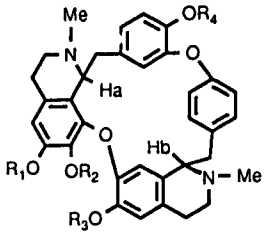
The twelve compounds used in this study were obtained from diverse sources.[17] Tetrandrine and fangchinoline, were extracted from the tubers of *Stephania tetrandra*,[18] which were purchased from Maruzen Seiyaku Company, Onomichi, Japan; cepharanoline, cepharanthine, isotetrandrine and cycleanine, extracted from the tuberous root of *Stephania cepharantha*,[19] were purchased from Kaken Shoyaku Company, Tokyo, Japan; berbamine and oxyacanthine were purchased from Carl Roth, Germany; aromoline was isolated from root cultures of *Stephania cepharantha*, as described previously;[20] homoaromoline and obaberine were converted from aromoline and oxyacanthine, respectively, by methylation with diazomethane; *N*-methylcoclaurine was prepared by chemical synthesis.[21]

All compounds (>97% pure) were identified for chemical structure by mass spectroscopy and ^{1}H-NMR spectroscopy against reference samples and literature values.[22] The HCl salts of the compounds were dissolved in RPMI 1640 medium at a concentration of 2 mg/ml, and these stock solutions further diluted in RPMI 1640 medium for the experiments.

Production of IL-1β and TNFα: The study was performed with monocytes–macrophages isolated from human blood.[10–12] Blood was drawn by venepuncture from three healthy non-smoking adults not on any medication for at least 2 weeks prior to the study. The heparinized blood was layered onto Hypaque–Ficoll medium of density 1.114 and centrifuged at 600 × ***g*** for 30 min. The



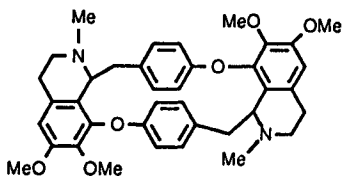
TYPE A



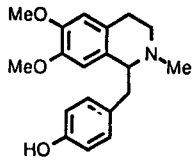
TYPE B

Derivative	R_1	R_2	R_3	R_4	Ha	Hb
Aromoline	Me	H	Me	H	R	S
Homoaromoline	Me	H	Me	Me	R	S
Oxyacanthine	Me	Me	Me	H	R	S
Obaberine	Me	Me	Me	Me	R	S
Cepharanoline	-CH_2-		Me	H	R	S
Cepharanthine	-CH_2-		Me	Me	R	S

Derivative	R_1	R_2	R_3	R_4	Ha	Hb
Tetrandrine	Me	Me	Me	Me	S	S
Fangchinoline	Me	H	Me	Me	S	S
Berbamine	Me	Me	Me	H	S	R
Isotetrandrine	Me	Me	Me	Me	R	S



Cycleanine



N-Methylcoclaurine

FIG. 1. Chemical structure of the bisbenzylisoquinolines: ten head-to-head conformation (Types A and B), one head-to-tail (cycleanine) and one half-molecule (*N*-methylcoclaurine). Ha and Hb designate absolute configuration (*R* or *S*).

mononuclear leukocytes (MNC) in the top band at the interface were harvested,[23] washed and resuspended in RPMI 1640 medium for the experiments.

Preparation of adherent MNCs, containing predominantly monocytes, were made in sterile plastic Petri dishes. After 3 h at 37°C, the non-adherent cells were washed off, and the adherent cells were later removed with the aid of a sterile rubber policeman, washed and re-suspended in RPMI 1640 medium containing 5% foetal calf serum, and the cell concentration adjusted by counting in a Neubauer haemocytometer. To 1 ml of 2×10^6 cells/ml was added 0.5 ml of drug solution. After incubation at 37°C for 15 min, 0.5 ml of 4×10^7 heat-killed, formalin-fixed, *Staphylococcus aureus* as a stimulant, were added into wells of 24-well cluster plates. The supernatants were harvested after 24 h, and kept frozen at −70°C for up to 2 weeks before being assayed for the presence of IL-1β and TNFα. The cells were checked for viability by Trypan-blue dye exclusion (>94%).

Quantitation of IL-1β and TNFα: Both IL-1β and TNFα in the culture supernatants were measured by a competitive binding radioimmunoassay (Amersham, UK).[12] The tracer was [^{125}I]IL-1β or TNFα (human, recombinant), and the antibody to human recombinant IL-1β or TNFα was made in rabbits. The antibody-bound IL-1β or TNFα was then reacted with the Amerlex-M second antibody reagent (donkey anti-rabbit serum) which contains a second antibody that is bound to magnetizable polymer particles. Separation of the antibody-bound fraction is effected by centrifugation of the Amerlex-M suspension and decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of labelled IL-1β or TNFα in the bound fraction to be calculated. The concentration of unlabelled IL-1β or TNFα in the sample is then determined by interpolation from a standard curve.

Determination of IC_{50} values: The concentration of drug (μM) which causes 50% inhibition of cytokine production (IC_{50}) was determined from graphical plots of inhibitor concentration vs. percentage inhibition.

Results

Effect on IL-1β and TNFα synthesis: Human monocytes-macrophages were incubated with 0, 1, 4, 10, 20 and 40 μg/ml of each of the twelve compounds. The results (Table 1) showed dose dependent inhibition of both IL-1β and TNFα production for all twelve compounds. Control values (fM/10^6 cells) for quantities of IL-1β and TNFα produced by cultures were 162.5 ± 8.2 and 81.2 ± 4.9 (mean ± S.E.M.) respectively, or approximately 2762 and 1380 pg/l/10^6 cells, respectively.

IC_{50} values: The IC_{50} values were measured from graphs of inhibitor concentration vs. % inhibition and are presented in Table 2. The results show that all IC_{50} values for the twelve compounds are within the same order of magnitude and the range 4–26 μM and 2–18 μM for IL-1β and TNFα respectively.

Discussion

Currently available drugs for therapy of chronic inflammatory and autoimmune diseases are unsatisfactory because of significant toxic and/or immunosuppressive side effects. There is therefore a great need to develop better and safer drugs to control inflammatory tissue destruction. The discovery of the central role of inflammatory cytokines in the pathogenesis of these diseases[1–9] opens up a new strategic target for drug development. The authors recently showed that the bisbenzylisoquinoline alkaloids, tetrandrine and berbamine, have the capacity to inhibit both the generation and action of IL-1β and TNFα,[10–12] and control disease progression in a number of animal models.[13–16] Although the mechanism of action of bisbenzylisoquinolines is not well understood, studies with tetrandrine have shown its capacity to block calcium channels,[24] interfere with transmembrane signalling,[25] and induce apoptosis.[26] Moreover, it is largely devoid of immunosuppressive properties,[27,28] and is non-toxic at therapeutic doses (20 mg/kg per day for 84 days) as assessed by appearance, behaviour, weight change, blood chemistry and organ histology of BB rats.[14] This compares well with the oral LD_{50} of 2230 mg/kg in rats,[29] which is more than 100 times the therapeutic dose. Furthermore, tetrandrine has been used empirically in humans for the treatment of silicosis at a dose of 300 mg/day for 3 years without serious toxic side effects.[30] Thus, bisbenzylisoquinolines may have significant advantages over existing drugs used in the treatment of chronic inflammatory and autoimmune diseases, such as corticosteroids, cytotoxic and anti-rejection drugs, which have substantial toxic and immunosuppressive side effects. As such, they may become the fore-runner of a new class of safe and effective drug suitable for long term clinical usage.

The results of the present study showed that all eleven bisbenzylisoquinoline alkaloids have inhibitory effects on IL-1β and TNFα synthesis by human monocytes–macrophages. Calculation of IC_{50} values showed that inhibitory concentrations of these eleven compounds were within the same order of magnitude. This was surprising as the structures depicted in Fig. 1 vary considerably. It was expected

Table 1. Effect of bisbenzylisoquinolines on production of IL-1β and TNFα by human monocytes–macrophages

Drug	Concentration (μg/ml)	Percent of control (±S.E.M.)		Drug	Concentration (μg/ml)	Percent of control (±S.E.M.)	
		IL-1	TNF			IL-1	TNF
Aromoline	1	89.7 ± 2.9	86.0 ± 6.6	Obaberine	1	95.3 ± 5.7	92.6 ± 2.6
	4	71.5 ± 5.2	75.3 ± 2.1		4	90.4 ± 2.6	64.9 ± 6.8
	10	45.7 ± 5.5	52.1 ± 12.2		10	69.0 ± 3.8	30.2 ± 5.1
	20	20.5 ± 7.1	39.3 ± 14.2		20	45.9 ± 5.6	11.3 ± 1.5
	40	8.2 ± 3.4	11.7 ± 3.6		40	13.1 ± 2.5	4.1 ± 0.3
Berbamine	1	87.9 ± 5.1	77.3 ± 3.2	Oxyacanthine	1	101.5 ± 4.8	100.3 ± 10.1
	4	83.4 ± 4.8	71.5 ± 11.0		4	80.9 ± 7.9	80.3 ± 10.6
	10	33.7 ± 11.9	24.0 ± 12.3		10	37.9 ± 12.4	32.2 ± 3.9
	20	7.0 ± 0.7	3.1 ± 1.3		20	16.9 ± 4.2	10.5 ± 1.1
	40	4.3 ± 1.0	1.5 ± 1.0		40	5.6 ± 0.1	3.2 ± 1.3
Cepharanoline	1	82.4 ± 7.0	63.3 ± 8.8	Tetrandrine	1	84.3 ± 5.6	72.3 ± 8.4
	4	63.9 ± 5.0	41.1 ± 13.7		4	64.9 ± 4.2	39.6 ± 6.3
	10	40.8 ± 10.0	23.6 ± 11.0		10	16.6 ± 6.0	1.2 ± 0.7
	20	23.0 ± 8.8	4.5 ± 0.5		20	13.0 ± 7.9	1.7 ± 1.2
	40	17.1 ± 5.7	1.9 ± 0.9		40	10.1 ± 6.6	0.3 ± 0.0
Fangchinoline	1	94.3 ± 4.9	91.7 ± 7.2	Cepharanthine	1	98.7 ± 11.2	53.2 ± 6.3
	4	36.0 ± 6.3	44.0 ± 21.2		4	84.4 ± 7.7	28.7 ± 1.6
	10	3.8 ± 1.0	2.4 ± 0.9		10	49.8 ± 11.5	10.7 ± 0.9
	20	3.0 ± 0.9	2.3 ± 0.8		20	20.5 ± 5.8	3.1 ± 1.1
	40	1.7 ± 0.4	1.0 ± 0.6		40	5.7 ± 1.1	2.1 ± 1.1
Homoaromoline	1	74.3 ± 9.5	84.9 ± 6.3	Cycleanine	1	84.5 ± 3.9	74.0 ± 5.0
	4	49.6 ± 8.9	66.9 ± 10.7		4	78.0 ± 4.2	41.2 ± 10.8
	10	36.4 ± 10.8	32.4 ± 2.9		10	64.0 ± 12.6	22.5 ± 10.2
	20	13.6 ± 3.5	7.5 ± 1.1		20	47.0 ± 14.1	12.7 ± 3.5
	40	8.1 ± 3.1	2.2 ± 1.56		40	36.6 ± 8.5	3.3 ± 1.5
Isoterandrine	1	73.9 ± 9.3	75.8 ± 6.5	*N*-methyl-coclaurine	1	92.6 ± 9.5	96.6 ± 3.8
	4	70.0 ± 10.3	43.2 ± 6.1		4	51.8 ± 8.7	82.4 ± 12.2
	10	46.5 ± 3.3	6.7 ± 1.1		10	1.5 ± 3.7	15.2 ± 6.3
	20	12.0 ± 4.2	1.8 ± 0.7		20	1.5 ± 3.0	7.7 ± 2.1
	40	5.1 ± 0.8	1.0 ± 0.9		40	1.6 ± 3.3	5.5 ± 1.3

The results represent the mean ± S.E.M. of three experiments using cells from three individuals. The quantity (fM/10^6 cells) of IL-1β and TNFα produced by control cultures were 162.5 ± 8.2 and 81.2 ± 4.9 (mean ± S.E.M.), respectively.

Table 2. Inhibition of IL-1β and TNFα production by bisbenzylisoquinolines: IC_{50} values

Bisbenzylisoquinolines (HCl salts)	MW	IC_{50} (μM)	
		IL-1β	TNFα
Aromoline	667	13.6	18.3
Homoaromoline	681	8.7	9.8
Oxyacanthine	681	11.6	10.1
Obaberine	695	26.0	8.9
Berbamine	681	11.3	7.3
Isotetrandrine	695	13.4	4.3
Fangchinoline	681	4.6	4.6
Tetrandrine	695	7.2	4.6
Cepharanoline	665	12.3	3.8
Cepharanthine	679	15.0	2.2
Cycleanine	695	25.0	4.6
N-methylcoclaurine*	335.5	11.6	16.4

* half-molecule

that the locations and numbers of methoxy- vs. hydroxy-substituents (types A and B, cycleanine) would have significantly influenced interaction with (unknown) receptors responsible for blocking synthesis of the specific cytokines in question, especially if the dimeric form is maintained intracellularly. Similarly the chirality differences would lead to different molecular orientations of the isoquinolines during interaction with receptor(s). However, the similarity in inhibitor potencies suggested that the dimer structure as such may not be the principal activity determinant.

A very significant finding may be that *N*-methylcoclaurine, a non-cyclic benzylisoquinoline quite different from the cyclic bisbenzylisoquinolines, has equivalent inhibitory effects on IL-1β and TNFα production. There are at least two possible interpretations of these findings in terms of structure–activity relationships. One interpretation would be that the bisbenzylisoquinolines and benzylisoquinolines act on different receptors, even though both are equipotent inhibitors of inflammatory cytokine production. A more likely interpretation is that the monomers, benzylisoquinolines, may be the active forms of the bisbenzylisoquinolines. Bisbenzylisoquinolines like *N*-methylcoclaurine are known to chemically assemble in plants to form bisbenzylisoquinolines like tetrandrine. However, it is not known whether bisbenzylisoquinolines transform biologically into

monomeric benzylisoquinolines, although it has been shown chemically that cleavage of tetrandrine can be achieved using strong reducing conditions (metallic sodium in liquid ammonia) into two monomers, one of which is *N*-methylcoclaurine.[31]

One avenue for further investigation would be to determine whether the bisbenzylisoquinolines are metabolized under the conditions of these experiments and, if so, to identify the metabolites after *in vitro* and *in vivo* degradation. Another would be to determine whether bisbenzylisoquinolines and benzylisoquinolines have similar or different molecular modes of actions. Because of the importance of inflammatory cytokines in health and disease, further development of these classes of compounds may provide insights into the biology of inflammatory cytokines as well as to a better and safer drug for therapy of inflammatory diseases.

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