Evaluation of Sage Phenolics for Their Antileishmanial Activity and Modulatory Effects on Interleukin-6, Interferon and Tumour Necrosis Factor-α-Release in RAW 264.7 Cells

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A series of sage phenolics was tested for activity against a panel of *Leishmania* parasites and for immunomodulatory effects on macrophage functions including release of tumour necrosis factor (TNF), interleukin-6 (IL-6), and interferon (IFN)-like activities. For this, functional bioassays were employed including an *in vitro* model for leishmaniasis in which macrophage-like RAW 264.7 cells were infected with *Leishmania* parasites, an extracellular *Leishmania* growth-inhibition assay, a fibroblast-lysis assay for TNF-activity, a cell proliferation assay using IL-6 sensitive murine B9 hybridoma cells, and a virus protection assay for IFN-like activity. Whereas none of the test samples exhibited marked activities against extracellular *Leishmania* promastigotes (IC_{50} > 700 to > 2800 nM; > 500 μg/ml), caffeic acid, salvianolic acids K and L as well as the methyl ester of salvianolic acid I showed pronounced antileishmanial activities against intracellular amastigote stages within RAW cells (IC_{50} 3–23 nM vs. 10–11 nM for the reference Pentostam®). Noteworthy, the phenolic samples showed no cytotoxicity against the host cells (IC_{50} > 600 to > 2200 nM; > 400 μg/ml). Tested sage phenolics activated Leishmania-infected RAW 264.7 for release of TNF ranging 22–117 U/ml and IL-6 ranging 3–42 U/ml. In contrast, their TNF- or IL-6-inducing potential in experiments with non-infected host cells was negligible. Furthermore, caffeic acid and salvianolic acid K induced a modest release of IFN-like activity (5–9 and 2–4 U/ml, respectively) as reflected by inhibition of the cytopathic effect of encephalomyocarditis virus on L929 cells. The results support the emerging picture that plant polyphenols may be credited for the profound health-beneficial properties of various herbal medicines and agricultural products.

Key words: Salvia officinalis, *Leishmania*, Immunomodulation

Introduction

Leishmaniasis, causing extensive morbidity and mortality in most developing countries, has traditionally been considered rather exotic elsewhere. However, due to facilitated international travel, this group of parasitic diseases is beginning to have a major impact on human populations of the developed world as well. Besides the development of resistant parasite strains, a clinical problem that has emerged in recent years is *Leishmania*/HIV co-infection especially in southern Europe, where 25–70% of potentially fatal visceral leishmaniasis (VL) cases are associated with HIV infection, and 2–9% of AIDS cases suffer from newly acquired or reactivated VL (Alvar *et al.*, 1997). Approximately 12 million people are infected worldwide, and it is estimated that some 350 million people live at risk of infection with *Leishmania* parasites (Ashford *et al.*, 1992). Taking into account the annual incidence of about 2 million new cases and the deficiencies in current drug therapy, their is an urgent need for new and innovative antileishmanial drugs.

In recent studies, we have shown that polyphenols have favourable antileishmanial activity *in vitro* and might be considered as beneficial immunological response modifiers (Kiderlen *et al.*, 2001; Kolodziej *et al.*, 2001a, b). Continuing our research program to identify and functionally characterise

novel antileishmanial compounds, a series of caffeic acid-derived metabolites, obtained from *Salvia officinalis* (Lu and Foo, 1999; Lu *et al.*, 1999), was tested for direct leishmanicidal activity against a panel of *Leishmania* parasites as well as immunomodulatory effects on macrophage functions.

Materials and Methods

Test compounds

Compounds **1**–**7** were isolated from *Salvia officinalis* (Lu and Foo, 1999; Lu *et al.*, 1999), their identity being proved by MS and NMR spectroscopy. Sodium stibogluconate (Pentostam®; kindly provided by Dr Goldbach, Glaxo Wellcome, Germany) was used for comparison as standard drug of antileishmanial activity. Recombinant murine interferon-γ (rIFN-γ), expressed in *E. coli*, was produced by Genentech, San Francisco, USA and kindly provided by Bender & Co., Wien, Austria. Bacterial lipopolysaccharide (LPS), extracted from *Salmonella abortusequi* was kindly provided by Prof. Dr. O. Holst, Forschungsinstitut Borstel, Germany.

General procedures and assays for leishmanicidal and cytotoxic activity

General experimental procedures and assays for extra- and intracellular leishmanicidal activity and for cytotoxicity against host cells are fully described elsewhere (Kiderlen and Kaye, 1990; Kayser *et al.*, 1999). The murine macrophage-like cell line, RAW 264.7, was kindly supplied by Dr. M. Masihi, Robert Koch-Institut, Berlin, Germany, the IL-6 dependent B9 hybridoma cells were kindly provided by Dr. L. A. Aarden, Red Cross transfusion Service, Amsterdam, The Netherlands, and the L929 fibroblast cells were from Dr. Lohmann-Matthes, Fraunhofer Institute of Toxicology, Hannover, Germany.

Induction of cytokine release and assays for TNF and IFN-activity

Non-infected and *Leishmania*-infected murine RAW 264.7 cells were seeded at 5×10^6/well in 2 ml RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum (herein designated R5) in 12-well flat-bottom microtiter plates and incubated at 37 °C in 6% CO_2-enriched, humidified atmosphere. After 2 h non-adherent cells were removed by gentle washing and the medium replaced by 2 ml/well fresh R5 containing the test samples or controls indicated in the Results and Discussion section. Test sample concentrations corresponded to their respective IC_{50} values for intracellular antileishmanial activity shown in Table I. After incubating another 18 h (for TNF) or 24 h (for IL-6 and IFN) 100 µl aliquots were removed and tested for TNF- and IFN-activity as fully described elsewhere (Kolodziej *et al.*, 2001a and b) and for IL-6 as described below.

Interleukin-6 (IL-6) detection assay

Aliquots of the RAW 264.7 cell culture supernatants described above were diluted 1:2 in R5 6-times across a 96-well flat-bottom microtiter plate (Falcon, BD Labware, Franklin Lakes, USA). IL-6 sensitive murine B9 hybridoma cells were added at 1×10^4/100 µl R5/ well. Following an incubation period of 72 h at 37 °C in 6% CO_2-enriched, humidified atmosphere, the relative number of viable cells per well was assessed colorimetrically. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well at 500 µg/ml and the plates incubated another 4–5 h allowing viable cells to transform yellow soluble MTT into blue formazan crystals. The reaction was stopped and formazan solubilised by adding 50 µl sodium dodecyl sulfate (20%, pH 6.4)/well over night. Relative optical densities per well, correlating with the relative amount of originally viable cells/well was measured at 570 nm in an ELISA-reader (Spectra Fluor, Tecan, Grödig/Salzburg, Austria). IL-6 units/ml were calculated as the reciprocal values of the supernatant dilution that would cause 50% maximal proliferation of B9 hybridoma cells. These values were correlated with a defined laboratory IL-6 standard (70 U/ml) to account for fluctuation in assay sensitivity.

Results and Discussion

Salvia species (Lamiaceae) are used in folk medicines for the treatment of a variety of diseases, including infectious conditions. Studies on their chemical constituents have been mainly confined to the terpenoids. Recently, the polar components have also attracted considerable attention as a po-

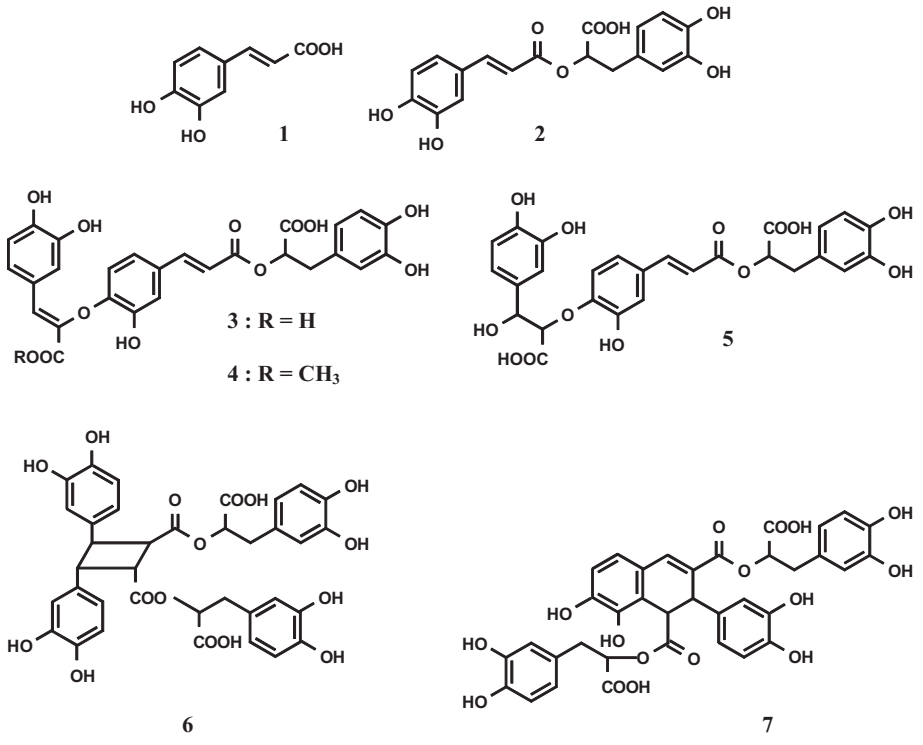

Fig. 1. Structures of caffeic acid (**1**), rosmarinic acid (**2**), salvianolic acid I (**3**), methyl ester of **3** (**4**), salvianolic acid K (**5**), salvianolic acid L (**6**), and sagerinic acid (**7**).

tentially valuable resource for drug discovery (Lu and Foo, 2002). These polyphenols are apparently constructed from the caffeic acid building block via a variety of condensation reactions, with antiviral and antimicrobial rosmarinic acid as the most abundant representative (Gerhardt and Schroeter, 1983). Having in mind the promising antiparasitic and immunomodulatory effects recently demonstrated for a variety of polyphenols (Kolodziej *et al.*, 2001a, b), we extended our studies to a series of these structural variants.

Starting with the antileishmanial activity of the polyphenolic samples, the activity of compounds **1–7** (Fig. 1) was assessed against both extracellular promastigotes and intracellular amastigotes of a panel of *Leishmania* species (Table I), with *L.*

Table I. Toxicity for RAW host cells and antileishmanial activity against a panel of *Leishmania* promastigotes (PM) and amastigotes (AM) of compounds **1–7** (IC_{50} values in nM).

No	Compound	Toxicity for RAW cells	PM	Leishmanicidal activity AM			
				L. major	*L. donovani*	*L. guyanensis*	*L. killicki*
(**1**)	caffeic acid	>2200	>2800	4.4	6.1	6.6	3.9
(**2**)	rosmarinic acid	>1100	>1400	59.2	74.4	84.2	69.4
(**3**)	salvianolic acid I	>700	>900	160.4	175.8	151.5	167.8
(**4**)	methyl ester of **3**	>700	>900	10.8	18.6	15.2	13.6
(**5**)	salvianolic acid K	>700	>900	18.3	18.2	13.3	14.5
(**6**)	salvianolic acid L	>700	>900	20.3	15.4	22.6	13.0
(**7**)	sagerinic acid	>600	>700	128.7	122.1	141.5	154.8
	Pentostam	not det.	3500	11.2	10.6	10.1	10.9

major and *L. donovani* as causative agents of Old World cutaneous and visceral leishmaniasis, respectively. As a parameter for antileishmanial activity, the IC_{50} value, *i. e.* the sample concentration causing 50% reduction in survival/viability of the parasites, was used. As can be seen, none of these phenolic samples showed selective toxicity when tested against the promastigote stages of the four *Leishmania* species (IC_{50} > 700 to > 2800 nM; > 500 μg/ml). In contrast, caffeic acid (**1**), methyl ester of salvianolic acid I (**4**) as well as salvianolic acids K (**5**) and L (**6**) reduced the intracellular survival of *Leishmania* amastigotes within RAW 264.7 cells (IC_{50} 3.9–22.6 nM) in our *in vitro* experiments, when compared with the IC_{50} values 10.1–11.2 nM of the therapeutically used antileishmanial drug, Pentostam®. Rosmarinic acid (**2**), salvianolic acid I (**3**) and sagerinic acid (**7**) exhibited only moderate antileishmanial activity (IC_{50} > 59 nM). These findings suggested either an amastigote-specific activity of the polyphenols, provided that these compounds reach the parasitic vacuole within the host cell, or they indicated activation of macrophage effector functions. Notably, all samples appeared to be non-toxic against the host cells (IC_{50} 600 to > 2200 nM; > 400 μg/ml).

Concerning structure-activity relationships, it would appear that neither the degree of oligomerisation nor the varying condensation reactions provide structural clues in this group of compounds. Conspicuously, the most powerful candidate is represented by the simple caffeic acid (**1**), being twice as potent as the standard reference.

Tumour necrosis factor (TNF)-α, produced principally by activated macrophages and monocytes, plays a crucial role in the host defence against various pathogens (Beutler *et al.*, 1989). As shown in Table II, the amounts of TNF induced by the tested sage phenolics in non-parasitised RAW 264.7 was negligible (< 5 U/ml for **3**, **4** and **7**) or below detection limit (**1**, **2**, **5** and **6**). In *Leishmania*-parasitised RAW 264.7 cells, however, tested compounds induced moderate to fairly high amounts of TNF (19–117 U/ml) compared to IFN-γ + LPS as positive control (200–215 U/ml). Although distinct structural features are difficult to define, the TNF-inducing potential of **1–7** seemingly decreases with increasing condensation level, as reflected by the TNF-production of monomer **1** > dimer **2** > trimers **3**, **5** and **6** > tetramer **7** (Table II). The relatively weak inducing potential of **4** may be attributable to the esterification of the carboxyl group of the terminating caffeoyl unit.

Several lines of evidence indicate that IL-6, a pleiotropic cytokine produced by various types of lymphoid and non-lymphoid cells, is involved in the battery of non-specific immune defence mechanisms in intimate and causal relationship with IL-1 and to some extent with TNF (Théze, 1999). Given the pivotal role of IL-6 in the proliferation and differentiation of immune cells, this factor was assessed in a functional bioassay employing the selective dependency of murine B9 hybridoma cells on IL-6 for survival and proliferation. Appropriate controls were performed using medium alone for minimal and a defined laboratory IL-6 standard (70 U/ml) for maximal cell proliferation. The results listed in Table II showed that all compounds stimulated RAW 264.7 cells to some extent for IL-6 release. As with TNF, activation of non-parasitised cells was only marginal (1–8 U/ml) whereas especially compounds **1**, **3** and **6** activated *Leishmania*-parasitised RAW 264.7 cells to release more IL-6 (19–42 U/ml) than the stimulus IFN-γ/LPS (12–16 U/ml).

Next, we assessed the potential of compounds **1–7** to activate RAW 264.7 for IFN-release, measured as protection of IFN-sensitive murine L929 fibroblasts from the cytopathic effect of encephalomyocarditis virus (EMCV) (Kolodziej *et al.*, 2001a, b). Macrophage functions are intimately related to the IFN system (Billiau, 1995), consistent with our recent finding that stimulated RAW 264.7 cells express IFN-γ mRNA, as evidenced by RT-PCR-analysis (Radtke *et al.*, 2001). The IFN-like activities found in supernatants of phenolic-treated infected RAW cells ranged from below detection level to modest (Table II). Of the tested compounds, only caffeic acid (5–9 U/ml) and salvianolic acid K (2–4 U/ml) induced antiviral effects.

In conclusion, our study not only provides first evidence that caffeic acid derived metabolites contribute to the crucial transformation of macrophage-like RAW 264.7 cells from host cells to leishmanicidal effector cells, but also, although not yet understood, supports the emerging picture that plant polyphenols may be credited for the profound beneficial properties of various herbal medicines and agricultural products. The prominent

Table II. TNF-, IL-6 and IFN-inducing potential of compounds **1–7** in macrophage like RAW 264.7 cells.

Compound	Tumour necrosis factor [a]					IL-6[b]					IFN[c]			
	Non-infected RAW 264.7	*L. major*	*L. donovani*	*L. guyan.*	*L. killicki*	Non-infected RAW 264.7	*L. major*	*L. donovani*	*L. guyan.*	*L. killicki*	*L. major*	*L. donovani*	*L. guyane n.*	*L. killicki*
(1) caffeic acid	n.d.	117	106	117	100	2	19	25	22	19	6	*5*	*8*	*9*
(**2**) rosmarinic acid	n.d.	100	100	110	92	2	14	10	10	12	n.d.	n.d.	n.d.	n.d.
(**3**) salvianolic acid I	5	40	44	37	31	> 1	35	39	36	42	n.d.	n.d.	n.d.	n.d.
(**4**) methyl ester	4	26	22	29	19	1	9	6	5	4	n.d.	n.d.	n.d.	n.d.
(**5**) salvianolic acid K	n.d.	70	70	83	78	1	18	27	17	22	2	3	3	3
(**6**) salvianolic acid L	n.d.	92	87	83	87	6	35	35	32	34	n.d.	n.d.	n.d.	n.d.
(**7**) sagerinic acid	> 1	36	35	31	31	4	4	4	3	5	n.d.	n.d.	n.d.	n.d.
IFN (100 U/ml) + LPS (10 ng/ml)	218	206	212	215	200	8	14	14	12	16	n.d.	n.d.	n.d.	n.d.

[a] U/ml, calculated as the reciprocal of the values of the macrophage supernatant dilution that would cause 50% maximal lysis of L929 fibroblasts.
[b] U/ml, calculated as the reciprocal of the values of the macrophage supernatant dilution that would cause 50% maximal proliferation of B9 hybridoma cells.
[c] U/ml, calculated as the reciprocal of the values of the macrophage supernatant dilution that would cause 50% maximal inhibition of the cytopathic effects of EMC virus on L929 fibroblasts; for non-infected RAW cells, IFN activity was below detection level.
n.d., not detectable; below detection limit.

in vitro antileishmanial activity and TNF-inducing potential of caffeic acid (**1**) is worth noting. Furthermore, that (**1**) displayed such potencies demonstrates that interesting biological activities are not confined to exotic molecules and that simple known compounds are still attractive from a therapeutic point of view. In view of the abundance and possible medicinal properties of plant polyphenols, this field deserves continuing studies to ascertain any actual therapeutic benefits in humans.

Alvar J., Canavata C., Gutierrez-Solar B., Jimenez M., Laguna F., Lopez-Velz R., Molina R., and Moreno J. (1997), *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin. Microbiol. Rev. **10**, 298–318.

Ashford R. W., Desjour P., and DeRaadt P. (1992), Estimation of population at risk of infection and number of cases of leishmaniasis. Parasitol. Today **8**, 104–105.

Beutler B., and Cerami A. (1989), The biology of cachectin/TNF: a primary mediator of host response against a human tumour necrosis factor-α receptor. Annu. Rev. Immunol. **7**, 625–655.

Billiau A. (1995), Interferon-γ:biology and role in pathogenesis. Adv. Immunol. **62**, 61–130.

Gerhardt U., and Schroeter A. (1983), Rosmarinic acid – a naturally occurring antioxidant in spices. Fleischwirtschaft **63**, 1628–1630.

Kayser O., Kiderlen A. F., Folkens U., and Kolodziej H. (1999), *In vitro* leishmanicidal activity of aurones. Planta Med. **65**, 316–319.

Kiderlen A. F., and Kaye P. M. (1990), A modified colorimetric assay of macrophage activation for intracellular cytotoxicity against *Leishmania* parasites. J. Immunol. Methods **127**, 11–18.

Kiderlen A. F., Kayser O., Ferreira D., and Kolodziej H. (2001), Tannins and related compounds: killing of amastigotes of *Leishmania donovani* and release of nitric oxide and tumour necrosis factor α in macrophages *in vitro*. Z. Naturforsch. **56 c**, 444–454.

Kolodziej H., Kayser O., Kiderlen A. F., Ito H., Hatano T., Yoshida T., and Foo L. Y. (2001), Proanthocyanidins and related compounds: antileishmanial activity and modulatory effects on nitric oxide and tumor necrosis factor-α-release in the murine macrophage-like cell line RAW 264.7. Biol. Pharm. Bull. **24**, 1016–1021.

Kolodziej H., Kayser O., Kiderlen A. F., Ito H., Hatano T., Yoshida T., and Foo L. Y. (2001), Antileishmanial activity of hydrolyzable tannins and their modulatory effects on nitric oxide and tumour necrosis factor-α release in macrophages *in vitro*. Planta Med. **67**, 825–832.

Lu Y., and Foo L. Y. (1999), Rosmarinic acid derivatives from *Salvia officinalis*. Phytochemistry **51**, 91–94.

Lu Y., and Foo L. Y. (2002), Polyphenolics of *Salvia* – a review. Phytochemistry **59**, 117–140.

Lu Y., Wong H., and Foo L. Y. (1999), Sagecoumarin, a novel caffeic acid trimer from *Salvia officinalis*. Phytochemistry **52**, 1149–1152.

Radtke O. A., Kiderlen A. F., Kayser O., and Kolodziej H. (2001), Cytokine gene expression in *Leishmania major*-infected macrophage-like RAW cells treated with gallic acid. Intern. Symp. on Immunomodulation by Parasites, Berlin, Germany, Abstract P3.

Théze J. (1999), The Cytokine Network and Immune Functions. Oxford University Press, Oxford.