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Original article

Anti-neuroinflammatory properties of synthetic cryptolepine in human neuroblastoma cells: Possible involvement of NF-κB and p38 MAPK inhibition

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ABSTRACT

Cryptolepis sanguinolenta and its bioactive alkaloid, cryptolepine have shown anti-inflammatory activity. However, the underlying mechanism of anti-inflammatory action in neuronal cells has not been investigated. In the present study we evaluated an extract of *C. sanguinolenta* (CSE) and cryptolepine (CAS) on neuroinflammation induced with IL-1β in SK-N-SH neuroblastoma cells. We then attempted to elucidate the mechanisms underlying the anti-neuroinflammatory effects of CAS in SK-N-SH cells. Cells were stimulated with 10 U/ml of IL-1β in the presence or absence of different concentrations of CSE (25–200 μg/ml) and CAS (2.5–20 μM). After 24 h incubation, culture media were collected to measure the production of PGE_2 and the pro-inflammatory cytokines (TNFα and IL-6). Protein and gene expressions of cyclooxygenase (COX-2) and microsomal prostaglandin synthase-1 (mPGES-1) were studied by immunoblotting and qPCR, respectively. CSE produced significant ($p < 0.05$) inhibition of TNFα, IL-6 and PGE_2 production in SK-N-SH cells. Studies on CAS showed significant and dose-dependent inhibition of TNFα, IL-6 and PGE_2 production in IL-1β-stimulated cells without affecting viability. Pre-treatment with CAS (10 and 20 μM) was also found to inhibit IL-1β-induced protein and gene expressions of COX-2 and mPGES-1. Further studies to determine the mechanism of action of CAS showed inhibition of NF-κBp65 nuclear translocation, but not IκB phosphorylation. At 10 and 20 μM, CAS inhibited IL-1β-induced phosphorylation of p38 MAPK. Studies on the downstream substrate of p38, MAPK-activated protein kinase 2 (MAPKAPK2) showed that CAS produced significant ($p < 0.05$) and dose dependent inhibition of MAPKAPK2 phosphorylation in IL-1β-stimulated SK-N-SH cells. This study clearly shows that cryptolepine (CAS) inhibits neuroinflammation through mechanisms involving inhibition of COX-2 and mPGES-1. It is suggested that these actions are probably mediated through NF-κB and p38 signalling.

1. Introduction

Neuroinflammation is a critical aspect of neurodegenerative diseases. A wide variety of inflammatory mediators contribute to the mechanisms involved in neuroinflammation. Prostaglandin (PG) E_2 is an inflammatory mediator which plays an important role in neuroinflammation and the inflammatory process involved in neurodegenerative disorders like Alzheimer's disease [1]. In the brain, PGE_2 can be synthesised by two isoforms of the cyclooxygenase (COX), named COX-1 and COX-2 [2]. However, COX-2, but not COX-1, has been shown to be induced through inflammatory cytokines [3] and in various neurodegenerative disorders [4]. Microsomal PGES-1 (mPGES-1) is an inducible enzyme that has been shown to be linked with COX-2. COX-2 and mPGES-1 are both regulated at the transcription levels and both enzymes are essential for the synthesis of PGE_2 during inflammation [5].

The regulation of these enzymes might be controlled by different intracellular signalling pathways, such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK).

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NF-κB is a transcription factor which has been considered to be the critical central regulator of the inflammatory process. The transcription factor regulates gene expressions of the pro-inflammatory cytokines (IL-6, TNFα, β, IL-2 etc.), chemokines (IL-8, MCP-1, RANTES, etc.), adhesion molecules (ICAM, VCAM, E-selectin), inducible enzymes (COX-2 and iNOS), growth factors, some acute phase proteins and immune receptors [6]. NF-κB has therefore become a critical molecular target in drug discovery, and several natural and synthetic compounds are currently being investigated for their potential to inhibit NF-κB [7].

MAPKs are intracellular enzymes which make it possible for cells to respond to stimuli such as inflammatory cytokines, from their extracellular environment [8]. This group of kinases includes extracellular signal-regulated kinases (ERK 1/2), c-Jun N-terminal kinases (JNK) and p38 isoforms. Importantly, p38 has been closely associated with pathologies involved in neurodegenerative disorders [9,10]. Several studies have demonstrated roles for p38 MAPK in microglia- and astrocyte-driven neuroinflammation. Evidence suggests that patients with neurodegenerative disorders like Alzheimer's disease might benefit from p38 MAPK inhibitors [8]. Therefore, there is a need to develop new compounds targeting this kinase. MAPK-activated protein kinase 2 (MAPKAPK2) is a kinase which acts as a substrate for the action of p38. It has been linked to microglial cell activation and microglial-mediated neuronal cell death [11]. It is therefore important to determine involvement of MAPKAPK2 in the action of novel pharmacological agents on p38 during neuroinflammation.

The human neuroblastoma (SK-N-SH) cells are significantly similar to primary neuronal cells and are therefore useful in investigating potential compounds for neurodegenerative disorders. Fiebich et al. [3] have shown that interleukin (IL)-1β induces COX-2 mRNA and protein synthesis and the release of PGE_2 in the SK-N-SH cell line. They further demonstrated that both a free radical scavenger and an inhibitor of p38 MAPK reduce IL-1β-induced synthesis of COX-2 in these cells.

Cryptolepis sanguinolenta (Family Asclepiadaceae) is an herb that is employed extensively in Traditional African Medicine for malaria and other infectious diseases, pain and inflammation [12]. Cryptolepine (5-methyl,10*H*-indolo[3,2-*b*]quinoline) (Fig.1) is an indoloquinoline bioactive alkaloid in *C. sanguinolenta*. Earlier, we showed that this compound produces *in vitro* and *in vivo* anti-inflammatory effects [13,14].

In an attempt at establishing the mechanisms of anti-inflammatory potential of *C. sanguinolenta*, we showed that an extract of the plant (CSE) inhibited cytokine and PGE_2 production from IL-1β-stimulated SK-N-SH cells. Experiments conducted to further understand the anti-inflammatory action of *C. sanguinolenta* show that the synthetic form of its active component, cryptolepine (CAS) reduced cytokine and PGE_2 production from SK-N-SH neuronal cells and inhibited COX-2 and mPGES-1 protein and gene expressions in SK-N-SH cells. Here we demonstrated that these anti-neuroinflammatory effects may be dependent on NF-κB and p38 inhibition.

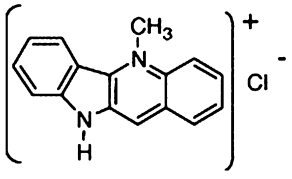

Fig. 1. Cryptolepine hydrochloride.

2. Results

2.1. CSE reduced PGE_2 and cytokine production from IL-1β-stimulated SK-N-SH cells

Initially, we evaluated the effects of CSE on the production of PGE_2, TNFα and IL-6 in IL-1β-stimulated SK-N-SH cells. Results show that CSE produced significant ($p < 0.05$) and dose-related inhibition of PGE_2 production from SK-N-SH cells (Fig. 2C). Fig. 2A and B shows that IL-1β caused marked increase in TNFα and IL-6 production in SK-N-SH cells. However, pre-treatment with CSE resulted in significant ($p < 0.05$) and dose-related reductions in both cytokines. ATP assay for cell viability showed that none of the concentrations of CSE used produced cytotoxicity to SK-N-SH cells following incubation for 24 h (data not shown).

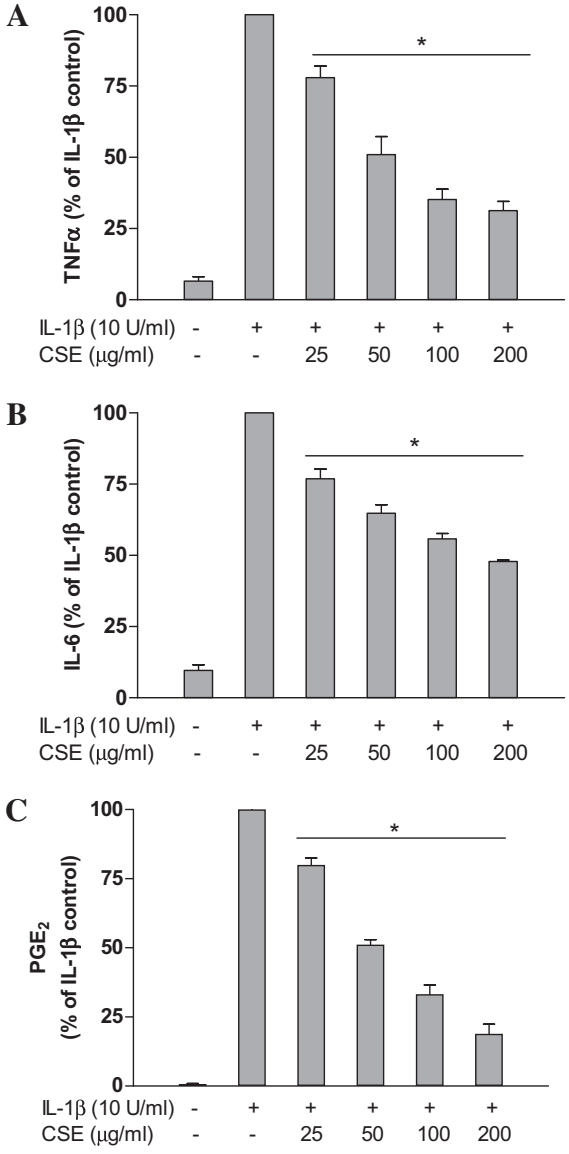

Fig. 2. Effect of CSE on cytokine and PGE_2 production from SK-N-SH cells. CSE reduced TNFα (A), IL-6 (B) and PGE_2 (C) production in IL-1β-stimulated SKN-SH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CSE (25–200 μg/ml) for 24 h. At the end of the incubation period, supernatants were collected for TNFα, IL-6 and PGE_2 measurements. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. *$p < 0.05$ in comparison with IL-1β control.

2.2. CAS reduced PGE_2 and cytokine production from IL-1β-stimulated SK-N-SH cells

Having established anti-neuroinflammatory effects with CSE, we next evaluated CAS, the bioactive compound in CSE, for effects on PGE_2 production in IL-1β-stimulated SK-N-SH cells. PGE_2 production was not detectable in unstimulated cells. However, treatment with IL-1β caused marked secretion of PGE_2 which was significantly ($p < 0.05$) reduced in a dose-dependent fashion by 2.5–20 μM of CAS (Fig. 3C).

We also investigated whether CAS inhibits the production of pro-inflammatory cytokines (TNFα and IL-6) in IL-1β-stimulated SK-N-SH cells. CAS significantly ($p < 0.05$) inhibited the production of TNFα and IL-6 (Fig. 3A and B). ATP assay for cell viability showed that none of the concentrations of CAS used produced cytotoxicity to SK-N-SH cells following incubation for 24 h (data not shown).

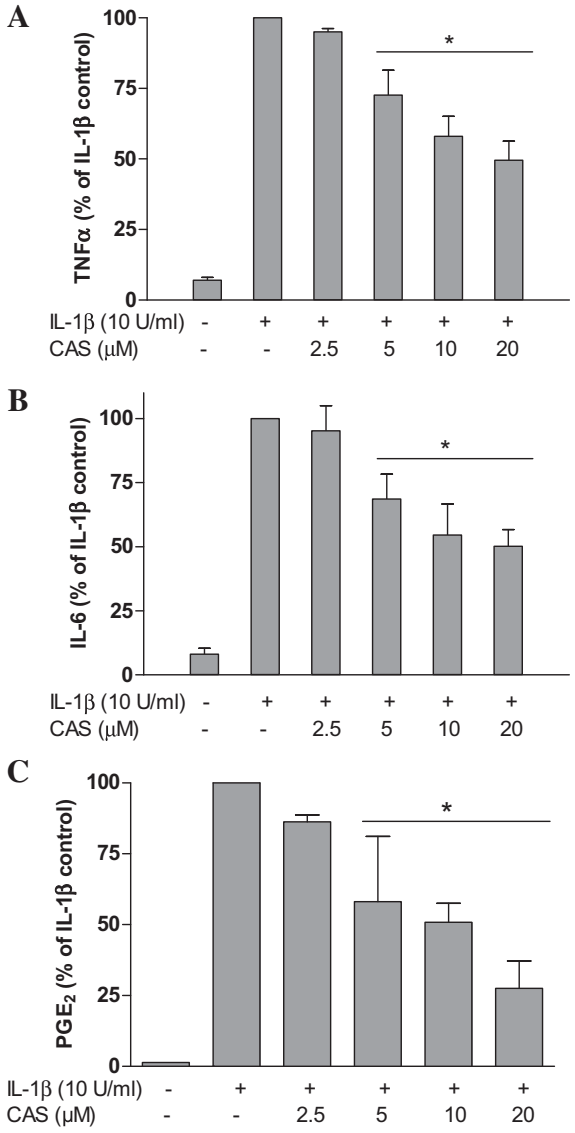

Fig. 3. Effect of CAS on cytokine and PGE_2 production from SK-N-SH cells. CAS reduced TNFα (A), IL-6 (B) and PGE2 (C) production in IL-1β-stimulated SKN-SH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 24 h. At the end of the incubation period, supernatants were collected for TNFα, IL-6 and PGE2 measurements. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. *$p < 0.05$ in comparison with IL-1β control.

2.3. Inhibition of PGE_2 synthesis by CAS is mediated by reduction of COX-2 protein and gene expressions

The neuronal induction of COX-2 has been linked to neuroinflammatory aspects of Alzheimer's disease (AD) [3]. We therefore investigated if the effect of CAS on PGE_2 production in SK-N-SH cells is due to inhibition of COX-2 protein and gene expressions. COX-2 protein was not detectable in unstimulated controls, whereas stimulation of SK-N-SH cells with IL-1β induced the synthesis of COX-2 proteins (Fig. 4A and B) and genes (Fig. 5). At 2.5 μM of CAS, there was no reduction in COX-2 protein expression; results show a slight increase in COX-2, when compared with IL-1β control. However, statistically significant ($p < 0.05$) inhibition of COX-2 protein expression was exhibited at 5–20 μM of CAS. We observed a slightly different outcome with COX-2 gene expression experiment. In this case, statistical significant inhibition of COX-2 gene in IL-1β-stimulated SK-N-SH cells was evident with only 10 and 20 μM of CAS. These results suggest that higher concentrations of CAS are effective in reducing PGE_2 production through the regulation of the gene transcriptional levels of COX-2 in stimulated neuronal cells.

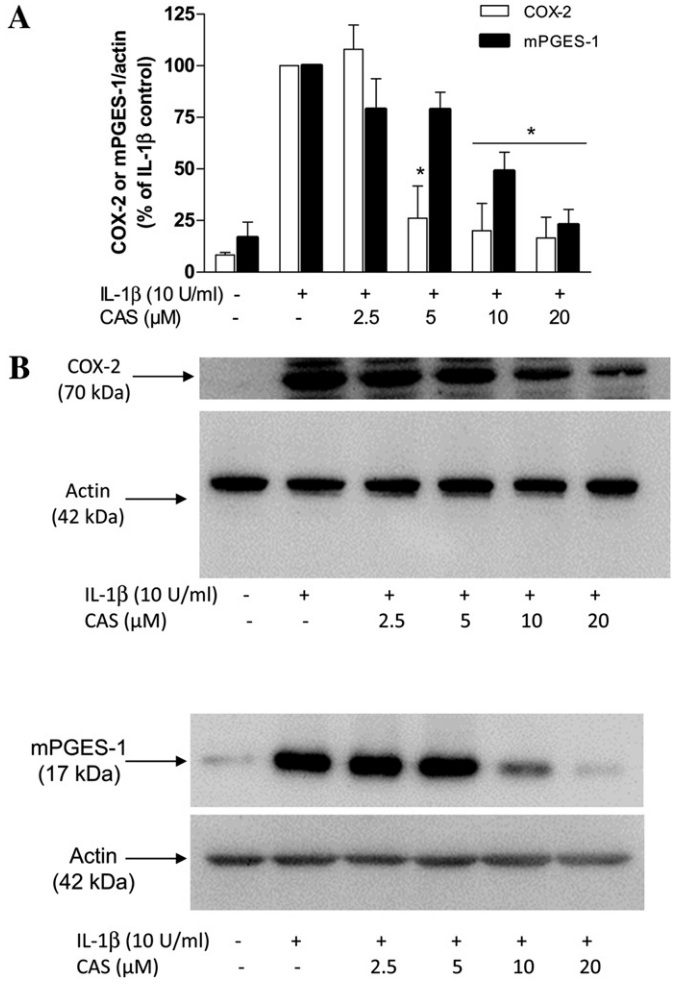

Fig. 4. Effects of CAS on COX-2 and mPGES-1 immunoreactivity. CAS inhibited COX-2 and mPGES-1 protein expressions in IL-1β-stimulated SK-NSH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 24 h. At the end of incubation period, COX-2 and mPGES-1 protein expressions were determined using western blot with specific anti-COX-2 and anti-mPGES-1 antibodies. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. *$p < 0.05$; in comparison with IL-1β control.

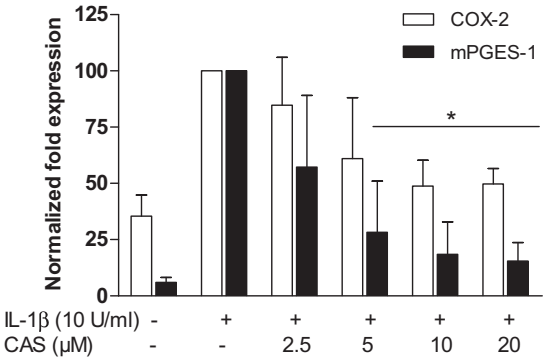

Fig. 5. Effect of CAS on COX-2 and mPGES-1 gene expressions. CAS inhibited COX-2 and mPGES-1 gene expressions in IL-1β-stimulated SK-NSH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 4 h. At the end of incubation period total RNA was extracted and subjected to RTPCR. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. $^{*}p < 0.05$; in comparison with IL-1β control.

2.4. CAS inhibited of mPGES-1 expression IL-1β-stimulated SK-N-SH cells

It is widely known that mPGES-1 is involved in regulating COX-2-dependent PGE_2 synthesis and that transcriptional regulation of COX-2 and mPGES-1 are essential for the synthesis of PGE_2 during inflammation. Consequently, we investigated whether the observed effect of CAS on PGE_2 is related to inhibition of mPGES-1 protein and gene expressions. Stimulation of SK-N-SH cells with IL-1β resulted in marked expression of mPGES-1 protein and gene (Figs. 4A, B and 5). Pre-treatment with 2.5 μM CAS did not have an effect on either mPGES-1 protein or gene, while 5 μM of the compound produced significant effect on gene, but not protein expression. However, with 10 and 20 μM of CAS, there were significant inhibitions of mPGES-1 at the protein and transcriptional levels.

2.5. CAS inhibited NF-κBp65 subunit translocation, but not IκB phosphorylation

We also investigated whether CAS acts to block the activation of the NF-κB pathway, which has been shown to be the critical factor in the transcriptional regulation of inflammatory mediators in IL-1β-stimulated SK-N-SH cells [15]. CAS dose-dependently inhibited IL-1β-induced translocation of the NF-κB subunit into the nucleus (Fig. 6B), indicating a potential role of NF-κB in the mechanism of CAS suppressing PGE_2 and pro-inflammatory cytokines in activated SK-N-SH cells. Strangely, inhibition of IL-1β-induced IκB phosphorylation was not inhibited by CAS (Fig. 6A).

2.6. CAS inhibited phosphorylation of p38 MAPK and MAPKAPK2

Based on the weak effects of CAS on IκB phosphorylation, we were interested to know if its effect on NF-κB might be linked to inhibition of p38 MAPK phosphorylation. p38 has been shown to be part of the intracellular signal transduction pathways leading to IL-1β-induced expression of COX-2 [15]. We found that 10 and 20 μM of CAS significantly ($p < 0.05$) inhibited IL-1β-induced phosphorylation of p38 MAPK in SK-N-SH cells. This result suggests that inhibition of p38 MAPK contributes to transcriptional inhibition of pro-inflammatory proteins by CAS (Fig. 7A and B).

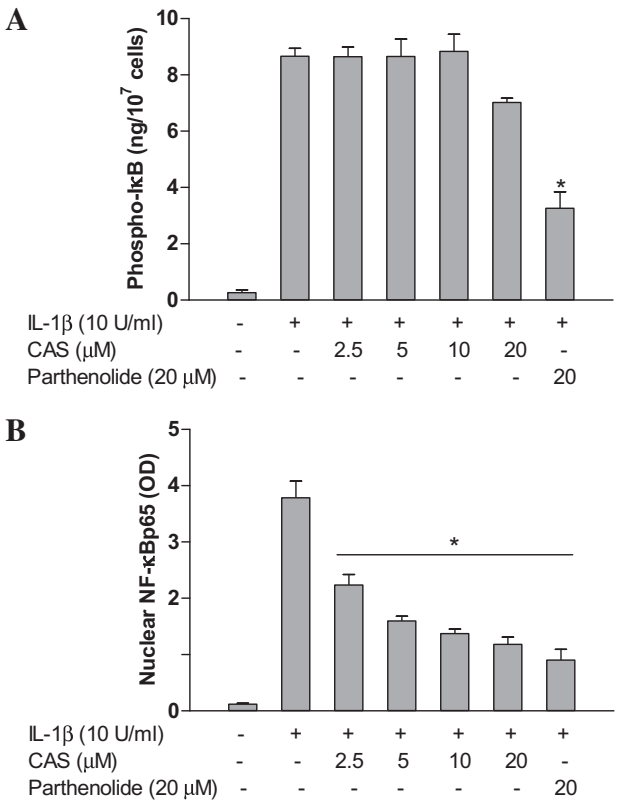

Fig. 6. Effect of CAS on IκB phosphorylation and NF-κBp65 nuclear translocation induced by IL-1β. CAS inhibited IL-1β-induced nuclear translocation of NF-kB (B) but not IkB phosphorylation (A) in SK-N-SH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 15 min. Cell nuclear extracts were then analysed for total NF-kBp65 using NF-κBp65 ELISA kit. All values are expressed as mean ± SEM for 3 independent experiments. Optical densities were measured at 450 nm with a microplate reader. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. $^{*}p < 0.05$ in comparison with IL-1β control.

We further investigated whether or not CAS inhibit the phosphorylation of MAPKAPK2, which is the important downstream enzyme substrate of p38 in the production of pro-inflammatory factors. CAS (2.5–20 μM) produced significant and dose-dependent inhibition of MAPKAPK2 phosphorylation induced by IL-1β (Fig. 8A and B).

3. Discussion

In the present study, it has been shown that *C. sanguinolenta* and CAS inhibit inflammatory activation of SK-N-SH neuronal cells. In order to establish this, we stimulated SK-N-SH neuronal cells with IL-1β to induce production of inflammatory mediators. It has been shown that IL-1β induced COX-2 expression and PGE_2 secretion in SK-N-SH cells and suggested that there is interplay between glial derived IL-1β and neuronal upregulation of COX-2 expression in neurodegenerative diseases such as AD [16].

C. sanguinoleta root extract significantly reduced PGE_2 and cytokine production in IL-1β-stimulated SK-N-SH cells. These observations seem to justify the use of the plant in treating inflammatory disorders. A major challenge in understanding the therapeutic potentials of plant extracts usually relates to lack of information on the pharmacological profile of its bioactive substances. Consequently, we investigated a semi-synthetic bioactive alkaloid *of C. sanguinolenta*-cryptolepine, in IL-1β-stimulated SK-N-SH cells.

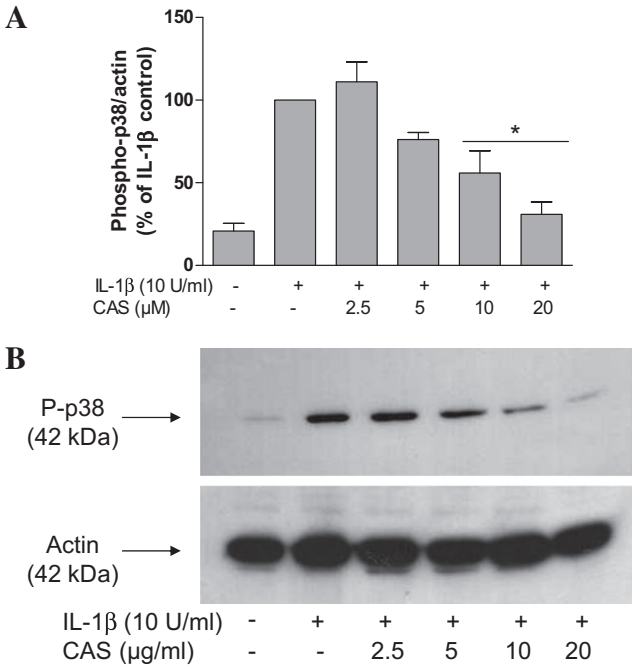

Fig. 7. Effects of CAS on p38 phosphorylation induced by IL-1β. CAS inhibited p38 phosphorylation in IL-1β-stimulated SK-N-SH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 1 h. At the end of incubation period, phospho-p38 protein expressions were determined using western blot with specific anti-phospho-p38 antibody. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. $^{*}p < 0.05$; in comparison with IL-1β control.

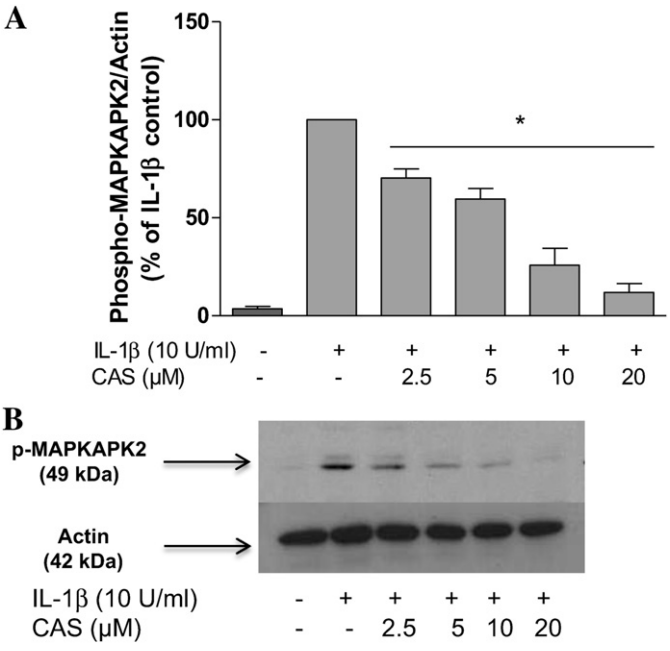

Fig. 8. Effects of CAS on MAPKAPK2 phosphorylation induced by IL-1β. CAS inhibited MAPKAPK2 phosphorylation in IL-1β-stimulated SK-N-SH cells. Cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 1 h. At the end of incubation period, phospho-p38 protein expressions were determined using western blot with specific anti-phospho-MAPKAPK2 antibody. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman–Keuls test. $^{*}p < 0.05$; in comparison with IL-1β control.

CAS significantly reduced PGE_2, TNFα and IL-6 production from IL-1β-stimulated SK-N-SH cells. We showed that CAS inhibits LPS-mediated NO production in RAW 264.7 macrophages [13]. Our group and other workers have shown that cryptolepine exhibits *in vivo* anti-inflammatory effects through the inhibition of the carrageenan-induced paw oedema and pleurisy in rats [14,17]. Results obtained from this study have added to existing observations on the anti-inflammatory effect of cryptolepine.

Experiments conducted to gain a better understanding of the mechanism of action of the anti-inflammatory actions of cryptolepine showed that the alkaloid produced anti-neuroinflammatory effects in IL-1β-stimulated neuronal cells by inhibiting COX-2 and mPGES-1 protein and gene expressions. Studies by Fiebich et al. [3] and Hoozemans et al. [16] showed that the IL-1β induced PGE2 secretion by SK-N-SH cells is mediated by COX-2. It can therefore be concluded that the observed reduction of PGE_2 production by cryptolepine is mediated through its inhibitory effect on COX-2 expression, albeit an inhibitory effect in mPGES-1 expression might also be important.

The inducible prostaglandin E synthase (mPGES-1) is an enzyme downstream of COX-2 in the biosynthesis of PGE_2. We investigated whether the reduction of PGE_2 by cryptolepine might also be related to an action on this enzyme in neuronal cells. Our results show that cryptolepine inhibited mPGES-1 protein and gene expressions, suggesting that the alkaloid possibly causes reduction in PGE_2 production by targeting both COX-2 and mPGES-1.

NF-κB has been shown to be one of the most important upstream modulators for pro-inflammatory cytokines and COX-2 in many inflammatory cells. NF-κB activation has also been demonstrated in SK-N-SH cells stimulated with IL-1β [3]. We have earlier shown that cryptolepine blocked DNA binding of activated NF-κB and transcription of NF-κB-regulated pro-inflammatory protein in RAW 264.7 macrophages [13]. In this study, we have shown that cryptolepine significantly inhibited translocation of the p65 subunit, thereby confirming the earlier observation in RAW cells. Similar to our observation with RAW cells, cryptolepine failed to prevent phosphorylation of IκB in SK-N-SH cells.

Different MAPK might be involved in the regulation of COX-2 and mPGES-1 expressions induced by inflammatory stimuli [18,19]. In this sense, it has been shown that p38 contributes to COX-2 gene expression and mRNA stability [20]. Despite this, evidences have suggested that the p38 MAPK signalling plays a critical role in neuroinflammation and neurodegenerative disorders like AD [8]. We therefore investigated the possible involvement of this MAPK in the anti-neuroinflammatory activity of cryptolepine. Cryptolepine produced significant and dose-related inhibition of p38 phosphorylation in SK-N-SH cells stimulated with IL-1β, indicating that the compound targets p38 signalling in neuronal cells. This inhibition might be important for reduction of COX-2 protein stabilization. Moreover, Fiebich et al. [15] reported that the IL-1β-induced NF-κB activation is independent of p38 MAPK, suggesting that most likely two separate signal transduction cascades are involved in IL-1β-regulated COX-2 synthesis in SK-N-SH cells. It is therefore suggested that cryptolepine possibly acts by targeting both NF-κB and p38 to mediate IL-1β-induced COX-2 expression in SK-N-SH cells. We further investigated the role of MAPKAPK2 in the action of CAS and showed a dose-related inhibition of its phosphorylation, suggesting that the compound inhibited p38-mediated phosphorylation of this kinase in IL-1β-stimulated SK-N-SH cells.

Our results suggest that cryptolepine produces anti-neuroinflammatory effects in neuronal cells by targeting NF-κB and p38 mediated PGE_2/COX-2 synthesis. It is not clear yet, how cryptolepine inhibits NF-κB activity independent of IκB. These data also seem to indicate that cryptolepine might serve as a potential

template for the design of novel compounds in neurodegenerative disorders.

4. Materials and methods

4.1. Cell culture

SK-N-SH cells were obtained from the American Type Culture Collection (HTB-11, Rockville, USA) and were grown in MEM-Eagle's medium (PAA, Cölbe, Germany), which does not contain any anti-inflammatory substance. Medium was supplemented with 5% foetal calf serum (PAN, Aidenbach, Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, 40 units/ml penicillin/streptomycin (PAA Laboratories, Cölbe, Germany), 0.4% MEM vitamins and 0.4% MEM nonessential amino acids (both purchased from Invitrogen GmbH, Karlsruhe, Germany). Confluent monolayers were passaged routinely by trypsinisation. Cultures were grown at 37 °C in 5% CO_2 until 80% confluence, and the medium was changed the day before treatment.

4.2. Extraction of C. sanguinolenta

Dried powdered roots of *C. sanguinolenta* were extracted with methanol three times by shaking at room temperature for 15–30 min. The resulting extracts were combined, filtered, and evaporated to dryness under reduced pressure (50 °C) using a rotary evaporator.

4.3. Synthesis of cryptolepine

Cryptolepine (in the hydrochloride salt form) was synthesised by methylation of quindoline as described earlier [13] and based on the methodology of Holt and Petrow [21]. Spectral data for cryptolepine (^{1}H and ^{13}C NMR) were identical to published values [12]. Purity was confirmed by means of NMR spectroscopy. The alkaloid was prepared in sterile water for biological studies and kept at −20 °C.

4.4. PGE_2 determination in cell supernatants: enzyme immunoassay (EIA)

SK-N-SH cells were pre-treated for 30 min with CSE (25–200 μg/ml) and CAS (2.5–20 μM). After pre-stimulation, cells were incubated with 10 U/ml IL-1β for 24 h. Supernatants were then centrifuged at 10,000 *g* for 10 min and levels of PGE_2 in the media were measured by enzyme immunoassay (EIA) (Assay Designs Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions. Standards from 39 to 2500 pg/ml were used.

4.5. Determination of cytokine production (TNF-α and IL-6) in cell supernatants

SK-N-SH cells were pre-treated for 30 min with CSE (25–200 μg/ml) and CAS (2.5–20 μM). After pre-stimulation, cells were incubated with 10 U/ml IL-1β for 24 h. The concentrations of tumour necrosis factor (TNF)-α and interleukin (IL)-6 released into the media were measured by a specific TNF-α and IL-6 ELISA kits following manufacturer's recommendations (Biolegend, distributed by Cambridge Biosciences, UK).

4.6. Immunoblotting

In order to further establish the mechanisms involved in the anti-inflammatory action of *C. sanguinoleta* in the CNS, the effects of its active component cryptolepine were investigated on protein expressions of key inflammatory targets. For COX-2 and mPGES-1 immunoblotting, SK-N-SH cells were left untreated or treated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 24 h. For phospho-p38 MAPK and phospho-MAPKAPK2 western blots, cells were pre-treated for 30 min with CAS (2.5–20 μM) and then stimulated for 1 h with IL-1β (10 U/ml). At the end of each experiment, cells were washed with phosphate-buffered saline (PBS) and lysed in 1.3× sodium dodecyl sulphate (SDS)-containing sample buffer without 1,4-dithio-DL-threitol (DTT) or bromophenol blue containing 100 μM orthovanadate. Protein contents were measured using the bicinchoninic acid method (BCA protein determination kit from Pierce, distributed by KFC (Chemikalien, Munich, Germany) according to the manufacturer's instructions. Bovine serum albumin (BSA, Sigma) was used as a standard. Before electrophoresis, bromophenol blue and DTT (final concentration, 10 mM) were added to the samples. For Western blotting, 40 μg of total protein from each sample was subjected to SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by semi-dry blotting. The membranes were blocked overnight at 4 °C using Rotiblock (Roth, Karlsruhe, Germany) and for another hour at room temperature before incubation with the antibodies. Primary antibodies were goat anti-COX-2 (1:500), rabbit anti-p38 (1:500), rabbit anti-mPGES-1 (Cayman, 1:500), rabbit anti-phospho-p38, rabbit anti-phospho-MAPKAPK2 (Assay Biotech, 1:1000) and rabbit anti-actin (Sigma, 1:5000). Primary antibodies were diluted in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 1% BSA. Membranes were incubated with the corresponding primary antibody for 2 h at room temperature. After extensive washing (three times for 15 min each in TBS-T), proteins were detected with horseradish peroxidase-coupled rabbit anti-goat IgG (Santa Cruz, 1:100,000 dilution) or goat anti-rabbit IgG (Amersham, 1:25,000 dilution) using chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Freiburg, Germany). Equal protein loading and transfer were assessed by subjection of each sample to a Western blot for actin (rabbit anti-actin IgG, diluted 1:5000. All Western blot experiments were carried out at least three times.

4.7. NF-κBp65 nuclear translocation

SK-N-SH cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 15 min. After the stimulation period, nuclear extracts were prepared using Cayman nuclear extraction kit (Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions. Briefly, cells were collected by scraping and washed twice with cold PBS. Cells were centrifuged for 5 min at 4 °C, the supernatants discarded and cell pellet re-suspended in 5 ml of ice-cold PBS. The centrifugation procedure was repeated twice and the cell pellets placed on ice and allowed to swell in 500 μl of 1× hypotonic buffer, followed by addition of 10% NP-40 with gentle mixing. The suspension was centrifuged and the supernatants which contained the cytosolic fractions were stored at −80 °C for subsequent analysis of cytoplasmic NF-κB. The pellet was re-suspended in 100 μl of ice-cold Complete Nuclear Extraction Buffer, vortexed and rocked gently for 15 min. The samples were then centrifuged and the supernatants (nuclear fractions) were collected. Cytoplasmic and nuclear fractions were measured for levels of NF-κB using human NF-κBp65 ELISA kit (Invitrogen, California, USA), according to the manufacturer's instructions. Parthenolide (20 μM), a compound that inhibits NF-κB activity, was used as a positive control.

4.8. Determination of IκB phosphorylation

SK-N-SH cells were stimulated with IL-1β (10 U/ml) in the presence or absence of CAS (2.5–20 μM) for 15 min. After the stimulation period, nuclear extracts were prepared using Cayman nuclear extraction kit (Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions. Briefly, cells were collected by scraping and washed twice with cold PBS. Cells were centrifuged for 5 min at 4 °C, the supernatant discarded and cell pellet re-suspended in 5 ml of ice-cold PBS. The centrifugation procedure was repeated twice and the cell pellets placed on ice and allowed to swell in 500 μl of 1× hypotonic buffer, followed by addition of 10% NP-40 with gentle mixing. The suspension was centrifuged and the supernatants which contained the cytosolic fractions were stored at −80 °C for subsequent analysis of cytoplasmic NF-κB. The pellet was re-suspended in 100 μl of ice-cold Complete Nuclear Extraction Buffer, vortexed and rocked gently for 15 min. The samples were then centrifuged and the supernatants (nuclear fractions) were collected. Cytoplasmic and nuclear fractions were measured for levels of NF-κB using NF-κBp65 ELISA kit (Cell Signalling Technology, Inc), according to the manufacturer's instructions. Parthenolide (20 μM) was used as a positive control.

4.9. qPCR for COX-2 and mPGES-1

Cells were pre-incubated for 30 min with CAS at different concentrations (2.5–20 μM), subsequently IL-1β (10 U/ml) was added for total 4 h. RNA preparation was done by using RNAspin mini RNA isolation kit (GE Healthcare, Freiburg, Germany) and for cDNA synthesis 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase and random hexamers (Promega, Mannheim, Germany). The synthesised cDNA was the template for the real-time polymerase chain reaction (PCR) amplification which was carried out by the CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc.) using iQ™ SYBR™ Green supermix (Bio-Rad Laboratories GmbH, Munich, Germany). Specific primers were designed by using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) program and primers were obtained from Biomers (Ulm, Germany). Reaction conditions were 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 50 °C, and 45 s at 72 °C and every cycle was followed by plate reading. After that 1 min at 95 °C, 1 min at 55 °C, followed by melt curve conditions of 65–95 °C with increment of 0.5 °C for 5 s followed by final plate reading. GAPDH served as an internal control for sample normalisation and the comparative cycle threshold Ct method was used for data quantification as described previously [22]. The following Primer sequences were used in the present study. COX-2: Fwd 5′-GCCCAGCACTTCACGCATCAGT-3′; Rev 5′-AAGTCCACCCCATGGCCCAGC-3′. mPGES-1: Fwd 5′-TGCAGCACGCTGCTGGTCAT-3′; Reverse 5′-GGCAAAGGCCTTCTTCCGCAG-3′. GAPDH: Fwd 5′-GTCGCCAGCCGAGCCACATC-3′; Reverse: 5′-CCAGGCGCCCAATACGACCA-3′.

4.10. Statistical analysis

Data from at least three independent experiments were used for analysis. Original data were converted into percentage-values of IL-1β control and mean ± SEM were calculated. Values were compared using *t*-test (two groups) or one-way ANOVA with post-hoc Student–Newman–Keuls test (multiple comparisons).

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