Immunomodulatory activity of cucurbitacins from *Picrorhiza scrophulariiflora*

8.1 Immunomodulatory effects on T lymphocytes and monocytes *in vitro*

8.2 Immunomodulatory effects *in vivo*

Chapter 8
Introduction

In the previous chapter, the isolation of two bioactive cucurbitacins from the rhizomes of *Picrorhiza scrophulariiflora* was described. Both compounds potently inhibited mitogen-induced proliferation of T cells. In this chapter, experiments are delineated that pertain to the underlying mechanisms of this immunomodulatory effect.

As described in chapter 5, the activation of T cells requires signal transduction that leads to the release of IL-2 and the expression of IL-2 receptor (IL-2R), ultimately leading to proliferation. In normal situations, cell proliferation and cell death are in balance. However, autoimmune diseases are associated with an increased survival of potentially autoreactive T lymphocytes. Activated T lymphocytes that are differentiated into pro-inflammatory effector cells release cytokines, which sustain cell-mediated immune responses (Swain, 1999). Within the articular synovium, these T cells activate synovial macrophages that produce a range of pro-inflammatory cytokines, which induce proliferation of synoviocytes, enhancing the local inflammatory process. These involve lymphocytes, chondrocytes, fibroblasts, and osteoclasts, inducing damage of bone, cartilage, and other connective tissue components (Feldmann *et al.*, 1996, Vaishnaw, *et al.*, 1997, Starkebaum, 1998).

Therapeutic measures of rheumatoid arthritis (RA) often focus on immunomodulatory activity, by means of nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, or disease-modifying antirheumatic drugs (DMARDs; AMR, 1996a). While NSAIDs only afford symptomatic relief, without altering the course of the disease or preventing joint damage, glucocorticoids and DMARDs interfere with the release of pro-inflammatory cytokines. As such, they have the potential to reduce and prevent joint damage and to preserve joint integrity and function (Shevach, 1995, Cronstein, 1996). A major disadvantage of these drugs, however, is their toxicity, which is usually proportional to their efficacy (AMR, 1996b, Jackson and Williams, 1998). These side effects imply an ongoing search for lead compounds that are able to selectively interfere with pathological processes underlying chronic inflammation.

8.1 *In vitro* immunomodulatory effects on T lymphocytes and monocytes

Materials and methods

Materials

Picracin and deacetylpicracin were isolated from *P. scrophulariiflora* Pennell as described earlier (chapter 7). Cucurbitacin E was obtained from Extrasynthese (Genay, France). M199 medium, RPMI 1640 medium, and heat-inactivated fetal calf serum (FCS) were obtained from Gibco BRL, Life technologies (Pailsey, Scotland); Phytohemagglutinin (PHA) from Murex Diagnostics (Dartford, GB); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-carboxyfluorescein diacetate (CFDA), propidium iodide (PI), and cyclophosphamide from Sigma Chemicals (St. Louis MO, USA); and sodium dodecyl sulphate (SDS) from ICN Biomedicals. Sheep
red blood cells (SRBC) were obtained from citrated arterial blood supplied by Charles River (Someren, The Netherlands) and were washed thrice before use.

**IL-2 release**

Peripheral blood lymphocytes (PBLs) were prepared from buffycoat residues, kindly provided by the Blood bank Midden-Nederland (Utrecht, Netherlands), using Ficoll-Hypaque centrifugation, according to the supplier’s instructions (Amersham Pharmacia). Cells were diluted to 2.10^6 cells/ml RPMI 1640 supplemented with 10 % FCS and dispensed in 96-wells microtiter plates (50 µl/well). Proliferation was induced using 50 µl PHA (0.1 mg/ml), and cells were incubated at 37 °C with 50 µl of test samples in appropriate dilution ranges. After incubation, 50-µl samples of supernatants were transferred to flatbottom plates and IL-2 levels were determined using an ELISA-kit (CLB, Amsterdam, Netherlands). Controls consisted of stimulated PBLs without sample (100% activity), non-stimulated PBLs without sample (0% activity), and non-stimulated PBLs with samples.

**Pro-inflammatory cytokine release**

Mononuclear cells were obtained from buffycoat residues (Bloedbank Midden-Nederland) using Ficoll-Hypaque centrifugation, according to the manufacturer’s instructions (Amersham Pharmacia). Under sterile conditions, 100-µl samples of cell suspension were dispensed in 96-wells flatbottom microtiter plates, and monocytes were allowed to adhere overnight at 37 °C under 5 % CO2-atmosphere in a humidified incubator, at a concentration of 10^6 cells/ml M199. After removal of non-adhering cells, 50-µl sample solutions in appropriate dilution ranges were added. Cells were stimulated by adding 50 µl LPS (0.5 µg/ml M199 medium), and incubated overnight at 37 °C under 5 % CO2 atmosphere in a humidified incubator. After incubation, 50-µl samples of supernatants were transferred to flatbottom microtiter plates, and used for the detection of IL-1β and TNFα, using ELISA-kits (CLB, Amsterdam, Netherlands). IC_{50}-values of the test compounds were calculated in µg/ml. Controls consisted of non-stimulated cells incubated with sample solutions.

**Toxicity assays**

For the assessment of cytotoxic effects of picracin and deacetylpicracin, cells were labeled with CFDA and PI to distinguish between viable and dead cells. After 4 days of incubation (37 °C), 50 µl of a 1 µl/ml CFDA dilution in RPMI medium was added. After incubation in the dark (15 min, room temperature), 50 µl PI in ink was added. The plates were inspected under an upside-down fluorescence microscope and the ratio of viable/dead cells was assessed. Controls consisted of cells incubated with medium only.

**Apoptosis**

PBLs (2 × 10^6 /ml), obtained as described above, were added to equal volumes of picracin or deacetylpicracin in different concentrations and incubated in a humidified incubator (36 °C). After incubation, cells were washed with phosphate-buffered saline (PBS) and incubated shed from light for 15 min in binding buffer containing FITC-conjugated annexin V (1:100). Propidium iodide (PI; 10 µg/ml) was added just before
analysis to detect dead cells. T cells were detected using FITC-conjugated anti-CD3 antibodies (1:100). Fluorescence of stained cells were measured by flow cytometry on a FACScan apparatus (Becton Dickinson) and analyzed with WinMDI (vs. 2.8; Scripps Institute, La Jolla, USA). T cells that were annexin V positive and PI negative were regarded as apoptotic cells.

Results

IL-2 release

Control experiments revealed maximum IL-2 release by PHA-activated T cells about 24 h after activation. This incubation time was used in determining the effects of picracin and deacetylpicracin on IL-2 release of activated PBLs. Picracin, deacetylpicracin, and cucurbitacin E inhibited IL-2 release by activated T cells after 24 h at IC50 values of 5 ± 2 µM, 16 ± 7 µM, and 5 ± 1 µM, respectively (figure 1). Activated T cells that were stained with CFDA and PI, following exposure to either of the compounds, revealed a direct toxic effect of cucurbitacin E, while cells treated with picracin or deacetylpicracin did not differ substantially from controls, as estimated by fluorescent microscopy.

![Figure 1](image)

*Figure 1. Effects of picracin, deacetylpicracin (DAP), and cucurbitacin E (cuc E) on IL-2 release by PHA-activated T lymphocytes after 24 h incubation, as determined by ELISA. Data were expressed as means ± SEM of 6 (picracin) or 3 (DAP and cuc E) separate experiments.*

IL-1β, TNFα release

To investigate additional immunomodulatory activities of picracin and DAP on the development of chronic inflammation, we assessed the effect of both compounds on the release of pro-inflammatory cytokines by monocytes. After stimulation of monocytes with LPS, cells were incubated for 24 h with different concentrations of picracin or DAP. Upon analysis of the supernatants of these cells, using ELISA, we found that picracin inhibited the release of both IL-1β and TNFα at IC50-values of 7 ± 2 µM and 10 ± 4 µM, respectively. Cucurbitacin E inhibited TNFα release more potently (IC50 = 3 ± 2 µM), while IL-1β-inhibiting activity was a factor 5 lower (IC50 = 16 ± 3 µM). Contradictorily, deacetylpicracin did not show inhibitory activity on the release of IL-1β up to 200 µM, the
highest concentration tested, while it moderately inhibited TNFα release (IC₅₀ = 120 ± 36 µM). Dexamethasone showed similar activity as deacetylpicracin (figure 3). Staining of activated monocytes, exposed to either of the compounds, with CFDA and PI, did not reveal any direct toxic effect after 24 h, as determined by analysis with fluorescent microscopy.

![Figure 2](image-url)  
*Figure 2. Effects of picracin, deacetylpicracin (DAP), cucurbitacin E, and dexamethasone on the release of pro-inflammatory cytokines by LPS-activated monocytes. Data were expressed as means ± SEM of 6 (picracin and DAP) or 3 (cucurbitacin E and dexamethasone) separate experiments.*

**Apoptosis of T cells**

To assess whether picracin and deacetylpicracin were able to induce apoptosis in non-stimulated T cells, PBLs were exposed to the two compounds. After incubation for 2, 4, 6, or 24 h, cells were stained with FITC-conjugated annexin V and propidium iodide (PI) and analyzed by flow cytometry. Stained cells that were annexin V-positive and PI-negative were regarded as apoptotic. Flow cytometric analysis of stained T cells that were exposed to picracin, showed a significant induction of apoptosis (70-85 % of gated T cells) after 4, 6, and 24 h, at concentrations of 20 µM and 200 µM (figure 2A). Deacetylpicracin, however, only slightly induced apoptosis (30-35 %) after 4, 6, and 24 h, at 200 µM, the highest concentration tested (figure 2).
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Figure 3. Flow-cytometric analysis of picracin- (A) and deacetylpicracin (DAP; B)-induced apoptosis in non-stimulated T-cells. After incubation, cells were stained with FITC-conjugated annexin V (FL1; x-axis) and propidium iodide (FL2; y-axis). Results are representative of two separate experiments. C. Percentage of T cells treated with picracin or deacetylpicracin (DAP) undergoing apoptosis (annexin-V positive and propidium iodide negative). Data were expressed as means ± SEM of two separate experiments and statistically analyzed by Student’s t-test. P-values are < 0.05 (*).
Discussion

In the previous chapter, the isolation of two cucurbitacins that inhibit T-cell proliferation from rhizomes of *Picrorhiza scrophulariiflora* was described. In this chapter, possible underlying mechanisms are investigated. As described earlier, proliferation of activated T-cells is mediated by the release of IL-2 and the subsequent interaction with the IL-2 receptor (IL-2R), which is only expressed in T cells that are activated. Control experiments show that IL-2 release by mitogen-activated peripheral blood T lymphocytes is optimal at 24 h after induction, which is in agreement with literature (Swain, 1999). Incubation of activated T cells with picracin or deacetylpicracin showed a marked inhibition of IL-2 release after 24 h, at IC50 values of 5 and 16 µM, respectively, which could not be ascribed to a direct toxic effect. Furthermore, cucurbitacin E, a cucurbitacin known to inhibit the proliferation of endothelial cells (Duncan and Duncan, 1997), inhibited IL-2 release at an IC50 value of 5 µM. However, fluorescence microscopical analysis following staining of cells with CFDA and PI demonstrated that in this case a direct cytotoxic effect was involved. These data may explain the antiproliferative activity of picracin and cucurbitacin, since IL-2 is responsible for signaling T-cell proliferation (Smith, 1988). The difference observed between picracin and deacetylpicracin regarding inhibition of IL-2 release might be ascribed to differences in lipophility between the two compounds.

In addition to the effects on T lymphocytes, the effects of picracin and deacetylpicracin on the release of the pro-inflammatory cytokines IL-1β and TNFα by activated monocytes was investigated. These cytokines play an important role in the pathogenesis of RA, because they enhance the release of metalloproteinases by chondrocytes and fibroblasts, inducing cartilage destruction (Dayer *et al.*, 1986) and stimulating bone erosion by osteoclasts (Thomas *et al.*, 1987). Incubation of human monocytes with picracin for 24 h inhibited the release of IL-1β and TNFα at IC50-values of 7 µM and 10 µM, respectively. Deacetylpicracin, however, did not inhibit the release of IL-1β up to 200 µM, the highest concentration tested, and slightly inhibited TNFα release (IC50 = 120 µM). Cucurbitacin E, which differs from picracin in the presence of carbonyl functionalities at C3 and C11, and a C1-2 double bond (chapter 4), inhibited the release of IL-1β at IC50 = 16 µM and TNFα release at IC50 = 3 µM. This suggests the involvement of at least the acetyl group in the side chain in the inhibition of IL-1β and TNFα release, although other functional differences between the two compounds were not taken in consideration.

The interference of picracin and deacetylpicracin with IL-2 release by activated T lymphocytes could be the result of a specific interaction with signal transduction pathways leading to the release of IL-2, or due to the induction of apoptosis, which renders the T cell incapable of releasing IL-2. To assess the apoptotic effect of picracin and deacetylpicracin, PBL were stained with FITC-conjugated annexin V, a phospholipid-binding protein with high affinity to phosphatidylserine, a marker of early apoptosis (Martin *et al.*, 1995, Vermes *et al.*, 1995). Because phosphatidylserine is exposed in necrotic cells as well, cells were double stained with propidium iodine (PI). Cells that were annexin V-positive and PI-negative were regarded as apoptotic. Cytometric analysis of T cells, exposed to picracin, and stained with annexin V and PI, showed a significant induction of apoptosis (70-85 %)
after 4, 6, and 24 h, at concentrations of 20 µM and 200 µM. Deacetylpicracin, however, was about three times less effective, and induced apoptosis for 30-35 % after 4, 6, and 24 h, at 200 µM, the highest concentration tested, which was not significantly different from untreated cells. Nevertheless, preliminary experiments suggest the induction of apoptosis in T cells, two days after incubation with deacetylpicracin. The effects observed might be due to the lower lipophilicity of deacetylpicracin, which could result in a less pronounced interaction with cell membranes, and, consequently, a delayed capacity to induce apoptosis. The observation that deacetylpicracin was about three times less effective in decreasing the release of IL-2 by T cells after 24 h of incubation, while its capacity to inhibit T-cell proliferation after 4 days of incubation was not inferior to picracin, are in support of this hypothesis.

### 8.2 Immunomodulatory effects in vivo

#### Material, animals, and methods

##### Animals

Specific-pathogen-free female BALB/c mice of 7-9 weeks were obtained from the Central Animal Laboratory (Utrecht, The Netherlands), maintained under standard housing conditions, and provided with food and water ad libitum. At the start of the experiments, the mice were 8-10 weeks old.

##### DTH response

Delayed-type hypersensitivity was induced in BALB/c mice according to Kerckhaert et al. (1974). Before priming, the mice were pretreated with 5 mg cyclophosphamide in phosphate buffered saline (PBS) intraperitoneally to suppress a B-cell mediated response. Eight hours after pretreatment, the mice were primed by intraperitoneal injection of 10⁸ SRBC in 200 µl PBS. After 10 days, the mice were challenged by injecting 10⁸ SRBC in 25 µl PBS subplantar in the left paw. Picracin or deacetylpicracin (10, 30, or 100 mg/kg) was administered, either intraperitoneally 1 hour before challenge (200 µl), or subcutaneously along with the challenge (25 µl). Dexamethasone served as a positive control. The locally administered samples, including the controls, contained 20 % DMSO in order to dissolve the drug properly. A separate control experiment was performed with primed mice, which were not challenged with antigen, and treated with subcutaneous injection of picracin or deacetylpicracin (0.1, 1, 10 µg/ml PBS), subplantar in the left paw. Swelling of the paws was measured 24 h after challenge using a computerized micrometer device (University Medical Center, Utrecht, The Netherlands), and compared to the control group. The differences between the experimental groups and the control group were statistically analyzed using the Students t-test.
Results

A delayed-type hypersensitivity (DTH) model was used to assess the immunomodulatory activity of picracin and deacetylpicracin *in vivo*. Intraperitoneal injection of sensitized mice, one hour before challenge with either picracin or DAP, showed significant inhibition of DTH responses for 100 mg/kg picracin (45 %) and for 30 mg/kg DAP (33 %). Dexamethasone (30 mg/kg i.p.) served as a control and inhibited DTH for 99 % (figure 4).

![Figure 4. Effects of picracin, deacetylpicracin (DAP), and dexamethasone on DTH responses in mice after intraperitoneal administration. Results are representative of two independent experiments. Data were expressed as means ± SEM (n = 6 per group) and statistically analyzed by the Student’s t-test. P values were < 0.05 (*), 0.01 (**), or < 0.001 (***).](image)

While these results were obtained by intraperitoneal injection of picracin or DAP, another experiment was performed to determine whether the inhibitory activity was enhanced after local administration of either of the two compounds. For this, a mixture of sheep erythrocytes with picracin, deacetylpicracin, or dexamethasone was given by means of subplantar injection in the hind paw. In this experiment, however, an increase in swelling rather than a decrease was observed in groups treated with picracin or DAP, suggesting an irritating or toxic response for both compounds at all concentrations tested (figure 5).
Figure 5. Effects of picracin, deacetylpicracin (DAP), and dexamethasone on DTH-response in mice after subplantar administration. Results are representative of two independent experiments. Data were expressed as means ± SEM (n = 6 per group) and statistically analyzed by the Student’s t-test. P values are < 0.01 (**) or < 0.001 (**). To confirm the direct irritating or toxic effect of picracin or DAP, sensitized mice were given a subplantar injection of picracin or DAP (10, 1.0, or 0.1 µg/ml), suspended in PBS, in the absence of antigen. After 24 h, the paw swelling was measured. Picracin (10 µg/ml and 1.0 µg/ml), and to a lesser extend DAP (10 µg/ml), showed a significant irritating effect (figure 6).

Figure 6. Direct irritant effects of picracin and deacetylpicracin (DAP) after subplantar administration. Data were expressed as means ± SEM (n = 5 per group) and statistically analyzed by the Student’s t-test. P values are < 0.05 (*), < 0.01 (***), or < 0.001 (****).
Discussion

Delayed-type hypersensitivity (DTH) is a valuable model to demonstrate cell-mediated immune responses, which are mediated mainly by T cells. Kerckhaert et al. (1974) showed that a single injection of cyclophosphamide (300 mg/kg) 8 h before immunization resulted in an enhanced DTH-response 10 days after immunization, and a decreased B-cell response during the whole experiment. Later research demonstrated the selective inactivation of suppressor T cells or their precursors by cyclophosphamide (Kaufmann et al., 1980, Bovbjerg et al., 1986), and the selective induction of apoptosis in differentiating B cells as compared with T cells in vivo (Hemendinger and Bloom, 1996). Therefore, this model was chosen to determine the effects of picracin or deacetylpicracin in vivo, with dexamethasone serving as a positive control (Schrier et al., 1985). The significant decrease in paw edema upon systemic administration of picracin or deacetylpicracin suggests that these substances are immunosuppressive. However, their effects were less prominent than the effects of dexamethasone, which is considered an anti-inflammatory rather than an immunosuppressive drug. To determine whether local administration would enhance the effects observed, the compounds were injected directly within the area of inflammation. The inhibitory effects of both cucurbitacins, however, could not be enhanced by local, subcutaneous administration. Instead, an increased swelling was observed. Control experiments revealed a concentration-dependent direct inflammatory response of picracin, which was significant at low concentrations (1 µg/ml and 10 µg/ml), and a minor but significant effect of deacetylpicracin at 10 µg/ml, the highest concentration tested.

The inflammatory and immunosuppressive effects observed for picracin and deacetylpicracin suggest a dualistic effect of these compounds. Upon local administration, they might induce a direct inflammatory response, while upon systemic administration (i.p.) they might be metabolically converted into compounds that exhibit immunosuppressive effects instead. Alternatively, the effects might be related to the induction of T-cell apoptosis in the paw. Apoptosis is usually not associated with inflammation, because the apoptotic bodies, which are the result of apoptosis, are ingested by surrounding phagocytes and any leakage of their contents is thus avoided (Savill et al., 1993). However, Uchimura et al. (1997) demonstrated that apoptotic CTL-L2 cells, cocolonated with peritoneal exudate cells, resulted in the production of both anti-inflammatory and pro-inflammatory cytokines. Culture supernatants induced the accumulation of neutrophils in vivo (Uchimura et al., 1997). These findings suggest that apoptosis is associated with leukocyte infiltration in vivo. Whether this effect is associated with edema and inflammation remains unclear. However, the intraperitoneal administration of high doses of picracin or deacetylpicracin, e.g. 30 and 100 mg/kg, might induce a strong influx of leukocytes secondary to T-cell apoptosis. Leukocytes might as well be withheld from the paw, leaving behind a reduced number of inflammatory cells to elicit a local immune response after subcutaneous injection of antigen. A similar phenomenon has been described before as counter-irritation, e.g. inflammation in progress at one site decreases edema formation at a second and separate inflammatory focus (Normann et al., 1985).

In conclusion, picracin and deacetylpicracin, two cucurbitacins isolated from Picrorhiza scrophulariiflora, which inhibit mitogen-induced proliferation of T cells, are
potent inhibitors of IL-2 release. Furthermore, picracin potently induced apoptosis of non-stimulated T cells, while deacetylopicracin was much less active. The induction of apoptosis by picracin might explain its inhibitory effects on the proliferation of T cells and its inhibition of IL-2 release. The inferior effects of deacetylopicracin in inhibiting IL-2 release and inducing apoptosis might be due to the lower lipophilicity of deacetylopicracin. The inactivity of deacetylopicracin and the activity of picracin to inhibit the release of IL-1β and TNFα in LPS-stimulated monocytes upon 24-h co-incubation, suggest that these effects might be ascribed to the induction of apoptosis as well, although further research has to confirm any apoptosis-inducing effect on monocytes. Furthermore, picracin and deacetylopicracin moderately inhibited delayed-type hypersensitivity responses in mice upon intraperitoneal administration, while subplantar injection in the paw elicited a local inflammatory response. These observations suggest the attraction of phagocytes, secondary to T-lymphocyte apoptosis in vivo, and producing a counter-irritant effect upon intraperitoneal administration of these compounds.

Although our findings do not favor the usefulness of picracin and deacetylopicracin in autoimmune disorders such as rheumatoid arthritis, these compounds might be useful tools in studying the effects of apoptosis induction in vivo. Furthermore, the effect of picracin on T-cell proliferative disorders needs study, as well as its effect on cell arrest extended to other cell types and relevant diseases. Picracin might serve as a lead compound for the development of pharmacotherapeutics, effective in such diseases.

References


