Prevention of plant crown gall tumor development by the anti-malarial artesunate of Artemisia annua

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Abstract
The antimalarial drug artesunate, a sesquiterpene trioxane lactone derivative of artemisinin from Artemisia annua L., is known for its extraordinary inhibitory effects on plasmodia and trematodes and also for suppressing the proliferation of human tumor cells. In the present study the effect of artesunate was investigated on rapidly dividing plant cells in Agrobacterium tumefaciens-induced crown gall tumor and wound callus cells at the model plant Ricinus communis. Low concentrations of artesunate (10 µM) were sufficient to completely suppress crown gall development upon permanent application. Within three weeks the shoots of artesunate-treated plants attained about double the size of the tumor-bearing plants and showed abundant, healthy and larger leaves. Moreover, artesunate retarded wound callus development and induced superficial necroses. However, artesunate did not prevent or inhibit infections of cucumber leaves by powdery (Podosphaera xanthii) or downy mildew (Pseudoperonospora cubensis). Young cucumber leaves showed symptoms of phytotoxicity upon treatment with very high artesunate concentrations of 100 µM and higher. Lower concentrations (50 µM or less) did not cause visible necrotic lesions. These novel findings suggest a general and conserved basic mode of action of artesunate in human, animal and plant cells, except of phytopathogenic fungi. A possible application of artesunate for biological control of crown gall development in grapevine and precious fruit trees is discussed.

Key words: Agrobacterium tumefaciens, crown gall, Cucumis sativus, downy mildew, growth promotion, powdery mildew, Ricinus communis, vascularization

Zusammenfassung

Stichwörter: Agrobacterium tumefaciens, Cucumis sativus, Echter Mehltau, Falscher Mehltau, Mauke, Ricinus communis, Vaskularisierung, Wachstumsförderung

Introduction

Extracts of Artemisia annua L. have been used to cure chills and fever in China for more than 2000 years (Klayman, 1985). Searching for potent anti-malarial drugs, between 1972 to 1979 compounds of the glandular secretory trichomes of A. annua, such as the sesquiterpene trioxane lactone endoperoxide artemisinin and its derivatives including the most potent of them, artesunate, were identified as being active against malaria caused by Plasmodium falciparum and P. vivax infections (Klayman, 1985). It is active against chloroquine- and mefloquine-resistant strains with an excellent tolerance in patients and negligible side effects (Ribiero and Olliaro, 1994; Bazzi et al., 1999; Creasap et al., 2005; Zauner et al., 2006; Otten et al., 2008). By analogy to mammalian tumors, vascularization, the development of a dense net of vascular bundles, consisting of functional phloem and xylem strands, is a precondition for plant tumor development (Fig. 1a). Therefore, the aim of the present investigation was to test whether or not artesunate is also active against plant tumors. Disease control of crop plants by natural plant-derived agents is an increasing requirement for organic farming, such as the particularly efficient plant-derived extract of Reynoutria sachalinensis (Herger et al., 1988). Because of its efficacy in very divergent organismic kingdoms, the action of artesunate was studied also on the oomycete Pseudoperonospora cubensis, the downy mildew, and on the ascomycete Podosphaera xanthii, the powdery mildew, of cucumber.

Materials and Methods

Plant material
Castor bean (Ricinus communis L. var. gibsonii cv. Carmencita; Walz Samen, Stuttgart, Germany) was grown in standard potting soil LD 80 and kept in the greenhouse with 16 h light and 8 h dark cycles at 28 °C/20 °C. 120-old seedlings were wounded with razor blades 1 cm below the cotyledons by 2 longitudinal cuts of 5 mm length and inoculated with bacterial pellet.

Cucumber (Cucumis sativus L.) cv. Chinesische Schlange, was cultivated in LD 80 potting soil and kept in a growth chamber at 22 °C and at 70% RH at 18 h light and 6 h dark. 3 weeks after sowing the 2 remaining first leaves, left over after removing side shoots, were pre-treated with artesunate (10 and 50 µM in 0.1% dimethylsulfoxide (DMSO)) or with 0.1% DMSO and 24 h later both leaves were inoculated with spores or sporangia.

Pathogens
The nopaline wild-type strain Agrobacterium tumefaciens (Smith and Townsend) C58, obtained from the Max-Planck-Institut Köln, Germany, was grown in yeast extract broth (YEB) as described earlier (Aloni et al., 1995).

For inoculation with powdery mildew, Podosphaera xanthii (Castagne), conidia from infected cucumber plants were collected and adjusted in 0.0125% Tween 20 in de-ionized water to a density of 1 x 10^6 spores ml^-1. Sporangia of downy mildew, Pseudoperonospora cubensis (Berct et Curt.), were adjusted to a density of 5 x 10^3 sporangia ml^-1.

Artesunate treatment
Ricinus: To investigate the effect of artesunate on tumor growth and wound callus development, artesunate was...
applied at the site of infection or wounding 1 d after inoculation or wounding. A Terostat funnel (Teroson, Heidelberg, Germany) was mounted around the wound and was kept filled up with 10 µM artesunate in 0.1% DMSO (Saokim Co. Ltd., Hanoi, Vietnam) or only 0.1% DMSO as control, following prior test series on the optimal effective concentration avoiding general phytotoxicity.

Cucumber: In preliminary experiments artesunate concentrations of 1 µM to 13 mM were tested on phytotoxicity in cucumber leaves. 100 µM and higher concentrations rapidly induced severe necroses in the younger leaves within 24 h (Fig. 2 j). Since leaves did not develop necrotic symptoms upon application of 50 µM artesunate and lower concentrations, in the further experiments 10 and 50 µM artesunate in 0.1% DMSO or 0.1% DMSO as control were protectively and thoroughly sprayed until run off on both the upper, adaxial, and the lower, abaxial, side of cucumber leaves using a glass atomizer (Desaga, Wiesloch, Germany). Treated plants were kept at 23°C for 24 h, and then spores of powdery mildew or sporangia of downy mildew were sprayed on the upper or the lower leaf sides. Plants inoculated with downy mildew sporangia were kept at 15°C in a humid and dark chamber for further 24 h and then at 20°C in a growth chamber for 14 d. Plants inoculated with spores of powdery mildew were transferred to the greenhouse and kept at 22°C for 12 d. All inoculated and control plants were daily thoroughly sprayed with artesunate or DMSO solution on both leaf sides.

Microscopy
After 2 to 3 weeks, 150 µm thick cross sections from tumor and wounded stem tissue were stained with 0.05% toluidine blue for 3 min or with 0.1% aniline blue (in 1 M glycine at pH 9.4) for 1 min and immediately viewed.
with an Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany) either under bright field or UV light (Leica, filter block A: excitation BP 340-380 nm, emission LP 430 nm). Micrographs were digitally reproduced from color slides taken with an Orthomat E camera system (Leica) on Kodak Ektachrome Elite 100 ASA daylight film.

Results

Stems of seedlings of *Ricinus communis* were used as an experimental model. They were wounded and infected with *Agrobacterium tumefaciens* strain CS8. They rapidly developed crown galls upon the integration and expression of the T-DNA of the bacterial Ti plasmid (ALONI et al., 1995; ULLRICH and ALONI, 2000; ALONI and ULLRICH, 2008). To avoid washing out the bacteria and to ensure successful transformation of the plant tissue, artesunate (10 µM in 0.1% DMSO) was administered in terostat funnels mounted around the wound and infection site 24 h after inoculation of the stem tissue (Fig. 1b). Whereas the DMSO-treated control plants rapidly developed crown galls (Fig. 1c, d, g, j), artesunate caused only brown necrotic lesions around the wound (Fig. 1e, f, h). Microscopic analysis revealed strong vascularization within the DMSO-treated control tumors, consisting of considerable masses of xylem (Fig. 1j) and phloem (Fig. 1k, aniline blue staining). In artesunate-treated tissue, characteristic histological T-DNA-dependent phytohormone effects of auxin and cytokinin (VESELOV et al., 2003; ALONI and ULLRICH, 2008) became obvious in small vascular bundle development, proving successful transformation of the plant tissue (Fig. 1i). Hence the agrobacteria were not directly affected, which is in line with the fact that Gram-negative and Gram-positive bacteria are artemisinin-resistant (KLAYMAN et al., 1984). Importantly, further proliferation was inhibited when the wounds were carefully and permanently covered with artesunate solution. Already two weeks after inoculation the artesunate-treated plants were taller than the DMSO-treated tumor-bearing plants (Fig. 1l).
weeks the shoots of artesunate-treated plants attained about double the size of the tumor-bearing plants and showed abundant, healthy and larger leaves (Fig. 1m).

Wounds of stems treated only with artesunate but not inoculated with \textit{A. tumefaciens} developed characteristic brown necroses around the wound (Fig. 2d), in contrast to wounds treated in the same way with water or 0.1% DMSO or without any solution (Fig. 2a-c). However, unwounded tissue was not affected by artesunate (Fig. 2e). Microscopic analysis confirmed that in DMSO- or water-treated wounds, the wound callus regenerated the different tissues (Fig. 2f, g), while artesunate suppressed the complete regeneration of superficial wound callus cells and resulted in small necrotic cavities (Fig. 2h, i).

Artesunate induced necroses on younger cucumber leaves only at high concentrations (100 \( \mu \text{M} \)), but not on slightly older leaves. 50 \( \mu \text{M} \) and lower concentrations did not affect any healthy leaves (Fig. 2j, k). In spite of pre-treatment and continuous daily treatment with artesunate for 12 to 14 d growth of powdery and downy mildew remained unaffected. In this case an efficient detoxifying metabolic mechanism may be assumed, which raises problems also with many other known fungicides and biological agents.

Because of the similarities with so many other different organisms and the striking difference to phytopathogenic fungi, further detailed biochemical, molecular and physiological analyses, including the role of endogenous FeII ions in crown gall cells, are required to reveal the mode of action of artesunate in young differentiating plant cells, in particular in crown gall tissue. It is now important to examine the practical application of artesunate in grapevine crown gall prevention. Due to different anatomy and physiology of plants in contrast to animals and humans different application formulations have to be developed. Grapevine graftings or precious pome fruit trees may be protected or saved from crown gall disease by application of artesunate-containing wound dressing or pruning sealant formulations.

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