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The Plant-Derived Naphthoquinone Droserone Inhibits *In Vitro* Measles Virus Infection

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ABSTRACT

The naphthoquinone droserone (**1**) is a natural product occurring in dicotyledonous plants. We have now observed that the addition of **1** during infection of tissue culture cells with measles virus considerably reduced the infection. Interestingly, the infection was inhibited only when droserone (**1**) was added during virus entry, but not when added to the cells prior to virus uptake or after virus uptake. These findings suggest that **1** interacts with viral particles to reduce infectivity. The formation of progeny measles virus particles was inhibited to 50% by droserone (**1**) at a concentration (IC_{50}) of approximately 2 µM with a half-maximal cytotoxicity (CC_{50}) of about 60 µM for Vero cells. Other tested naphthoquinone derivatives, among them the likewise natural plumbagin (**2**), but also synthetic analogs, were either more cytotoxic or not as effective as **1**. Thus, our data do not support the development of naphthoquinone derivatives into antiviral compounds, but suggest that they may be interesting research tools to study measles virus entry into cells.

ABBREVIATIONS

eGFP	enhanced green fluorescent protein
MV	measles virus
pfu	plaque-forming units

Introduction

MV, of the family Paramyxoviridae and the genus *Morbillivirus*, is one of the most contagious aerosol-transmitted viruses. Worldwide, it still causes more than 100 000 deaths per year. After entering the upper respiratory tract, MV exhibits a pronounced tropism for mono- and lymphocytic cells using the cellular receptor CD150 for virus entry [1, 2]. Dendritic cells of the respiratory tract transport the virus to draining lymph nodes, and soon after this the initial spread viral replication is detected in other secondary lymphoid organs [3]. Following replication in lymphoid tissues, the virus spreads to various organs and can be detected in the skin, gastrointestinal tract, eyes, and lungs, where it is released after infection of epithelial cells using the receptor nectin-4 [4–6]. It can also enter the brain, where it may cause acute and long-lasting complications (for a review, see [7]). In immunocompetent patients, MV infection is usually cleared by the virus-specific immune response, while the number of lymphocytes in the blood is considerably reduced and the general immune response to other infectious agents is suppressed from several weeks up to years [8]. The very successful available live measles vaccine does

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not only protect from acute infection, but also prevents complications, including various forms of encephalitis [9]. Furthermore, vaccination prevents long-term measles-induced immunomodulation and thus reduces overall childhood infectious disease mortality [10, 11].

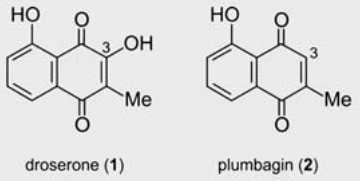
However, children can only be vaccinated when the concentration of maternal anti-MV antibodies has decreased, 6 to 12 months after birth. This leads to an unavoidable window of susceptibility. Since MV is one of the most contagious aerosol-transmitted viruses, it might be helpful to reduce its contagiousity by prophylactic measures or treatment of virus releasing patients using natural substances with little side effects. We therefore began to screen natural substances isolated from plants for their potential to inhibit the MV infection.

The naphthoquinones droserone (**1**) and plumbagin (**2**; ▶ **Fig. 1**) are well-known secondary metabolites of two small palaeotropical plant families, the Ancistrocladaceae and the Dioncophyllaceae [12, 13], and of their closest phylogenetic neighbors, the Ebenaceae, Plumbaginaceae, Droseraceae, Drosophyllaceae, and Nepenthaceae, some of them showing the phenomenon of carnivory [13–15]. In the plants, droserone (**1**) and plumbagin (**2**) act as phytoalexins, and their formation is induced by all sorts of chemical, physical, or biotic stress [13]. For example, high quantities of pure crystalline **1**, even well-suited for X-ray structure analysis, were detected under the stem bark of insect-wounded parts of *Ancistrocladus robertsoniorum* (Ancistrocladaceae), produced as a weapon against herbivores [16]. Droserone (**1**) and plumbagin (**2**) were likewise found to be the main metabolites in the medium of liquid cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) [14]. The predominant release of **1** and **2**, instead of the usual naphthylisoquinoline alkaloids [13, 17], obviously was a response to stress, caused by the transfer of the calli of *T. peltatum* from solid to liquid culture conditions [12]. Droserone (**1**) and, in particular, plumbagin (**2**) are also known to display strong anticancer, antimicrobial, antiinflammatory, and antiprotozoal activities [15, 18, 19].

In this paper, we describe the screening of droserone (**1**) and plumbagin (**2**) together with a series of 15 further structurally related naphthoquinones (**3**–**17**) for potential antiviral activity against MV. The compounds exhibited moderate to good activities, with natural droserone (**1**) being the most active one (IC_{50} of ca. 2 µM), showing only moderate cytotoxicity (CC_{50} of ca. 60 µM). Thus, **1** might serve as a plant-derived antiviral additive or as a lead compound for the development of novel, more effective antivirals.

Results

In a first screening assay, we tested the capacity of the natural products droserone (**1**) and plumbagin (**2**; ▶ **Fig. 1**) to inhibit MV infection of tissue culture cells using a recombinant wild-type MV (rMV-IC323eGFP) expressing eGFP [20]. By utilizing the cellular receptors CD150 and nectin-4, this recombinant MV has the tropism and growth properties of wild-type isolates and is widely used as a model MV [6]. CD150-expressing Vero cells were infected in the presence of various concentrations of **1** or **2** or



▶ **Fig. 1** Chemical structures of the natural naphthoquinones droserone (**1**) and plumbagin (**2**).

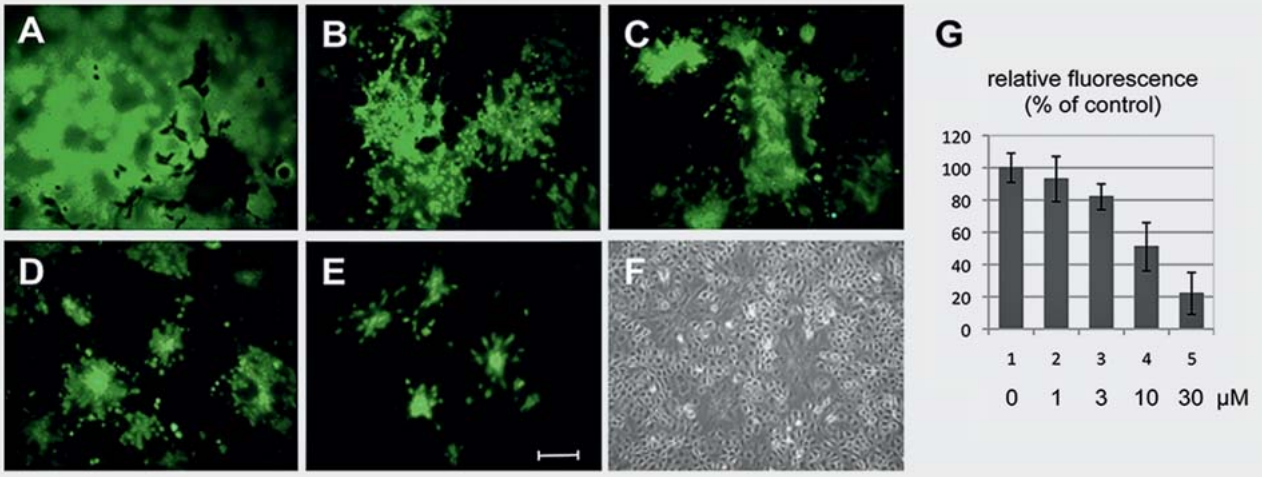
DMSO as a control. After 48 h, the eGFP autofluorescence in the tissue cultures, which depends on the transcriptional activity of the viral RNA-dependent RNA polymerase, was quantified by a fluorescence reader. As an example, photomicrographs of an experiment with droserone (**1**) are shown in ▶ **Fig. 2**, illustrating large fluorescent syncytia in the absence of an inhibitor (DMSO control; ▶ **Fig. 2A**), and reduced-sized syncytia with increasing concentrations of droserone (**1**; ▶ **Fig. 2B–E**). Quantification of the fluorescence showed 50% inhibition at approximately 10 µM of **1** (▶ **Fig. 2G**), thus revealing droserone (**1**) to be a potent inhibitor of MV infection. Plumbagin (**2**), by contrast, was found to be less effective against MV, while simultaneously displaying an enhanced cytotoxicity. Up to 1 µM plumbagin (**2**) did not inhibit MV transcription, and concentrations above 1 µM were cytotoxic (not shown).

This result made it rewarding to test the antiviral potential of a series of some analogs derived from **1** and **2**. For this purpose, a small library of structurally closely related naphthoquinones (**3**–**10**; ▶ **Fig. 3A**) was synthesized (see Supporting Information), comprising compounds with an additional hydroxy or methyl group at C-6 (compounds **3**–**5**), or without a hydroxy function at C-5, but possessing a functional group (a hydroxy, a methyl, or a trifluoromethanesulfonyloxy substituent) at C-6 (compounds **6**–**10**).

The quantification of the infections in the presence of droserone (**1**), plumbagin (**2**), and the related naphthoquinones **3**–**10** are summarized in ▶ **Fig. 3B**. Among the tested compounds, droserone (**1**) showed the highest activity in inhibiting MV infection. All other compounds tested were either not as active as droserone (**1**) or were cytotoxic, as determined using the MTT test (for the results for **1**, see ▶ **Fig. 4**).

From this first test series, it became obvious that the introduction of an additional hydroxy group at C-6 led to decreased activities. This was evidenced by the fact that **3** and **4** were less active than **1** and **2**. Compounds **6** and **7** likewise showed lower activities than **8** and **9**, which bear a methyl group at C-6. These results achieved with only slightly modified droserone and plumbagin analogs suggested that a less polar substituent at C-6 had a positive influence on the bioactivity, which was seen by the comparison of **8** with **6** and **10**, and of **9** with **7**.

As to the substituent at C-3, it is interesting to note that in all pairs of the synthetic analogs (**3** vs. **4**, **6**, vs. **7**, and **8** vs. **9**), the 3-hydroxylated variants (i.e., **4**, **7**, and **9**) were less active than their deoxy analogs **3**, **6**, and **8**, while the situation was the opposite for

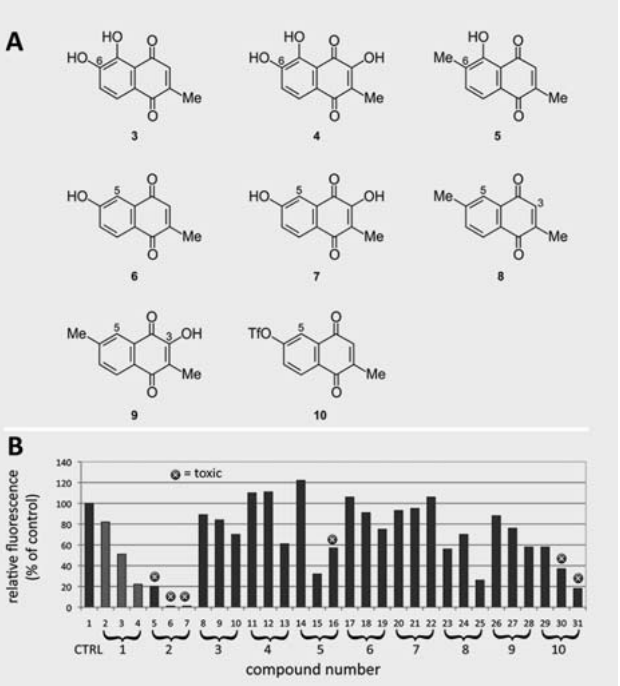


▶ **Fig. 2** Droserone (**1**) inhibits MV infection. Vero-hSLAM cells were infected with MV (rMV-IC323eGFP) at an MOI of 0.05 in the presence of DMSO (1 : 1000; **A**) or increasing concentrations of 1 (**B, C, D, E, F**: 1, 3, 10, 30, 30 µM) for 48 h. The eGFP autofluorescence (**A** to **E**) and bright-field (**F**) micrographs were taken using a UV microscope (100×). Vero-hSLAM cells (**G**) were infected with MV at an MOI of 0.01 in the presence of DMSO (1 : 1000) (entry 1) and increasing concentrations of droserone (**1**) as indicated (entries 2–5). After 48 h, the eGFP autofluorescence (MV infection) was measured using a fluorescence reader and values were normalized to the controls (n = 3).

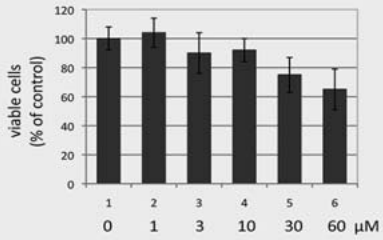
the natural products, where, as described above, droserone (**1**) inhibited the virus more efficiently than its oxygen-poorer analog plumbagin (**2**).

Since these small variations of the substitution patterns of droserone (**1**) or plumbagin (**2**) did not improve their activities, a second series of structurally more strongly modified naphthoquinones were synthesized. These compounds were additionally equipped with a chlorine substituent at C-6 (**11**–**13**; ▶ **Fig. 5A**) or were even more highly oxygenated, with further hydroxy or methoxy functions at C-6 and C-7 (**14**–**17**). Unfortunately, the naphthoquinones **11**, **14**, and **15** were inactive (▶ **Fig. 5B**), whereas **12**, **13**, **16**, and **17** did display strong antiviral effects, but simultaneously showed high cytotoxicities. The most toxic representatives were those with *ortho*-quinoid structures instead of the *para*-naphthoquinones, as seen from the pairwise comparison of **11** with **12** and of **15** with **16**. Thus, the natural product droserone (**1**) still remained the most potent inhibitor against MV.

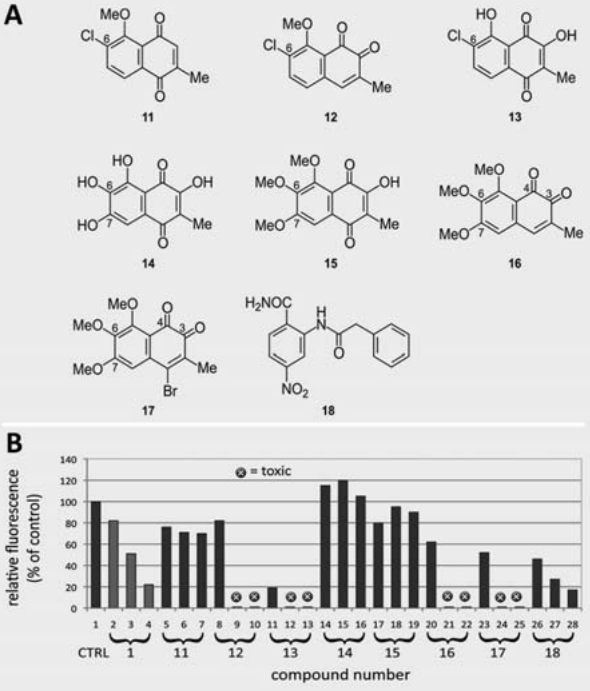
In order to get first information about the mode of infection inhibition induced by droserone (**1**), we determined if **1** affects the uptake of MV by cells or may act later in the intracellular replication phase of the infection. First of all, cells were incubated with MV in the presence of **1** for 2 h, then washed to remove the inoculum, and then further incubated for 48 h in the absence of **1** (▶ **Fig. 6A**, entries 1–5). Under these conditions, viral transcription and eGFP expression was reduced, demonstrating that droserone (**1**) acts during the uptake of MV by the cells. Incubation of the cells with **1** after the 2 h virus uptake phase, by contrast, did not significantly affect viral transcription (▶ **Fig. 6A**, entries 6–10). To compare the antiviral activity of droserone with that of a well-known MV-induced membrane fusion inhibitor, similar experiments were performed using the compound AS-48 [21–23] (**18**; ▶ **Fig. 5A**). Concerning the size of MV-induced syncytia and



▶ **Fig. 3** Naphthoquinones 3–10 structurally closely related to droserone (**1**) and plumbagin (**2**), evaluated for their inhibitory effects against MV infection (**A**). Infection-inhibition test using droserone (**1**), plumbagin (**2**), and its derivatives **3**–**10** (**B**). Vero-hSLAM cells were infected with MV at an MOI of 0.01 in the presence of DMSO (entry 1) and 3, 10, 30 µM concentrations, each, of droserone (**1**) and its nine analogs, **2**–**10**. After 48 h, the eGFP autofluorescence was measured using a fluorescence reader and values were normalized to the controls.



▶ **Fig. 4** Determination of cytotoxicity of droserone (**1**). Vero-hSLAM cells were incubated for 48 h in the presence of DMSO (1 : 1000) (entry 1) and increasing concentrations of droserone (**1**) as indicated (entries **2**–**6**). Cytotoxicity was measured using the MTT assay (n = 3). The CC_{50} was determined to be approximately 60 µM.



▶ **Fig. 5** Naphthoquinones bearing a chlorine substituent (**11**–**13**) and highly oxygenated naphthoquinones (**14**–**17**) studied for MV infection inhibition; the well-known fusion inhibitor AS-48 (**18**) was used for comparison (**A**). Infection-inhibition test using droserone (**1**), its derivatives **11**–**17**, and AS-48 (**18**; **B**). Vero-hSLAM cells were infected with MV at an MOI of 0.01 in the presence of DMSO (entry 1) and 3, 10, 30 µM concentrations, each, of droserone (**1**), its seven analogs **11**–**17**, and AS-48 (**18**). After 48 h, the eGFP autofluorescence was measured using a fluorescence reader and values were normalized to the controls.

fluorescence intensity of eGFP in the cultures, droserone (**1**) and AS-48 (**18**) led to similar results (▶ **Fig. 6A, B**).

To further clarify if droserone (**1**) acts on the virus or on the cells, we preincubated the cells with **1** for 2 h, washed the cells, and then infected them in the absence of **1** (▶ **Fig. 7**). Under these conditions, no significant inhibition was visible, suggesting that infection inhibition by droserone (**1**) is based on an interaction of **1** with virus particles.

Up to now, we had only measured the impact of droserone (**1**) on the virus-dependent eGFP expression in infected cells. To get an impression of how strong the inhibitory potential of **1** is with respect to virus replication and the production of progeny virus, we quantified the newly synthesized infectious viruses in dependence of the dose of droserone (**1**) using a plaque assay (▶ **Fig. 8**). Under these assay conditions, the newly synthesized virus was reduced to 50 % at approximately 2 µM of droserone (**1**). These results, in combination with the cytotoxicity assay, indicated that the IC_{50} was about 2 µM, the CC_{50} was ca. 60 µM, and thus the selectivity index (SI) was approximately 30. In addition, we determined the effect of AS-48 (**18**) for comparison under the same conditions. The inhibitory activity of **18** was very similar to that of droserone with an IC_{50} of approximately 1.5 µM.

Discussion

Our experiments have shown that the naturally occurring naphthoquinone droserone (**1**) must be present during the early infection phase in order to inhibit MV infection of target cells, when virus particles initiate pH-independent virus-cell membrane fusion and the viral ribonucleoprotein complex (RNP) is taken up by the cell. Preincubation of the target cells, and a subsequent addition of **1** during viral transcription and replication, did not lead to inhibition. We obtained very similar results using the established MV-induced membrane fusion inhibiting compound AS-48 (**18**) [21–23]. These findings suggest that droserone (**1**) may interact with viral structures involved in receptor recognition and/or membrane fusion induction. We do not yet know this viral structure, which obviously requires further experimental effort.

In our experiments described in this manuscript, as well as in unpublished experiments, we repeatedly observed that the effect of an inhibitor was stronger when the production of progeny infectious virions was measured as an end point compared to the expression of the viral GFP reporter. It is likely that the synthesis and titration of complete infectious virus particles and the fluorescence measurement of GFP are not linearly correlated. Nevertheless, due to the simplicity of the method, the GFP fluorescence measurement is the preferred method to screen for inhibitory compounds. However, for determination of the IC_{50}, the more sensitive titration assay should be used. In the case of droserone (**1**), this led to an IC_{50} value of approximately 2 µM.

Membrane fusion induced by Paramyxoviridae is an essential step in the viral infection cycle, which is mediated by the cooperation of both viral envelope glycoproteins, the attachment protein (H) and the fusion protein (F). After cleavage of the precursor F protein into F_1 and F_2 subunits, they form an active $H/F_{1/2}$ complex mediating virus-cell and cell-cell fusion. Small-molecule inhibitors such as **18** were designed to fit into a pocket of the MV F protein and prevent membrane fusion by blocking the natural interaction between the two essential heptad repeat regions within the F protein. These agents interfere with the conformational

changes of the F protein at the beginning of the fusion process [21–23]. We investigated similar small-molecule fusion inhibitors including *N*-(3-cyanophenyl)-2-phenylacetamide, which has a high capacity (IC_{50} = 3 µM; CC_{50} ≥ 300 µM) to inhibit the MV- and the related CDV-induced (CDV = canine distemper virus) membrane fusion, but not the Nipah virus-induced membrane fusion [24]. Compared to these known antiviral agents, droserone (**1**) displayed a good effect against MV infection with an IC_{50} value of 2 µM, but its cytotoxicity was higher than that of *N*-(3-cyanophenyl)-2-phenylacetamide. None of the analogs of droserone (**1**) and plumbagin (**2**) that were so far investigated showed improved inhibitory activities on MV infections. Most of them even displayed enhanced cytotoxicities. Thus, the search for further improved droserone-related inhibitors of MV infection, with minimized cytotoxicity, remains a challenging task for the future.

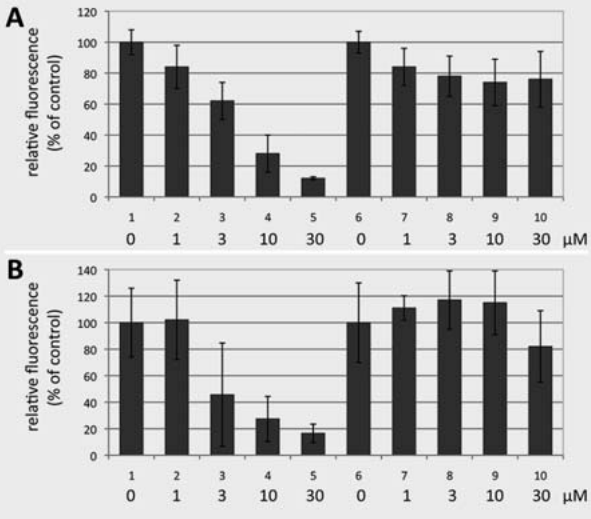
Materials and Methods

Cells, viruses, and virus titration

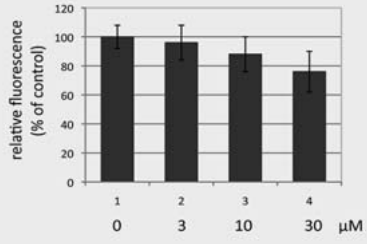
African green monkey Vero cells expressing human CD150 (Vero-hSLAM), and recombinant MV wild-type strain IC323-eGFP (rMV-IC323eGFP) were a gift of Dr. Y. Yanagi, Kyushu University, Fukuoka, Japan. Vero-hSLAM cells were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Recombinant MV-IC323eGFP was propagated and titrated using Vero-hSLAM cells as described earlier [20]. To titrate the newly synthesized virus, we performed plaque assays in 6-well plates. Briefly, 1.5×10^5 Vero-hSLAM cells were plated the day before infection on 6-well plates and infected with 1 mL virus dilutions (10^{-1} to 10^{-6}) in triplicate from infection inhibition assays for 1 h. Then, the medium was aspirated and cells were overlayed with 40 °C warm 0.75% Agar in MEM containing 5% FCS. After incubation for 5 days at 37 °C in the incubator, plates were treated with 0.016% neutral red solution (SIGMA) in PBS for 1 h, the solution was aspirated, and the plaques were counted.

Infection inhibition assay

The day before the start of the assay, 1×10^5 Vero-hSLAM cells were seeded in 6-well plates. Dilutions (1 : 1000) of inhibitors were mixed with medium and virus (MOI = 0.01) to achieve 0, 1, 3, 10, and 30 µM concentrations in an end volume of 1 mL medium and incubated for 5 min. The medium was aspirated from the 6-well plates and the inhibitor/virus mixtures were added to the cells. Alternatively, inhibitors or DMSO alone were added to the cells before the infection for 2 h, or after infection of the cells. After incubation for 48 h at 37 °C in the incubator, photomicrographs were taken (Leica DMi8), the eGFP autofluorescence was quantified using a fluorescence reader (Safire^2, Tecan), and plates were frozen at − 80 °C for subsequent determination of virus titers. Virus titers were determined by freezing/thawing complete cultures, removing cell debris by centrifugation at 10 000 rpm for 5 min in an Eppendorf centrifuge, and titration of supernatants by the plaque assay.



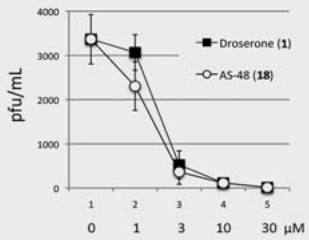
▶ **Fig. 6** MV infection is inhibited by droserone (**1**) (**A**) and AS-48 (**18**; **B**) when the inhibitors are present in the 2 h infection phase, but not when added afterwards. Vero-hSLAM cells were incubated with MV at an MOI of 0.01 in the presence of DMSO and increasing concentrations of droserone (**1**) or AS-48 (**18**) as indicated for 2 h, washed, and further incubated for 48 h (entries 1–5). Alternatively, cells were infected in the absence of the inhibitors for 2 h, washed, and then incubated for 48 h in the presence of DMSO and inhibitors as indicated (entries 6–10). The eGFP autofluorescence was measured using a fluorescence reader and values were normalized to the controls (n = 3).



▶ **Fig. 7** Pretreatment of cells with droserone (**1**) does not inhibit infection. Vero-hSLAM cells were incubated with DMSO + droserone (**1**) for 2 h, washed, and then infected with MV at an MOI of 0.01 (entries 1–4) and further incubated. After 48 h, the eGFP autofluorescence was measured using a fluorescence reader and values were normalized to the controls (n = 3).

Cell viability assays

The tetrazolium salt MTT (Sigma) is incorporated in cells and converted by cellular reduction systems into the purple water-insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). Only living, vital cells and cells in the very early phase of apoptosis can transform MTT to formazan. Vero cells (2.5×10^5) in a 6-well plate were preincubated for 20 h with the indicated chemical compounds. The medium was removed and cells incu-



▶ **Fig. 8** Virus production is effectively reduced by droserone (**1**) as well as by AS-48 (**18**). Vero-hSLAM cells were infected with MV at an MOI of 0.01 in the presence of DMSO (1 : 1000) (entry 1) and increasing concentrations of droserone (**1**) and AS-48 (**18**) as indicated (entries 2–5). After 48 h, the virus titer was determined by a plaque assay (n = 3). Data are presented as pfu/mL per 10^5 cells.

bated for 2 h with 1 mL/well fresh medium (MEM 5% FCS). After removal of the medium, the cells were incubated for 2 h at 37 °C with 750 µL MTT solution (0.25% MTT in PBS) per well. The solution was removed and the cells were incubated for 45 min at RT with 750 µL extraction solution (4% SDS, 10% 1 M HCl in DMSO). 12 × 50 µL Aliquots were transferred into a 96-well plate and evaluated using an ELISA reader measuring the absorbance at 570 nm. Results (mean values) are presented as % viability in relation to control cells treated with equivalent amounts of DMSO as used for the compounds.

Instrumentation and chemicals

Melting points were determined on a Kofler hot-stage apparatus (Reichert) and are uncorrected. IR spectra were measured using a Jasco FT/IR-410 spectrometer and are reported in wave numbers (cm^{-1}). NMR spectra were recorded on a Bruker AC 250, a Bruker AV 400, or a Bruker DMX 600 spectrometer at ambient temperature. The chemical shifts (δ) are given in parts per million (ppm) with the proton and carbon signals of the deuterated solvents as the internal reference for ^1H and ^{13}C NMR. Coupling constants *J* are given in Hertz (Hz). Mass spectra were recorded on a Finnigan MAT 8200 mass spectrometer at 70 eV for EI and on a Bruker Daltonics micrOTOFfocus for ESI. All reactions with air- and/or moisture-sensitive compounds were carried out in flame-dried glassware using the Schlenk-tube technique under inert a nitrogen or argon atmosphere. Organic solvents and all reagents used were of commercial quality. Reactions were monitored by TLC on aluminum plates with silica gel 60 F_{254} (Merck). Column chromatography was performed on Merck silica gel (63–200 µm). Plumbagin (**2**) was purchased from Sigma. Droserone (**1**) and dioncoquinone B (**4**) were isolated from cell cultures of *T. peltatum* as described earlier [12, 14]. The droserone analogs 5,6-dihydroxy-2-methyl-1,4-naphthoquinone (**3**) [12], 6-hydroxy-2-methyl-1,4-naphthoquinone (**6**) [12], 3,6-dihydroxy-2-methyl-1,4-naphthoquinone (**7**) [12], 2,6-dimethyl-1,4-naphthoquinone (**8**) [25], 2-methyl-6-[(trifluoromethanesulfonyl)oxyl]-1,4-naphthoquinone (**10**), 3,5,6,7-tetrahydroxy-2-methyl-1,4-naphthoquinone (**14**), 3-hydroxy-5,6,7-trimethoxy-2-methyl-1,4-naphthoquinone (**15**), and 5,6,7-trimethoxy-2-methyl-3,4-naphthoquinone (**16**) [26] and the precursors 1,5-dihydroxy-2,6-dimethylnaphthalene [27] and 5,6,7-trimethoxy-2-methyl-3,4-naphthoquinone [26] were synthesized as reported previously. For the synthetic procedures and the physical and spectroscopic data of **5**, **9**, **11**–**13**, and **17**, see Supporting Information. AS-48 [21–23] (**18**) was synthesized as described in the literature [24]. The purity of the compounds was determined by NMR spectroscopy (> 95%).

All of the compounds (**1**–**18**) were dissolved in DMSO at concentrations of 30, 10, 3, and 1 mM as stock solutions in order to use 1 : 1000 dilutions of these stocks in tissue culture medium as end concentrations in the infection inhibition assays. As control samples, 1 : 1000 DMSO in medium was always used.

Supporting information

Experimental procedures and physical and spectroscopic characterization (IR, ^1H and ^{13}C NMR, HR-ESI-MS) of compounds **5**, **9**, **11**–**13**, and **17** are available as Supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

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