

1 SARS-CoV-2 fears green: the chlorophyll catabolite Pheophorbide a is a potent antiviral

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16 **Abstract**

17 SARS-CoV-2 pandemic is having devastating consequences worldwide. Although vaccination
 18 advances at good pace, effectiveness against emerging variants is unpredictable. The virus has
 19 displayed a remarkable resistance to treatments and no drugs have been proved fully effective
 20 against Covid-19. Thus, despite the international efforts, there is still an urgent need for new
 21 potent and safe antivirals against SARS-CoV-2. Here we exploited the enormous potential of
 22 plant metabolism using the bryophyte *Marchantia polymorpha* and identified a potent SARS-
 23 CoV-2 antiviral, following a bioactivity-guided fractionation and mass-spectrometry approach.
 24 We found that the chlorophyll derivative Pheophorbide a (PheoA), a porphyrin compound
 25 similar to animal Protoporphyrin IX, has an extraordinary antiviral activity against SARS-CoV-
 26 2 preventing infection of cultured monkey and human cells, without noticeable cytotoxicity. We
 27 also show that PheoA prevents coronavirus entry into the cells by directly targeting the viral
 28 particle. Besides SARS-CoV-2, PheoA also displayed a broad-spectrum antiviral activity
 29 against (+) strand RNA viral pathogens such as HCV, West Nile, and other coronaviruses, but
 30 not against (-) strand RNA viruses, such as VSV. Our results indicate that PheoA displays a
 31 remarkable potency and a satisfactory therapeutic index, which together with its previous use in
 32 photoactivable cancer therapy in humans, suggest that it may be considered as a potential
 33 candidate for antiviral therapy against SARS-CoV-2.

34

35 **Introduction**

36 The pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is
 37 having devastating consequences, with more than 196M infected people and over 4M deaths
 38 worldwide (July 2021; <https://covid19.who.int/>). Besides the humanitarian cost, this pandemic
 39 carries a tremendous negative economic impact, a huge challenge for any government to
 40 overcome. The coronavirus disease 2019 (Covid-19), the respiratory illness caused by SARS-

41 CoV-2 (Genus betacoronavirus; Subgenus sarbecovirus), has displayed a remarkable resistance
 42 to treatments and no drugs have been proved fully effective against the virus. Moreover, the
 43 Covid-19 pandemic has made evident the need for a global strategy to fight similar situations
 44 that may appear in the future.

45 Current efforts to eradicate Covid-19 are focused on the development of vaccines and the
 46 search for antiviral lead compounds, mainly repurposing of existing drugs. Although the
 47 vaccination campaign seems to advance at good pace, its effectiveness against some of the
 48 present and future strains of SARS-CoV-2 is hard to predict due to the existence of different
 49 strains that could drastically reduce the vaccine efficiency¹. In addition, the best anti-Covid-19
 50 drugs approved so far (e.g. Remdesivir, Favipiravir or its derivative Avifavir, etc), have shown
 51 only a mild effect against the virus, slightly reducing hospitalization time of patients². Other
 52 treatments, such as Chloroquine and Hydroxychloroquine appear to help at least a subgroup of
 53 patients, but, possible negative side effects of these drugs remain under investigation³.
 54 Although, some compounds (e.g., Aplidin, Mefloquine, Nelfinavir, Protoporphyrin IX and
 55 Verteporfin) have shown potential on *in vitro* assays, and some of them also in animal models<sup>4-
 56 6</sup>, there is an urgent need for new potent and safe antivirals against SARS-CoV-2. Noteworthy,
 57 new pathogens, including viruses, are expected to emerge in coming decades, which puts an
 58 enormous pressure on society in order to be ready to fight back future pandemics with the
 59 proper chemical, biological, and engineering tools, including effective new antivirals.

60 For centuries, medical needs of society have been widely covered by plants, which have an
 61 extremely rich metabolism that provides them with a wide repertoire of chemical weapons to
 62 cope with environmental biotic stresses, including viruses^{7,8}. Originally recognized by
 63 traditional medicine, plants are the main source of compounds used today in pharmacology,
 64 from Aspirin (acetyl salicilate; from *Salix* sp.) to current anticancer drugs (e.g. Vinblastine and
 65 Vincristine from *Vinca* sp., or Taxol and Paclitaxel from *Taxus baccata*), simply to cite a few

66 successful examples^{9,10}. Therefore, the identification of new plant sources of enzymatic variants
67 and metabolites is essential to the discovery of new drugs and their optimization by metabolic
68 engineering. Aromatic and exotic vascular plants are commonly studied in order to identify
69 pharmacologically interesting compounds. In contrast, the metabolic richness of bryophytes
70 (non-vascular plants including mosses, liverworts and hornworts) has been little explored.
71 Bryophytes are rarely attacked by pathogens (fungi, bacteria, viruses) or herbivores (insects,
72 snails, mammals) in their natural habitats, which indicates that they are well protected by a
73 potent arsenal of secondary defense metabolites. However, studies on their chemical
74 constituents have been neglected until recently¹¹. Indeed, only around 5% of bryophyte species
75 have been metabolically explored, and results have shown an enormously rich diversity of
76 secondary metabolites, particularly in liverworts¹². Strikingly, more than 1600 terpenoids have
77 been reported in liverworts, whereas only about 100 terpenoids have been identified in the
78 medicinal plant *Cannabis sativa*^{11,13–16}. More importantly, several liverwort species of the order
79 Marchantiales, including *Marchantia polymorpha*, produce terpenoids and bisbibenzyls with
80 enormous potential for pharmaceutical applications since they show remarkable antimicrobial,
81 antioxidant, cytotoxic, anticancer and antiviral (anti-HIV) activities^{11,13,15–18}. Therefore, we
82 made use of our vast experience in vorology and *Marchantia*'s hormonal signaling and
83 secondary metabolism in order to explore this plant's potential as a source of antiviral
84 metabolites, particularly, against the SARS-CoV-2 virus.

85 In this study, we employed a set of *Marchantia* wild type plants, and signalling and metabolic
86 mutants to systematically study the pharmacological potential of this liverwort. We found that
87 total extracts from all the plants displayed a remarkable antiviral activity against SARS-CoV-2.
88 Using a bioactivity-guided chromatographic approach, in addition to mass-spectrometry (MS),
89 we identified the antiviral metabolite as Pheophorbide a (PheoA), a porphyrin chlorophyll
90 derivative very similar to animal Protoporphyrin IX, also described as an strong antiviral¹⁹. In

contrast to Protoporphyrin IX, which produces prophyria in humans, PheoA, however, was non toxic at all tested concentration. We also found that PheoA has a broad-range antiviral activity against positive strand RNA (+RNA) viruses, and acts as a virucidal by directly acting on the viral particle. PheoA is additive to remdesivir, which, together with its low toxicity, suggest its potential as candidate for antiviral therapy against SARS-CoV-2.

Results

Crude extracts of M. polymorpha show anti-SARS-CoV-2 activity

In order to explore for the presence of anti-SARS-CoV-2 metabolites in *M. polymorpha*, we prepared crude extracts of two different *M. polymorpha* subspecies (subsp. *ruderalis* from Japan and subsp. *polymorpha*, from Spain). The anti-SARS-CoV-2 activity of the resulting crude extracts was tested in Vero E6 cell monolayers infected with the SARS-CoV-2 NL2020 strain (Figure 1A). Infection was carried out at a multiplicity of infection (MOI) of 0.001 for 72 h. In the absence of antiviral activity, SARS-CoV-2 infection triggers cell death of Vero E6 cells and results in loss of cell biomass in the well, which is readily visualized as a strong reduction in crystal violet staining (DMSO, Figure 1A). Treatment of the cells during infection with serial dilutions of remdesivir (used as positive control), the only clinically approved antiviral for treatment of Covid-19 patients, protected the cell monolayers down to its reported EC₅₀ of 1.5 μ M (Supplementary Figure S1). Similarly, treatment of cell cultures with *Marchantia* crude extracts resulted in cell protection against virus-induced cytopathic effect without any signs of cytotoxicity in a broad dilution range (Figure 1A, Ex1 and Ex2), suggesting the presence of one or more *Marchantia* metabolites with strong antiviral activity.

Given that antiviral activity had been determined by an indirect measurement (Figure 1B), we set out to define if *Marchantia* extracts were indeed capable of interfering with viral spread in cell culture. Thus, Vero E6 cells were inoculated at MOI of 0.001 in the presence of control-solvent, remdesivir (25 μ M) and a 1:800 (v/v) dilution of the *Marchantia* extracts. Viral RNA

load, which in this experimental setup represents the degree of virus propagation, was determined 72 h post infection by RT-qPCR (Figure 1C). In the control, viral RNA accumulated six orders of magnitude above the assay background levels, whereas the viral RNA was undetectable in samples treated with the antiviral remdesivir. Importantly, in samples treated with *Marchantia* extracts, the viral RNA levels were comparable to those observed upon remdesivir treatment (Figure 1C). This outcome confirms the protective activity of the extracts observed in Figure 1A,B and suggest the presence of at least one antiviral compound.

Extract bioactivity does not depend on plant's secondary metabolism and is common to several plant species

Next, we explored whether the putative antiviral metabolite could belong to plant's secondary metabolism. Jasmonates (JAs) are a family of oxylipin-derived phytohormones regulating many aspects of plant development and growth; as well as mediating defense responses through transcriptional activation of the secondary metabolism, which includes several classes of compounds such as alkaloids, terpenoids and flavonoids^{20–22}. In *Marchantia*, secondary metabolites accumulate in specific organelles named oil bodies (OB), which are confined to scattered idioblastic OB cells distributed throughout the thallus²³. Therefore, we tested extracts from *M. polymorpha* WT, *Mpcoi1-2* [impaired in dn-OPDA perception, the active jasmonate in *Marchantia*²², thus, in defense metabolite induction], and *Mpc1hdz* plants (impaired in OB formation; *MpC1HDZ* is a transcription factor required for OB cells differentiation). *Mpc1hdz* mutants render plants defective in secondary metabolites, thus, susceptible to herbivory and microbes²⁴. To our surprise, all *Marchantia* extracts, WT or mutant, showed similar antiviral activity (Figure 2), indicating that the active antiviral should not belong to the plants's secondary metabolism. Indeed, data in Figure 2 suggests that the activity of extracts is due to the presence of a metabolite (or metabolites) that is constitutively synthesized and/or derived

from the plant's primary metabolism. Remarkably, regulation of primary metabolism is achieved in all plants by very similar conserved metabolic pathways^{25,26}. Therefore, we tested crude extracts of several plant species [sweet amber (*Hypericum androsaemum*), fern (*Blechnum spicant*), nettle (*Urtica dioica*), moss (*Physcomitrium patens*), tobacco (*Nicotiana benthamiana*) and thale cress (*Arabidopsis thaliana*)] for their capability of providing protection to Vero E6 cells against the SARS-CoV-2 virus. As shown in Figure S2, certain degree of protection was observed for most of the tested plant species; the clearest protecting activity was observed for *Marchantia* crude extracts.

Identification of the antiviral metabolite

In order to identify the bioactive metabolite(s), we followed a bioactivity-guided chromatographic fractionation of the *Marchantia*'s WT crude extracts, which showed strong antiviral effect in previous assays. Chromatographic fractions were obtained via flash column employing a solvent polarity gradient, starting at *n*-hexane (100%) up to AcOEt:MeOH (4:1, v/v). A total of 56 fractions were obtained; fractions of a similar composition, based on their thin layer chromatography (TLCs) profiles, were combined and evaluated as 12 new pooled fractions (1-12). Fractions 10, 11 and 12 showed antiviral activity in the monolayer protection assay (Figure 3A-B). To directly confirm their antiviral activity, the viral antigen load reduction after inoculation of cell cultures with SARS-CoV-2 (MOI = 0.01) was measured. In this experimental setup, viral antigen accumulates as a consequence of virus propagation and can be quantitated using automated immunofluorescence microscopy. Figure 3C shows a dose-dependent reduction of viral antigen accumulation and the absence of cytotoxicity, as confirmed by normal cell numbers, estimated by DAPI staining and image analysis, and cell viability studies performed in parallel, uninfected cultures by MTT assays (Figure 3C).

Interestingly, TLCs of the three active fractions presented red fluorescent spots (under long wave ultraviolet light, 365 nm; Supplementary Figure S3A), which are characteristic of plant chlorophylls, but with a smaller retention factor [$R_f = 0.36$, AcOEt:MeOH (9:1, v/v)] than that of chlorophyll ($R_f = 0.92$). At this point, we suspected that the active antiviral metabolite(s) could be related to plant chlorophylls, especially because a weak antiviral activity was observed at low dilutions of fractions 2 and 3 (Figure 3A), both containing chlorophyll. Indeed, these fractions, when re-chromatographed, yielded spots on the TLC with an R_f consistent with that observed in the active fractions 10 to 12, indicating that the active metabolite is a chlorophyll derivative (Supplementary Figure S3B). It is worth mentioning that heat notably accelerated the chlorophyll decomposition into the investigated metabolite.

Next, we employed preparative TLC in order to better isolate and characterize the red-light emitting metabolite. Photosynthetic metabolites were extracted from *M. polymorpha* WT and *Mpc1hdz* plants, and the extracts subjected to preparative TLC. Three spots showed fluorescence in the vicinity of the expected R_f ; these spots were isolated, analysed by HPLC-UV-MS and the bioactivity assayed as fractions C, D and E (Figure 4A). Fraction D showed the strongest antiviral activity (Figure 4B), which corresponded with an enrichment of compound **1** in the UV chromatograms (Figure 4C). Careful analysis of the MS spectra (positive mode) revealed a molecular formula of $C_{35}H_{36}N_4O_5$ for compound **1**, as deduced by HR-ESI⁺-MS from its monoprotonated molecular ion, $[M+H]^+$, with a m/z of 593.2759. The identified molecular formula (containing four nitrogen atoms), the exact mass and the characteristic red fluorescence of the compound helped to identify **1** as the chlorophyll catabolite *Pheophorbide a* (PheoA, Figure 4C). The identity of PheoA was further confirmed by comparison with both a commercially available original standard (Santa Cruz Biotechnology) and a semisynthetic sample. PheoA was obtained semi-synthetically from *Marchantia* in good overall yield via a

solvent-free, thus, environmentally friendly method (Supplementary Figure S4 and M&M section).

Antiviral activity of PheoA.

To confirm the antiviral potential of PheoA, a commercially available PheoA stock solution was serially diluted and mixed with a virus stock to inoculate Vero E6 and Huh7-ACE2 cells (human hepatoma cells expressing ACE2). Cells were fixed 72 h post-inoculation and stained with crystal violet to visualize the integrity of the cell monolayer. Figure S5 shows consistent protective capacity of PheoA at concentrations above 40 ng/ml (67 nM) in both cell lines.

PheoA antiviral activity was further confirmed by immunofluorescence microscopy, to estimate virus propagation, and an MTT assay to evaluate compound cytotoxicity. PheoA dose-response curves demonstrate PheoA's antiviral activity against SARS-CoV-2 in Vero E6 and human lung epithelial cells (A549-ACE2 and Calu3); in all three models, no cytotoxicity was observed (Figure 5A-C). This dataset was used to determine effective concentrations (EC₅₀ and EC₉₀) and cytotoxicity indexes. The estimated EC₅₀ and EC₉₀ values were around 14 ng/mL (25 nM) and 156 ng/mL (86 nM), with a wide therapeutic window in all tested cell lines (Table 1).

Table 1: Potency and cytotoxicity indexes of commercially available PheoA

Cell line	EC ₅₀ (nM)	EC ₉₀ (nM)	CC ₅₀ (nM)
Vero-E6 (green monkey)	25	86	>8420
A549-ACE2 (human lung)	52	263	2020
Calu3 (human lung)	34	232	>8420

To fully evaluate the extent to which PheoA interferes with SARS-CoV-2 infection (i.e. on viral replication) – given the narrow dynamic range of immunofluorescence –, we employed RT-qPCR to determine the extracellular viral RNA load in human cells inoculated with SARS-

CoV-2 (MOI = 0.001). In this experimental setup, remdesivir (5 μ M) reduced extracellular viral RNA by three orders of magnitude. Interestingly, PheoA significantly reduced viral spread even at the lowest tested concentration (150 ng/mL; 0.25 μ M), as shown by the three times log reduction in viral RNA accumulation after a 48h incubation period (Figure 5D). These results indicate that PheoA displays a remarkable potency and a satisfactory therapeutic index, and suggest that it may be considered as a potential candidate for antiviral therapy against SARS-CoV-2. Furthermore, results also suggest that PheoA is a major determinant of the antiviral activity observed in crude *Marchantia* extracts (Figure 1). This notion is underscored by the fact that the semisynthetic PheoA (88-94% by HPLC-UV/Vis) showed comparable potency to crude extracts in the different cell lines (Supplementary Figure S6). Nevertheless, other related chlorophyll metabolites may also contribute with antiviral activity. In fact, pyropheophorbide a (pPheoA), which was also found in antiviral fractions was tested to verify its antiviral potential in Vero E6 cells. The pPheoA showed antiviral activity in the absence of measurable cytotoxicity with an EC₅₀ of 185 nM (Supplementary Figure S7), suggesting lower potency than PheoA and further underscoring a major role for PheoA in the antiviral activity of *Marchantia* extracts.

Antiviral spectrum of Pheophorbide A

PheoA has previously been proven as an antiviral against the hepatitis C virus (HCV)²⁷ and virucidal against herpes simplex virus (HSV)²⁸. Thus, we determined the antiviral spectrum of PheoA on different enveloped +RNA viruses. First, we confirmed antiviral activity against HCV, using a surrogate model of infection by propagation-deficient, *bona fide* reporter virus bearing a luciferase reporter gene generated by trans-complementation (HCVtcp). Dose-response curves of the luciferase activity in HCVtcp-infected Huh7 cells indicated an EC₅₀ of

177 ng/mL (300 nM) for PheoA against HCV (Figure 6A), very similar to the previously reported EC_{50} ²⁷.

Next, we asked whether PheoA antiviral activity against SARS-CoV-2 could also be observed against other human coronaviruses such as hCoV-229E (Genus alphacoronavirus; Subgenus Duvinecovirus), which has been associated with mild respiratory infections²⁹. Huh7 cells were inoculated with a GFP reporter-expressing recombinant hCoV-229E (MOI = 0.01) and total GFP expression in the target Huh7 cells was evaluated by automated microscopy 48 h post-inoculation. Similar to the results with the HCV infection model, PheoA reduced viral spread (EC_{50} of 76 ng/mL; 128 nM) while no cytotoxicity was observed (Figure 6B). Strikingly, comparable results were obtained in an experimental model of infection by the West Nile Virus, a mosquito-borne zoonotic pathogen that may cause encephalitis in infected humans. A recombinant, di-cistronic infectious molecular clone expressing GFP in the second cistron³⁰ was used to inoculate Huh7 cells (MOI = 0.01). Cells were imaged 48 h post infection and the degree of virus propagation was determined via automated microscopy; dose response curves (Figure 6C) indicated antiviral activity for PheoA with an EC_{50} of 38 ng/mL (68 nM). Altogether, these results suggest that PheoA displays a broad-spectrum antiviral activity against +RNA viral pathogens. In view of these results, we decided to test PheoA antiviral activity against the vesicular stomatitis virus (VSV), a negative-strand RNA (-RNA) virus. Dose-response experiments, similar to those describe above, were performed employing A549-ACE2 cells. Interestingly, the VSV-GFP³¹ was not susceptible to PheoA doses largely exceeding those for which antiviral activity was observed against SARS-CoV-2 (Figure 6D). Collectively, these results suggest that PheoA is a broad-spectrum antiviral and that +RNA viruses are particularly susceptible.

Pheophorbide a can be employed in combination with remdesivir.

Once the broad antiviral activity of PheoA has been demonstrated, we studied whether the addition of PheoA to remdesivir treatment could result in a synergistic effect on viral infection. Thus, combination treatments were performed with increasing doses of PheoA and remdesivir. Drugs were mixed in different proportions, combined with infectious SARS-CoV-2 (MOI = 0.01) and the mixtures were used to inoculate Vero E6 cells. Twenty-four hours later, cells were fixed and processed to determine the infection efficiency as described in Figure 5. Individual treatment with either compound resulted in the expected dose-dependent inhibition of virus infection, achieving the EC_{50} at the expected doses (2000 nM for remdesivir and 40 nM for PheoA). Increasing concentrations of PheoA improved remdesivir efficacy and viceversa. However, full analysis of the combinations resulted in a synergy index close to three, indicating that the drug combination is mostly additive³², with an area of synergy at concentrations close to the EC_{50} s (Figure 7). These results suggest that combinations of PheoA with other antivirals may result beneficial, as it was observed by its additive effect in combination with remdesivir in cell culture infection models.

Characterization of Pheophorbide a mode of action on SARS-CoV-2 infection.

Next, antiviral efficacy of PheoA was compared when PheoA was (i) present at all times, (ii) added only during virus inoculation, or (iii) added only after virions had effectively penetrated the cells in single-cycle infection experiments (MOI = 5). Imatinib (15 μ M), for which antiviral activity at the level of virus entry has previously been demonstrated³³, was employed as the reference compound. Infection efficiency revealed the expected antiviral activity for imatinib and PheoA when maintained at all times in the experiment (Figure 8A). Imatinib showed comparable efficacy when added during the virus entry phase and greatly lost efficacy when added after virion internalization, as expected for an entry inhibitor. Similar results were obtained with PheoA, inhibition was nearly identical when maintained at all times or added

during viral entry, but only ca. 6% of the maximum efficacy was observed when added after virion internalization. These results suggest that PheoA is mainly acting at early stages of the infection, potentially at the level of viral entry.

In view of these results, we directly tested this hypothesis by determining the antiviral activity of PheoA in a surrogate model of infection recapitulating only aspects related with viral entry such as receptor recognition, virion internalization or membrane fusion. This system is based on reporter retroviral vectors pseudotyped with SARS-CoV-2 Spike protein (Spp) or VSV glycoprotein as a control (VSVpp). Infection efficiency in the presence of antiviral molecules is determined as the relative expression values of the reporter gene, in this case a Firefly luciferase³³. Relative infection efficiency was measured in the presence of the entry inhibitor imatinib (15 μ M) and antiviral doses of PheoA. As expected, imatinib selectively inhibited Spp and not VSVpp entry (Supplementary Figure S8). PheoA barely interfered with either retroviral pseudotype infection efficiency, with a maximum reduction of 40% at the maximum dose (400 ng/ml; 678 nM) (Supplementary Figure 8). These results suggest that, while time-of-addition experiments suggest that PheoA interferes predominantly with early aspects of the infection, surrogate models of viral entry indicate that it does not interfere substantially with molecular events leading to viral entry *per se*.

PheoA irreversibly interferes with the virion infectivity in HSV and influenza infection models²⁸, therefore, we explored whether PheoA could be virucidal for SARS-CoV-2 virions, a property that would be compatible with the observations made in the time-of-addition experiments. Thus, a known number of infectious particles (10^5 TCID₅₀) were mixed with increasing doses of PheoA [8 ng/mL(0.014 μ M) to 5000 ng/mL (8.45 μ M)] and incubated for 30 minutes before residual infectivity titer was calculated by endpoint dilution and TCID₅₀ determination. The dose of PheoA was kept below its effective concentrations during the titration experiment. Figure 8B shows how pre-incubation of the infectious virions with PheoA

(40 ng/mL or more) resulted in irreversible, dose-dependent inactivation of the virus infectivity. These results suggest that PheoA is virucidal for SARS-CoV-2 virions and that virion infectivity inactivation contributes to its overall antiviral effect.

Discussion

Due to their metabolic richness plants have been traditionally used as source of medicines. The potential of plant metabolites in pharmacology is still far from being saturated, particularly in certain plant clades. Indeed, bryophytes (non-vascular plants) are particularly rich in specialized metabolites that are rarely found in other plant lineages³⁴. Here, we explored this richness in order to find antiviral compounds against the SARS-CoV-2 virus by employing an activity-guided chromatographic method; and identified PheoA as a potent antiviral, very efficient not only against SARS-CoV-2 but also against several other enveloped viruses.

The first evidence for antiviral activity of PheoA derives from observations made on HSV infection models³⁵. In those initial reports, some degree of selectivity towards other viruses was reported, since adenovirus (Type VI), Japanese Encephalitis virus (JEV) or poliovirus were not affected by treatment with PheoA-enriched algal extracts³⁶. Subsequent studies suggested that PheoA and pPheoA display broad-spectrum antiviral activity against enveloped viruses, including influenza A²⁸ and HIV³⁷, but not against non-enveloped viruses²⁸. Ohta et al. reported that PheoA-containing preparations may display virucidal activity against HSV³⁶, a concept that was further supported by Bouslanu et al.²⁸. Our observations support that PheoA inactivates SARS-CoV-2 (enveloped +RNA virus) virion infectivity through a virucidal mode of action. First, time-of-addition experiments indicate that early aspects of the infection are targeted by PheoA. Second, the study of viral entry using retroviral pseudotypes did not reveal any measurable antiviral activity against SARS-CoV-2 entry, suggesting that receptor recognition by the Spike protein, particle internalization and Spike-mediated fusion are not affected by

PheoA. Similar models have been used to identify key SARS-CoV-2 entry factors as well as to study antibody neutralizing activity^{38,39}. Thus, irreversible inactivation of viral infectivity (virucidal activity) was tested as a possible mechanism reconciling these apparently contradicting observations. Pre-incubation of infectious SARS-CoV-2 virions with PheoA rendered the virions non-infectious even when PheoA-virus dilutions were performed below active PheoA concentrations. These observations are similar to those reported by other groups in other infection models^{28,36}. The virtual lack of activity of PheoA at post-entry levels may be explained by the fact that PheoA can only act on the viral particle, or that PheoA requires longer incubation periods to penetrate the cell and interfere with downstream steps of the virus lifecycle. Given that overall effectiveness of PheoA as virucidal is substantially stronger than during multiple cycle infection experiments, it is likely that virucidal activity is the main mechanism for interference with SARS-CoV-2 infection.

PheoA has been shown to integrate into biological membranes⁴⁰. Thus, it is possible that PheoA could insert into the viral envelope lipid bilayer, altering its biophysical properties, or even disrupting it, thus rendering the virion non-infectious. This would explain PheoA's virucidal activity and its broad-spectrum among enveloped viruses. However, some degree of selectivity was observed since PheoA doses that completely abolished infection by several +RNA viruses did not interfere with VSV or retroviral pseudotype (both also enveloped) infection. It is likely possible that the membrane's lipidic composition could play a key role for PheoA incorporation. In this sense, VSV and retroviral pseudotypes are assembled at the plasma membrane^{41,42}, while the rest of the tested virus particles are assembled in intracellular compartments^{43,44}, which display a very different membrane composition from that of the plasma membrane⁴⁵. PheoA is a plant derived porphyrin closely related to animal porphyrins, which have been widely described as broad-spectrum virucidals (reviewed in Sh. Lebedeva et al.⁴⁶). Virion inactivation is thought to occur through incorporation of porphyrins into the viral

envelope membrane and modifying its physico-chemical properties, thus interfering with host cell recognition and fusion processes. However, porphyrins such as protoporphyrin IX display antiviral activity independently of their virucidal activity at post-entry steps and have been proposed to interfere with receptor (ACE2) recognition in SARS-CoV-2 infection models¹⁹. The structural resemblance between PheoA and proporphyrin IX may explain their similar antiviral properties (broad spectrum and virucidal), but, at the same time, their differences may contribute to PheoA's increased tolerability and *in vivo* effectiveness, an issue that has extensively been explored for different PheoA applications as photosensitizer in photodynamic therapies against cancer^{47,48}.

One huge advantage of PheoA is that it is readily available from plant and algae chlorophyll. PheoA is the dephytylation and demetallation product of chlorophyll *a*, processes mediated by chlorophyllase and Mg-dechelataase, respectively^{49,50}. Chlorophyllase activity is favored by high temperatures (60-80 °C)⁴⁹ and its accumulation varies throughout plant development and in stress conditions. In this study, we also exploited stress conditions (heat) that favour PheoA accumulation and PheoA was semisynthetically prepared from *M. polymorpha* in good overall yield.

Another advantage of PheoA is that its combination with remdesivir has an additive effect with no cross inhibition in their antiviral activity, and a mild synergy. This, together with its low toxicity *in vivo*, represents an advantage that could be clinically exploited⁵¹.

Materials and Methods

Equipment and reagents

All solvents were of ACS quality unless stated otherwise. Commercially available PheoA was purchased from Santacruz Biotechnology (>90% by HPLC). A Geno Grinder Spex/SamplePrep 2010 was employed for tissue homogenization. Glass or aluminium supported Silica gel 60

(Merck) was used for preparative and analytical TLCs, respectively; for flash column purification, silica gel 60 $\square$, 230-400 mesh, 40-63 μm was employed. HPLC-UV-MS analysis was carried out by using a Waters Separations module Alliance e2695 system, a Waters QDa Detector Acquity QDa and a Waters Photodiode Array Detector 2996. HPLC was performed by using HPLC grade solvents and a Sunfire C18 (4.6 $\times$ 50 mm, 3.5 μm) column at 30 $^{\circ}\text{C}$, with a flow rate of 1 mL/min and a mobile phase gradient from 70 to 95 of A (formic acid 0.1% in CH_3CN) in B (0.1% of formic acid in H_2O) for 10 minutes. Electrospray in positive mode was used for ionization. The HR-MS analysis was carried out by using an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 quadrupole-time of flight (QTOF) mass spectrometer. $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (75:25, v/v) was used as the mobile phase at 0.2 mL/min. The ionization source was an ESI interface working in the positive-ion mode. The electrospray voltage was set at 4.5 kV, the fragmentor voltage at 150 V and the drying gas temperature at 300 $^{\circ}\text{C}$. Nitrogen (99.5% purity) was used as nebulizer (207 kPa) and drying gas (6 L/min). The scan range was 50–1100 m/z.

Preparation of crude M. polymorpha extracts

Plant material (10 g, fresh weight) was collected and dried in an oven (60 $^{\circ}\text{C}$) until constant weight. The dry tissue was ground to a fine powder using a Geno/Grinder (2x 2 min at 2700 rpm) and extracted two times at room temperature with 30 mL of $\text{CHCl}_3:\text{MeOH}$ (2:1, v/v) for at least 6 h each time. Extracts were combined and concentrated under a nitrogen flow. The remaining solid was dissolved in DMSO (1 mL) to create the stock solutions employed in the bioassays.

Chromatographic fractionation of extracts

Plants extracts were prepared as described above starting from ca. 20 g of plant material and directly subjected to silica gel flash column chromatography employing a solvent polarity gradient starting at *n*-hexane (100%) up to $\text{AcOEt}:\text{MeOH}$ (4:1, v/v). A total of 56 metabolite-

enriched fractions were obtained. Fractions were analyzed by TLC and those of similar composition were combined to render 12 new pooled fractions, which were screened for antiviral activity.

Preparative TLCs

Photosynthetic metabolites were extracted [two times, o/n, acetone (30 mL)] from fresh, finely grounded *M. polymorpha* thallus (ca. 40 g). The combined extracts were concentrated to a final volume of 10 mL, centrifuged (4000 rcf), filtrated (45 µm, Whatmann PTFE filters) and chromatographed on preparative TLC plates employing the solvent system AcOEt:MeOH (9:1, v/v).¹ Selected fluorescent spots (C, D and E) were scraped off, eluted (AcOEt:MeOH, 4:1, v/v) and dried under a nitrogen stream. Single components (C, D and E) were prepared at a 10 mg/mL and submitted to both antiviral assays, as described below, and HPLC-UV-MS analysis as described above.

Semisynthetic preparation of PheoA

Fresh *M. polymorpha* thallus (ca. 50 g) was oven dried to produce ca. 3.5 g of dry material, which was ground to a fine powder in the GinoGrinder as described above. The obtained powder (3 g) was extracted (3x, Acetone, 90 mL), the extracts combined and silica gel (6 g, ratio 2:1 by weight relative to the dried leaf powder) added. The solvent was evaporated under reduced pressure to produce an impregnated silica, which was heated (60 °C) overnight to further potentiate PheoA production. The obtained silica was directly loaded into a flash column that was run as follows: column ID = 3 cm, silica (70 g), *n*-hexane:AcOEt (1:1, 300 mL), AcOEt:MeOH (9:1, 300 mL; 4:1, 600 mL; 7:3, 300 mL), fractions of ca. 35 mL were collected. Fractions were analyzed by TLC and the obtained PheoA (3.9 mg, 0.13 % from oven dried leaf material).

¹ The TLC was heated (heat gun) vigorously at the extract application point before being developed; this procedure enhances Chlorophylls conversion into PheoA.

Cell culture

Vero E6 (ATCC) and Calu3 (ATCC) cell lines were kindly provided by Dr. Enjuanes (CNB-CSIC). A549 cells were kindly provided by Dr. Juan Ortín (CNB-CSIC) and Huh7 cells were kindly provided by Dr. Chisari (TSRI, La Jolla). A549 and Huh7 cells were transduced with a retroviral vector enabling expression of ACE2 in a di-cistronic expression cassette also conferring resistance to blasticidine. Transduced populations were selected using 2.5 µg/mL of blasticidine. All cell cultures were kept in complete media (DMEM) supplemented with 10 mM HEPES, 1X non-essential amino acids (gibco), 100 U/mL penicillin-streptomycin (GIBCO) and 10% fetal bovine serum (FBS; heat-inactivated at 56 °C for 30 min). Unless otherwise stated, all infection experiments were performed at 37°C in a CO₂ incubator (5% CO₂) the presence of 2% FBS and in the absence of selection antibiotics.

Viruses

SARS-CoV-2 (Orthocoronavirinae; Alphacoronavirus; Sarbecovirus; strain NL/2020) was kindly provided by Dr. R. Molenkamp, Erasmus University Medical Center Rotterdam. SARS-CoV2 stocks were produced and titrated in VeroE6 cells as described previously (PMID 33917313). VSV-GFP³¹ was kindly provided by Dr. Rodriguez (CNB-CSIC). WNV-GFP recombinant virus was rescued from cloned cDNA as reported previously³⁰. Trans-complemented defective reporter HCV virions (HCVtcp) were produced as described in (Steinmann et al., 2008)⁵². The hCoV-229E-GFP⁵³ was kindly provided by Dr. Thiel (University of Basel) and propagated in Huh7 cells at 33°C in a controlled 5% CO₂ environment.

Cytopathic effect protection assays in Vero E6 and Huh7-ACE2 cells

Vero E6 or Huh7-ACE2 cell monolayers were inoculated (MOI = 0.001) in the presence of a wide range of two-fold dilutions of the crude, or partially purified extracts, or pure compounds

and incubated for 72 h. Cytopathic effect and lack thereof was visualized by crystal violet staining, as previously described³³. Untreated and solvent-treated cells were included in each plate as controls.

Evaluation of the antiviral activity by immunofluorescence microscopy

Vero E6, A549-ACE2 or Calu3 were seeded onto 96-well plates as described above and infected in the presence of the indicated compound dose (MOI = 0.01). Twenty-four hours post infection (48 h for Calu3 cells), cells were fixed for 20 minutes at room temperature with a 4% formaldehyde solution in PBS, washed twice with PBS and incubated with incubation buffer (3% BSA; 0.3% Triton X100 in PBS) for 1 h. A monoclonal antibody against the N protein was diluted in the incubation buffer (1:2000, v/v; Genetex HL344) and incubated with the cells for 1 h; after this time, cells were washed with PBS and subsequently incubated with a 1:500 (v/v) dilution of a goat anti-rabbit conjugated to Alexa 488 (Invitrogen-Carlsbad, CA). Nuclei were stained with DAPI (Life Technologies) as recommended by the manufacturer during the secondary antibody incubation. Cells were washed with PBS and imaged using an automated multimode reader (TECAN Spark Cyto; Austria).

All the infection experiments were performed by mixing the virus and compound dilutions 1:1 (v/v) before addition to the target cells. In the time-of-addition experiments, Vero E6 cultures were inoculated (MOI from 0.5-1) for 1 h in the presence or absence of the compounds at 37 °C. Subsequently, virus-compound mixtures were left at all times, or removed and replaced with fresh 2% FBS complete media containing or not the tested compounds (see experimental scheme in Figure 8 for details). Cells were fixed 6 h post-inoculation.

Viral RNA quantitation by RT-qPCR

A549-ACE2 cell monolayers were inoculated at MOI = 0.001 in the presence of non-toxic concentrations of the compound. Forty-eight hours later, cell supernatants were collected and

heat-inactivated as described in (Smyrlaki et al., 2020)⁵⁴, and processed directly for RT-qPCR. Alternatively, cell lysates were prepared using the Trizol reagent (Thermo Scientific) and the viral RNA content was determined by RT-qPCR using previously validated sets of primers and probes specific for the detection of the SARS-CoV-2 E gene and the cellular 18S gene, for normalization purposes. Δ Ct method was used for relative quantitation of the intracellular viral RNA accumulation in compound-treated cells compared to the levels in infected cells treated with DMSO (set as 100%).

Cytotoxicity measurement by MTT assays

Cell monolayers were seeded in 96-well plates. The day after cells were treated with a wide range of compound concentrations and forty-eight hours later they were subjected to an MTT assays using standard procedures⁵⁵. The CC₅₀ values were graphically interpolated from dose-response curves obtained with three biological replicates.

Assessment of viral entry using retroviral pseudotypes

Retroviral particles pseudotyped with SARS-2-CoV spike envelope protein (Spp) were produced in HEK293T cells as previously described³⁹ with materials kindly provided by Dr. F. L. Cosset (INSERM, Lyon) and J. M. Casasnovas and J. G. Arriaza (CNB-CSIC) for the S protein cDNA. Particles devoid of envelope glycoproteins were produced in parallel as controls.

Statistical Analysis

Descriptive statistics were calculated using Microsoft Excel. One-way ANOVA and *post-hoc* tests were calculated using IBM SPSS Software Package (version 26). EC₅₀ and EC₉₀ values were obtained employing the PROBIT regression method⁵⁶ using IBM SPSS vs26.

Synergy analysis was carried out in the web-based platform Synergy Finder (<https://synergyfinder.fimm.fi/>)³².

Acknowledgements

This research was funded by CSIC (PIE-RD-COVID-19 ref 202040E236 to R.S., PIE-RD-COVID-19 ref. E202020E079 to P.G and U.G, and CSIC-CoV19-153 ref PIE-202080E221 to M.G-R.), EVA (European Virus Archive; grant agreement No 871029 to P.G.) and the European Union-NextGenerationEU. G.H.J-A. was supported by the Deutsche Forschungsgemeinschaft (Individual Research Grant JI 241/2-1). We thank Gema Calvo, Jennifer Moya, Laura Barbado and Enara San Sebastian for outstanding technical assistance.

References

1. Folegatti, P. M. *et al.* Safety and immunogenicity of the ChAdOx1 nCoV-19 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial. *Lancet* 1–13 (2020) doi:10.1016/S0140-6736(20)31604-4.
2. Grein, J. *et al.* Compassionate Use of Remdesivir for Patients with Severe Covid-19. *N. Engl. J. Med.* **382**, 2327–2336 (2020).
3. Li, X. *et al.* Is hydroxychloroquine beneficial for COVID-19 patients? (2020) doi:10.1038/s41419-020-2721-8.
4. Newman, D. J. & Cragg, G. M. Marine natural products and related compounds in clinical and advanced preclinical trials. *J. Nat. Prod.* **67**, 1216–1238 (2004).
5. Jan, J. T. *et al.* Identification of existing pharmaceuticals and herbal medicines as inhibitors of SARS-CoV-2 infection. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–8 (2021).
6. Gu, C. *et al.* Protoporphyrin IX and verteporfin potently inhibit SARS-CoV-2 infection in vitro and in a mouse model expressing human ACE2. *Sci. Bull.* (2020) doi:10.1016/j.scib.2020.12.005.
7. Wachtel-Galor, S. & Benzie, I. F. F. *Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs. Herbal Medicine: Biomolecular and Clinical Aspects* (2011).
8. Veeresham, C. Natural products derived from plants as a source of drugs. *J. Adv. Pharm. Technol. Res.* **3**, 200–201 (2012).
9. Nicolaou, K. C., Guy, R. K. & Potier, P. Taxoids: new weapons against cancer. *Sci. Am.* **274**, 94–98 (1996).
10. Efferth, T., Li, P. C. H., Konkimalla, V. S. B. & Kaina, B. From traditional Chinese medicine to rational cancer therapy. *Trends Mol. Med.* **13**, 353–361 (2007).
11. Asakawa, Y. & Ludwiczuk, A. Chemical Constituents of Bryophytes: Structures and Biological Activity. *J. Nat. Prod.* **81**, 641–660 (2018).

- 535 12. Asakawa, Y., Ludwiczuk, A. & Nagashima, F. Phytochemical and biological studies of
536 bryophytes. *Phytochemistry* **91**, 52–80 (2013).
- 537 13. Asakawa, Y., Ludwiczuk, A. & Nagashima, F. Phytochemical and biological studies of
538 bryophytes. *Phytochemistry* **91**, 52–80 (2013).
- 539 14. Asakawa, Y., Ludwiczuk, A. & Nagashima, F. *Chemical Constituents of Bryophytes*.
540 *Progress in the Chemistry of Organic Natural Products* vol. 1 (2013).
- 541 15. Chen, F. *et al.* Terpenoid Secondary Metabolites in Bryophytes: Chemical Diversity,
542 Biosynthesis and Biological Functions. *CRC. Crit. Rev. Plant Sci.* **37**, 210–231 (2018).
- 543 16. Davies, K. M. *et al.* The Evolution of Flavonoid Biosynthesis: A Bryophyte Perspective.
544 *Front. Plant Sci.* **11**, 1–21 (2020).
- 545 17. Komala, I., Ito, T., Nagashima, F., Yagi, Y. & Asakawa, Y. Cytotoxic, radical
546 scavenging and antimicrobial activities of sesquiterpenoids from the Tahitian liverwort
547 *Mastigophora diclados* (Brid.) Nees (Mastigophoraceae). *J. Nat. Med.* **64**, 417–422
548 (2010).
- 549 18. Asakawa, Y., Ludwiczuk, A. & Hashimoto, T. Cytotoxic and Antiviral Compounds from
550 Bryophytes and Inedible Fungi. *J. Pre-Clinical Clin. Res.* **7**, 73–85 (2014).
- 551 19. Gu, C. *et al.* Protoporphyrin IX and verteporfin potently inhibit SARS-CoV-2 infection
552 in vitro and in a mouse model expressing human ACE2. *Sci. Bull.* **66**, 925–936 (2021).
- 553 20. Wasternack, C. & Hause, B. Jasmonates: Biosynthesis, perception, signal transduction
554 and action in plant stress response, growth and development. An update to the 2007
555 review in *Annals of Botany*. *Ann. Bot.* **111**, 1021–1058 (2013).
- 556 21. Peñuelas, M. *et al.* Jasmonate-Related MYC Transcription Factors Are Functionally
557 Conserved in *Marchantia polymorpha*. *Plant Cell* **31**, 2491–2509 (2019).
- 558 22. Monte, I. *et al.* Ligand-receptor co-evolution shaped the jasmonate pathway in land
559 plants. *Nat. Chem. Biol.* **14**, 480–488 (2018).
- 560 23. Tanaka, M. *et al.* Direct evidence of specific localization of sesquiterpenes and
561 marchantin A in oil body cells of *Marchantia polymorpha* L. *Phytochemistry* **130**, 77–84
562 (2016).
- 563 24. Romani, F. *et al.* Oil Body Formation in *Marchantia polymorpha* Is Controlled by
564 MpC1HDZ and Serves as a Defense against Arthropod Herbivores. *Curr. Biol.* **30**, 2815-
565 2828.e8 (2020).
- 566 25. Rolin, D. Control of primary metabolism in plants. Annual Plant Reviews, Volume 22.
567 *Ann. Bot.* **98**, 1331–1332 (2006).
- 568 26. Maeda, H. A. Evolutionary diversification of primary metabolism and its contribution to
569 plant chemical diversity. *Front. Plant Sci.* **10**, 1–8 (2019).

- 570 27. Ratnoglik, S. L. *et al.* Antiviral activity of extracts from *Morinda citrifolia* leaves and
571 chlorophyll catabolites, pheophorbide a and pyropheophorbide a, against hepatitis C
572 virus. *Microbiol. Immunol.* **58**, 188–194 (2014).
- 573 28. Bouslama, L., Hayashi, K., Lee, J. B., Ghorbel, A. & Hayashi, T. Potent virucidal effect
574 of pheophorbide a and pyropheophorbide a on enveloped viruses. *J. Nat. Med.* **65**, 229–
575 233 (2011).
- 576 29. Hamre, D. & Procknow, J. J. A new virus isolated from the human respiratory tract.
577 *Proc. Soc. Exp. Biol. Med.* **121**, 190–193 (1966).
- 578 30. Pierson, T. C. *et al.* An infectious West Nile virus that expresses a GFP reporter gene.
579 *Front. Microbiol.* **334**, 28–40 (2005).
- 580 31. Ostertag, D., Hoblitzell-Ostertag, T. M. & Perrault, J. Overproduction of double-stranded
581 RNA in vesicular stomatitis virus-infected cells activates a constitutive cell-type-specific
582 antiviral response. *J. Virol.* **81**, 503–513 (2007).
- 583 32. Ianevski, A., Giri, A. K. & Aittokallio, T. SynergyFinder 2.0: visual analytics of multi-
584 drug combination synergies. *Nucleic Acids Res.* **48**, W488–W493 (2020).
- 585 33. Ginex, T. *et al.* Host-Directed FDA-Approved Drugs with Antiviral Activity against
586 SARS-CoV-2 Identified by Hierarchical In Silico/In Vitro Screening Methods.
587 *Pharmaceuticals (Basel)*. **14**, (2021).
- 588 34. Asakawa, Y. Biologically active compounds from bryophytes. *Pure Appl. Chem.* **79**,
589 557–580 (2007).
- 590 35. Chansakaow, S., Ruangrunsi, N. & Ishikawa, T. Isolation of pyropheophorbide a from
591 the leaves of *Atalantia monophylla* (ROXB.) CORR. (Rutaceae) as a possible antiviral
592 active principle against herpes simplex virus type 2. *Chem. Pharm. Bull. (Tokyo)*. **44**,
593 1415–1417 (1996).
- 594 36. Ohta, S. *et al.* Anti-herpes simplex virus substances produced by the marine green alga,
595 *Dunaliella primolecta*. *J. Appl. Phycol.* **10**, 349–356 (1998).
- 596 37. Zhang, H.-J. *et al.* Natural anti-HIV agents. Part IV. Anti-HIV constituents from *Vatica*
597 *cinerea*. *J. Nat. Prod.* **66**, 263–268 (2003).
- 598 38. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is
599 Blocked by a Clinically Proven Protease Inhibitor. *Cell* 1–19 (2020).
- 600 39. García-Arriaza, J. *et al.* COVID-19 vaccine candidates based on modified vaccinia virus
601 Ankara expressing the SARS-CoV-2 spike induce robust T- and B-cell immune
602 responses and full efficacy in mice. *J. Virol.* (2021).
- 603 40. RÖDER, B., HANKE, T. H., OELCKERS, S. T., HACKBARTH, S. T. & SYMIETZ, C.
604 H. Photophysical properties of pheophorbide a in solution and in model membrane
605 systems. *J. Porphyrins Phthalocyanines* **04**, 37–44 (2000).

41. Drokhlyansky, E., Soh, T. K. & Cepko, C. L. Preferential Budding of Vesicular Stomatitis Virus from the Basolateral Surface of Polarized Epithelial Cells Is Not Solely Directed by Matrix Protein or Glycoprotein. *J. Virol.* **89**, 11718–11722 (2015).
42. Morita, E. & Sundquist, W. I. Retrovirus budding. *Annu. Rev. Cell Dev. Biol.* **20**, 395–425 (2004).
43. De Wit, E., Van Doremalen, N., Falzarano, D. & Munster, V. J. SARS and MERS: Recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* **14**, 523–534 (2016).
44. Hirsch, A. J. *et al.* The Src family kinase c-Yes is required for maturation of West Nile virus particles. *J. Virol.* **79**, 11943–11951 (2005).
45. Meer, G. Membrane lipids, where they are and how they behave: Sphingolipids on the move. *FASEB J.* **24**, 112–124 (2010).
46. Sh Lebedeva, N., A Gubarev, Y., O Koifman, M. & I Koifman, O. The Application of Porphyrins and Their Analogues for Inactivation of Viruses. *Molecules* **25**, (2020).
47. Xodo, L. E., Rapozzi, V., Zacchigna, M., Drioli, S. & Zorzet, S. The chlorophyll catabolite pheophorbide a as a photosensitizer for the photodynamic therapy. *Curr. Med. Chem.* **19**, 799–807 (2012).
48. Bellnier, D. A. *et al.* Clinical pharmacokinetics of the PDT photosensitizers porfimer sodium (Photofrin), 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (Photochlor) and 5-ALA-induced protoporphyrin IX. *Lasers Surg. Med.* **38**, 439–444 (2006).
49. Yilmaz, C. & Gökmen, V. Chlorophyll. *Encycl. Food Heal.* 37–41 (2016) doi:10.1016/B978-0-12-384947-2.00147-1.
50. Saide, A., Lauritano, C. & Ianora, A. Pheophorbide A: State of the art. *Mar. Drugs* **18**, 1–12 (2020).
51. Hajri, A. *et al.* In Vitro and In Vivo Efficacy of Photofrin® and Pheophorbide a, a Bacteriochlorin, in Photodynamic Therapy of Colonic Cancer Cells¶. *Photochem. Photobiol.* **75**, 140 (2002).
52. Steinmann, E., Brohm, C., Kallis, S., Bartenschlager, R. & Pietschmann, T. Efficient trans-encapsidation of hepatitis C virus RNAs into infectious virus-like particles. *J. Virol.* **82**, 7034–7046 (2008).
53. Cervantes-Barragan, L. *et al.* Dendritic cell-specific antigen delivery by coronavirus vaccine vectors induces long-lasting protective antiviral and antitumor immunity. *MBio* **1**, 227 (2010).
54. Smyrlaki, I. *et al.* Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. *Nat. Commun.* **11**, 4812 (2020).
55. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63 (1983).

- 642 56. Cleophas, T. J. & Zwinderman, A. H. Probit Models for Estimating Effective
643 Pharmacological Treatment Dosages (14 Tests) BT - Machine Learning in Medicine –
644 A Complete Overview. in *Machine Learning in Medicine – A Complete Overview* (eds.
645 Cleophas, T. J. & Zwinderman, A. H.) 347–354 (Springer International Publishing,
646 2020).
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648 **FIGURES**

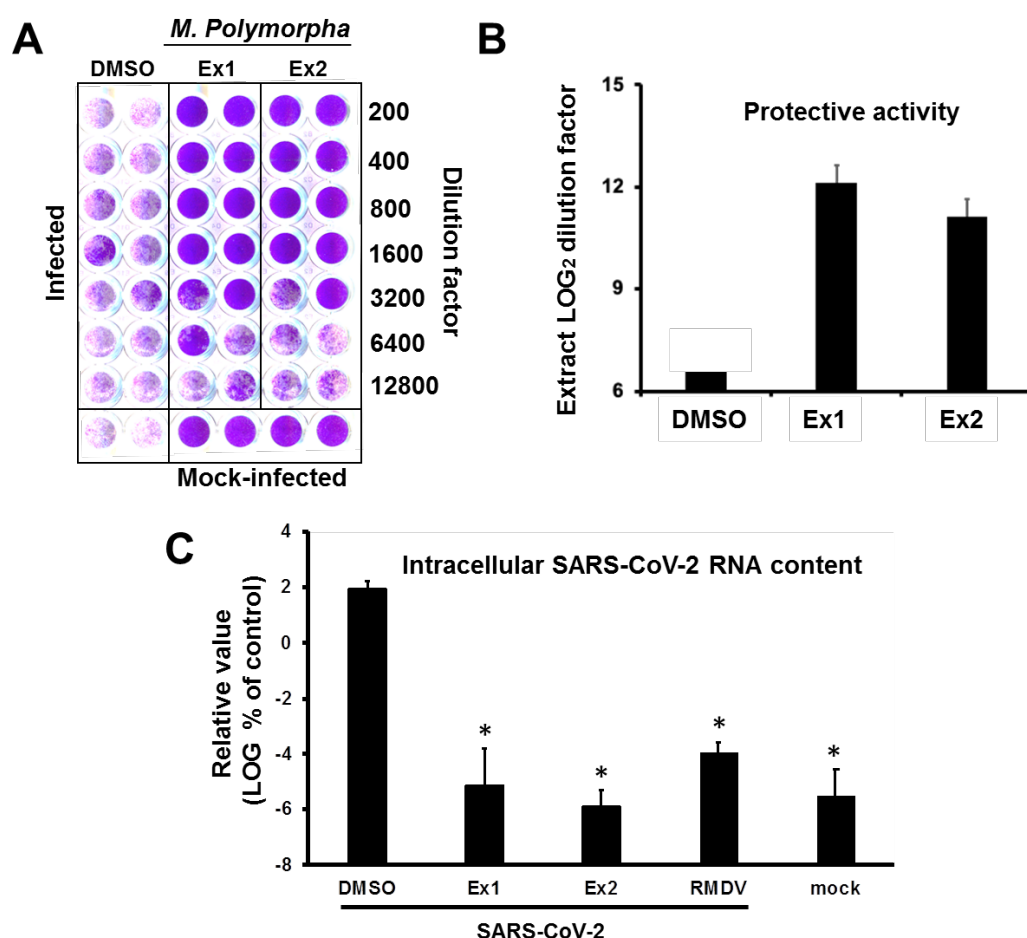


Figure 1: *Marchantia polymorpha* extracts interfere with SARS-CoV-2-induced cytopathic effect and virus propagation.

(A-B) Vero E6 cells were inoculated with SARS-CoV-2 (MOI = 0.001) in the presence of serial 2-fold dilutions of crude extracts from two different *M. polymorpha* ecotypes, *ruderalis* (Ex1) and *polymorpha* (Ex2) and incubated for 72 h, time after which they were fixed and stained with a crystal violet solution. Mock-infected cells were used as the control of the integrity of the cell monolayer. A) Image of an experimental plate showing dose-dependent protection of *M. polymorpha* extracts in comparison with vehicle (DMSO)-treated cells. B) Numeric expression of the protective capacity as the log₂ value of the highest dilution factor capable of full-monolayer protection. Data are shown as average and mean error of two biological replicates. (C) Vero E6 cells were inoculated with SARS-CoV-2 (MOI = 0.001) in the presence of vehicle (DMSO), remdesivir (RMDV; 25 mM) or a 1:800 (v/v) dilution of crude extracts. Parallel samples remained uninfected as control (mock). Total RNA was extracted 72 h post-infection and subjected to RT-qPCR to determine viral load. Normalized viral RNA levels are shown as percentage of the viral RNA found in vehicle-treated cells. Data are shown as mean ($\pm$ SD) of three biological replicates. Statistical significance was estimated using one-way ANOVA and a Dunnet's post-hoc test (* p <0.05).

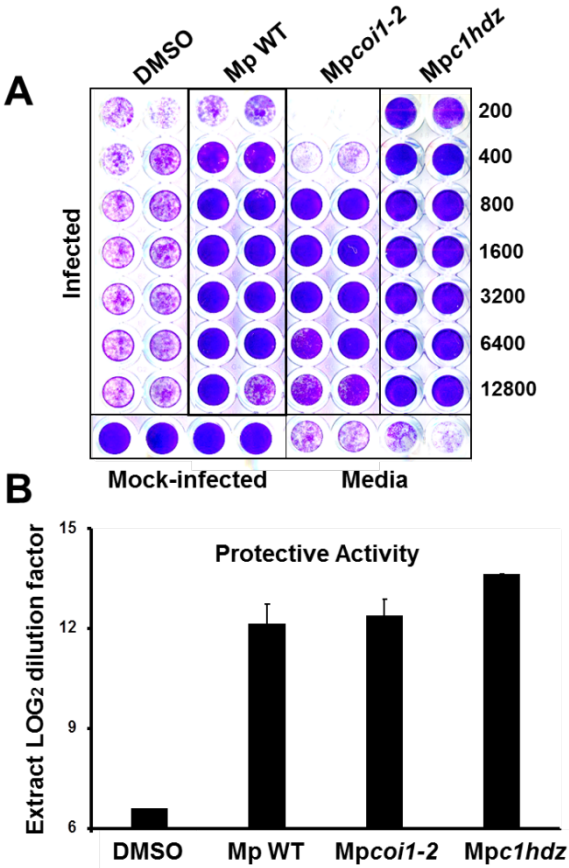


Figure 2: Antiviral candidates unlikely belong to *Marchantia*'s secondary metabolism.

Vero E6 cells were inoculated with SARS-CoV-2 (MOI = 0.001) in the presence of serial 2-fold dilutions of crude extracts from WT, Mpcoi1-2 or Mpc1hdz *Marchantia* plants. Cultures were incubated for 72 h, time after which they were fixed and stained with a crystal violet solution. Mock-infected cells were used as the control of the integrity of the cell monolayer. A) Representative experiment showing dose-dependent protection of plant extracts in comparison with vehicle (DMSO)-treated cells. B) Numeric expression of the protective capacity as the log₂ value of the highest dilution factor capable of full monolayer protection. Data are shown as average ($\pm$ mean error) of two biological replicates.

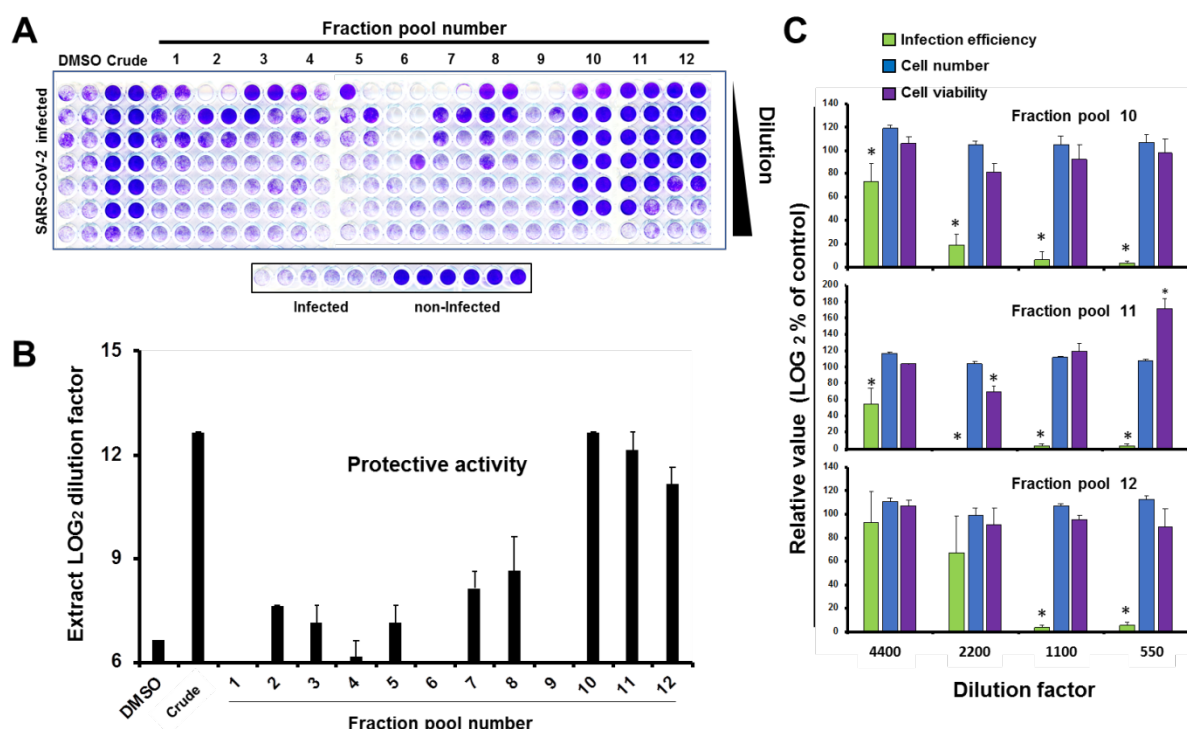


Figure 3: Extract fractionation and identification of antiviral fraction pools.

Vero E6 cells were inoculated with SARS-CoV-2 (MOI = 0.001) in the presence of serial 2-fold dilutions of vehicle (DMSO), a crude *Marchantia* and the fraction pools. Inoculated cultures were incubated for 72 h, time after which they were fixed and stained with a crystal violet solution. Mock-infected cells were used as the control of the integrity of the cell monolayer (non-infected). A) Experimental plates showing cell monolayer integrity (purple) in the presence of fractions containing the antiviral compound(s). B) Numeric expression of the protective capacity as the log₂ value of the highest dilution factor capable of full monolayer protection. Data are shown as average and mean error of two biological replicates. C) Vero E6 cells were inoculated (MOI = 0.01) in the presence of the indicated fraction dilutions and incubated for 24 h before fixation and processing for immunofluorescence microscopy and cytotoxicity assays as described in the materials and methods section. Data are shown as average ($\pm$ SD) of three biological replicates. Statistical significance was estimated using one-way ANOVA (Dunnet's post-hoc test, * $p < 0.05$).

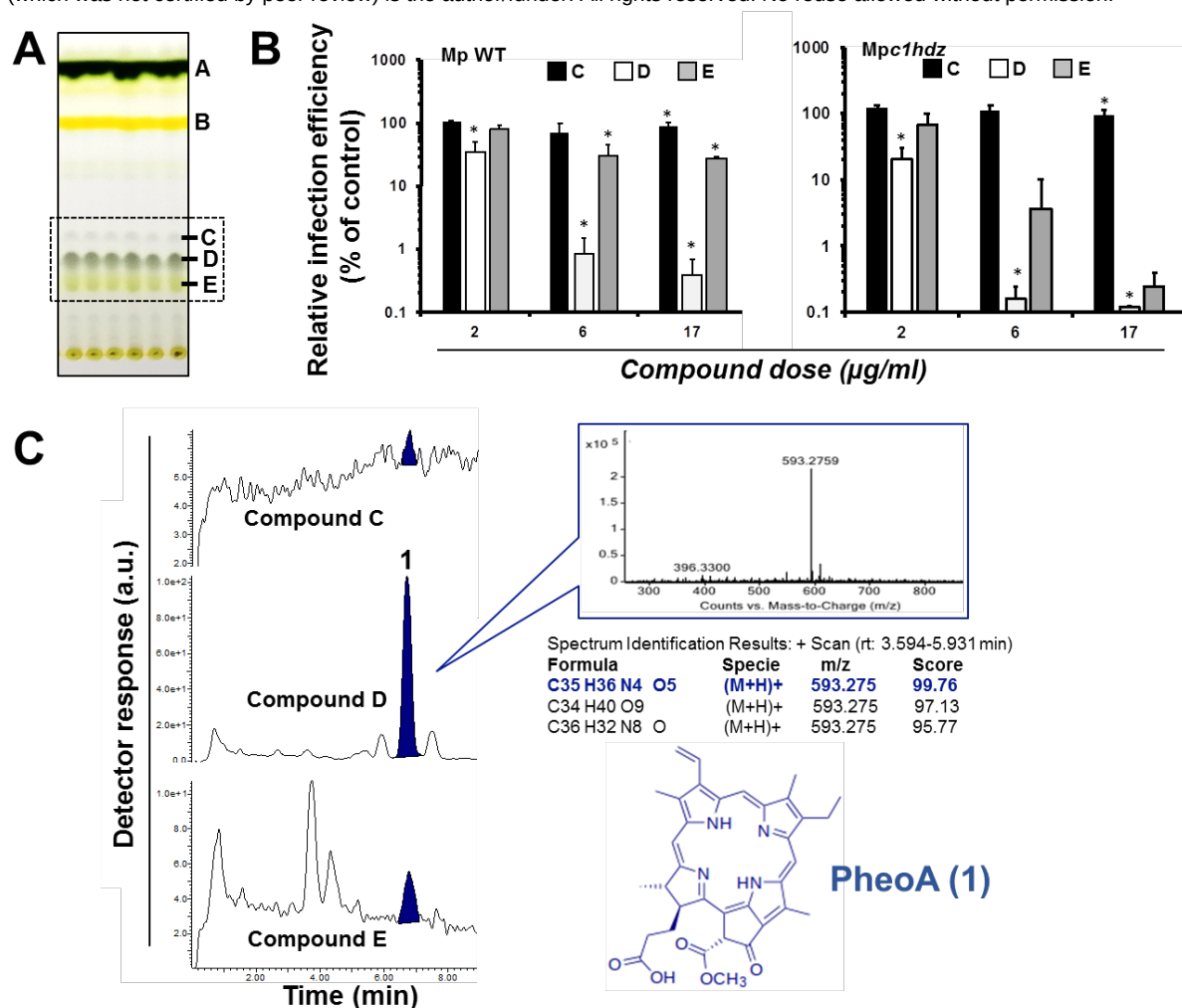


Figure 4: Identification of the main antiviral compound in *Marchantia* extracts.

A) Representative TLC of WT and *Mpc1hdz* *Marchantia* extracts. Compounds C, D and E (box) were tested for their antiviral potential. B) Vero E6 cells were inoculated (MOI = 0.01) in the presence of the indicated compounds and incubated for 24 h before fixation and processing for immunofluorescence microscopy. Data are shown as average and SD of three biological replicates. Statistical significance was estimated using one-way ANOVA and a Dunnet's post-hoc test (* $p < 0.05$). C) Representative HPLC/MS analysis (shown for WT) of fractions C, D, and E, including exact mass determination of the antiviral candidate **1**, and its inferred chemical structure.

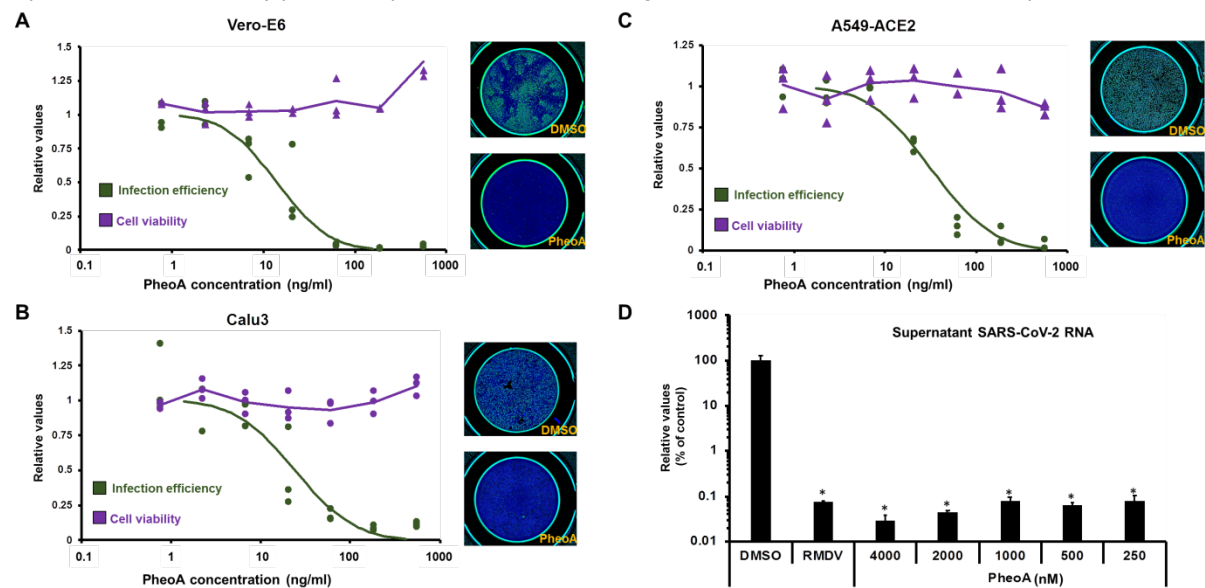


Figure 5: Pheophorbide a shows antiviral activity against SARS-CoV-2 in Vero E6 cells and human lung epithelial A549-ACE2 and Calu3 cell lines.

Commercially available PheoA was serially diluted and mixed (1:1) with SARS-CoV-2 preparations to achieve the indicated compound concentrations and a final MOI of 0.005 for (A) Vero E6 and (B) Calu3 and 0.01 for (C) A549-ACE2 cells. Cultures were incubated for 48 h, fixed and processed for automated immunofluorescence microscopy analysis. Parallel, uninfected cultures were processed for cytotoxicity evaluation using an MTT assay. Relative infection efficiency data (N=3 per dose) are shown as individual data and a PROBIT regression curve (green line) using the represented values. Cytotoxicity data (N=3 per dose) are shown as the individual data and a moving average trendline. D) A549-ACE2 cells were inoculated at MOI = 0.01 in the presence of increasing concentrations of PheoA or 5000 nM remdesivir and incubated for 48 h. Samples of the supernatants were collected, heat-inactivated and directly subjected to RT-qPCR to estimate overall infection efficiency. Data are expressed as relative values compared with the vehicle (DMSO)-treated cells and are shown as mean and standard deviation of three biological replicates (N=3). Statistical significance was estimated using one-way ANOVA and a Dunnet's post-hoc test (*p<0.05).

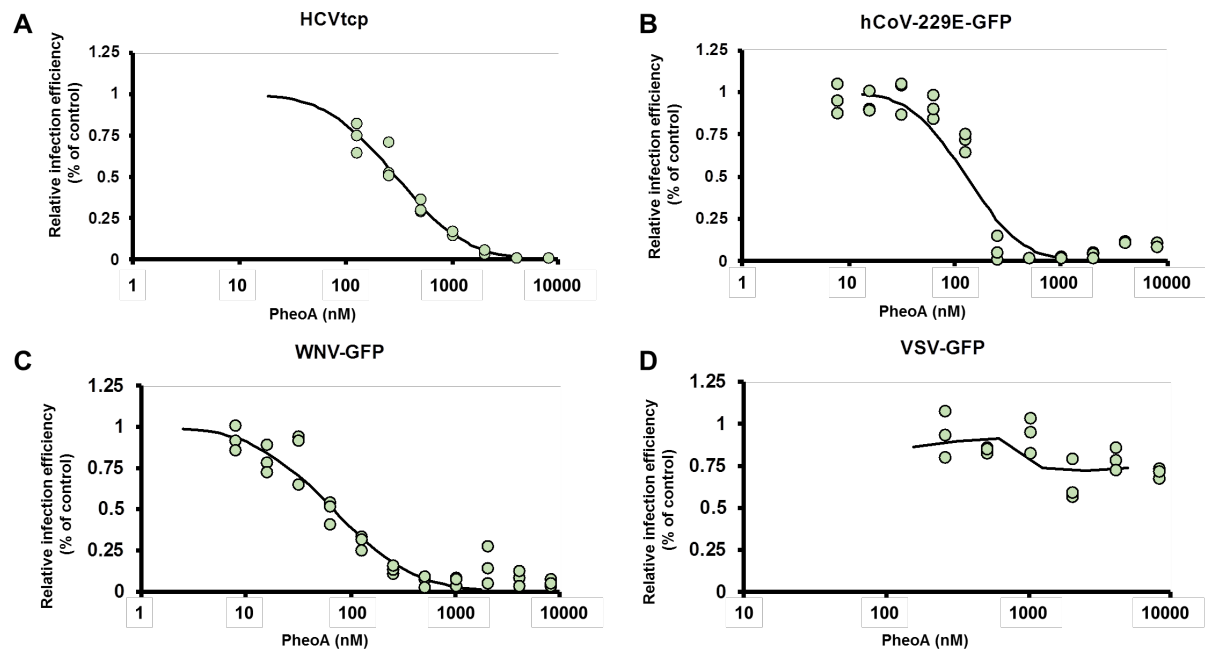


Figure 6: Antiviral spectrum of PheoA against different RNA viruses.

The effectiveness of PheoA was tested against four different recombinant RNA viruses expressing reporter genes. A) Huh7 cells were infected with HCVtcp in the presence of increasing PheoA doses and luciferase activity was determined 48 h post-inoculation. (B-D) Cells were inoculated in the presence of increasing concentrations of PheoA at MOI 0.01 and incubated to enable virus propagation. At the endpoint, cells were fixed and counter-stained with DAPI to control for unexpected cytotoxic effects. Relative infection efficiency was estimated using automated microscopy and is expressed as percentage of the infection efficiency observed in control wells. B) Huh7 cells were infected with hCoV-229E-GFP and fixed 48 h post-inoculation. C) Huh7 cells were infected with WNV-GFP and fixed 48 h post-inoculation. D) A549-ACE2 cells were inoculated with VSV-GFP and fixed 16 h post-inoculation. Individual replicate data are shown as green dots (N=3) and the PROBIT regression curve used to estimate EC50 values is shown.

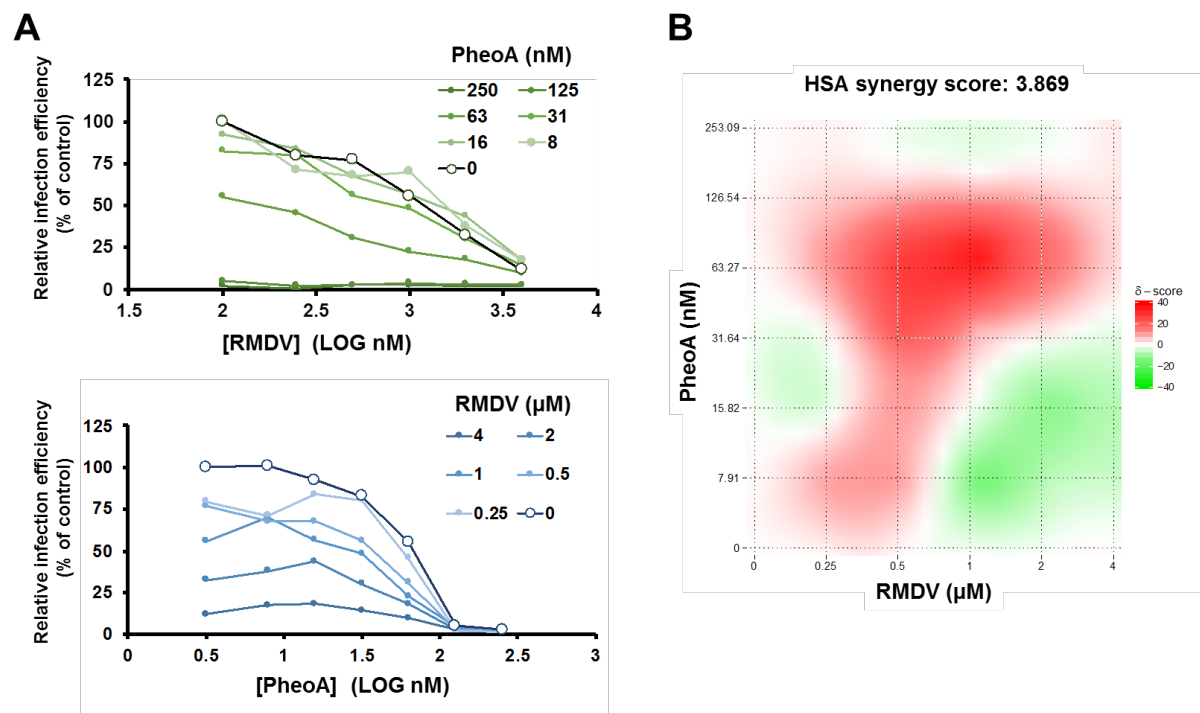


Figure 7: Combination treatment of Pheophorbide a with remdesivir.

Vero E6 cells were inoculated at MOI = 0.005 in the presence of increasing concentrations of PheoA in combination with increasing doses of remdesivir. Twenty-four hours post infection, cells were fixed and processed for automated immunofluorescence microscopy. Relative infection efficiency values were estimated as percentage of the values obtained in mock-treated cells. A) Data are shown as average of two biological replicates. B) Heatmap describing the areas of synergy within the combination treatments.

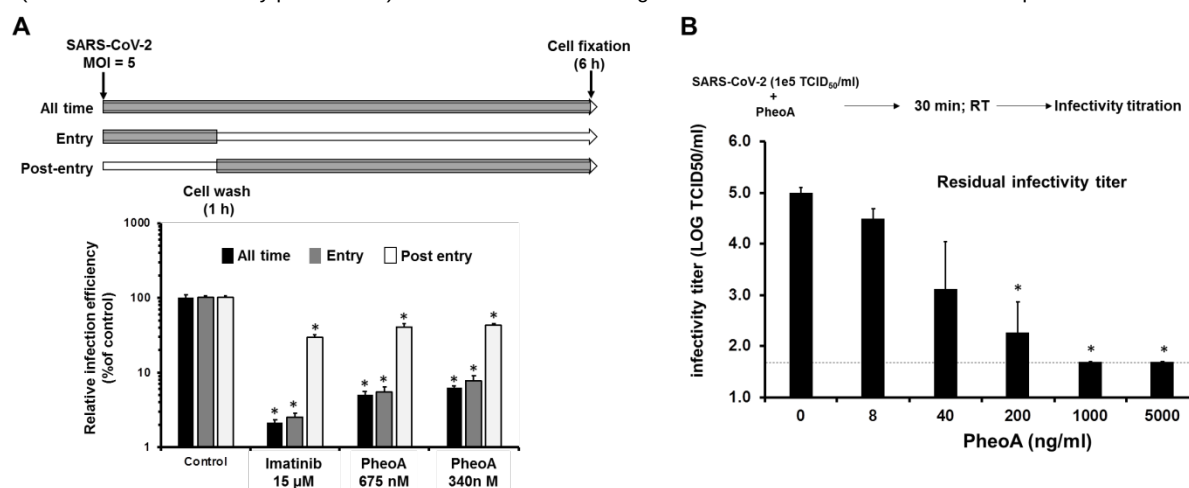


Figure 8: PheoA shows virucidal activity against SARS-CoV-2 interfering with early aspects of the infection.

A) Time-of-addition experiments indicate that PheoA interferes with early aspects of SARS-CoV-2 infection. Vero E6 cells were inoculated at MOI = 5 in the presence (gray) or absence (white) of the indicated doses of PheoA or imatinib as described in both the text and the scheme. Cells were incubated for 6 h in the presence (gray) or absence (white) before chemical fixation and processing for immunofluorescence microscopy. Infection efficiency is expressed as the percentage of that observed in vehicle DMSO-treated cells and is shown as average and standard deviation of three biological replicates (N=3). Statistical significance was estimated using one-way ANOVA and a Dunnet's post-hoc test (*p<0.05). **B) Pheophorbide A shows virucidal activity against SARS-CoV-2.** SARS-CoV-2 virus stocks were diluted to obtain 1×10^5 TCID₅₀/mL and were mixed with increasing concentrations of PheoA or the vehicle (DMSO). Virus-compound mixtures were incubated at room temperature for 30 minutes and were serially diluted to determine the remaining infectivity titer using endpoint dilution and determination of virus-induced cytopathic effect by crystal violet staining in Vero-E6 cells. Values are expressed as LOG TCID₅₀/mL and shown as the average and standard deviation of three independent experiments (N=3). Statistical significance was estimated using one-way ANOVA and a Dunnet's post-hoc test (*p<0.05).