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Artesunate derivative TF27 inhibits replication and pathogenesis of an oncogenic avian alphaherpesvirus

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ABSTRACT

Nucleoside analogues have been the cornerstone of clinical treatment of herpesvirus infections since the 1970s. However, severe side effects and emergence of drug resistant viruses raise the need for alternative treatment options. We recently investigated the broad and strong antiherpesviral activity of the optimized artesunate derivative TF27 *in vitro*. TF27 efficiently inhibited replication of the highly oncogenic Marek's disease virus (MDV), a virus that infects chickens, causes deadly lymphomas and threatens poultry populations worldwide. In this study, we used this natural virus-host model for herpesvirus-induced cancer by infecting chickens with MDV, and evaluated the protective efficacy of TF27 and the nucleoside analogue valganciclovir (VGCV) on virus replication and tumorigenesis. We could demonstrate that both drugs reduced viral load in the blood and prevented tumor development in a large portion of the animals. Antiviral treatment also had a positive impact on body weight gain, while no negative compound-associated side effects were observed. This research provides the first evidence that the artesunate derivative TF27 and VGCV can be used in avian species and that they inhibit MDV replication and tumorigenesis. In addition, our study paves the way for promising approaches in future antiherpesviral drug development.

To this day, antiherpesviral therapy is predominantly based on drug-induced inhibition of viral DNA synthesis. Herpesvirus-encoded thymidine kinases (TK) and protein kinases (PK) allow the activation of nucleoside analogues like acyclovir or penciclovir that are excessively used in antiherpesviral treatments. Another nucleoside analogue is valganciclovir (VGCV) that was shown to have an antiviral activity against various herpesviruses (Aoki, 2015). These drugs are incorporated into viral DNA where they act as chain terminators, resulting in a block of viral DNA replication. Nevertheless, mutations in the viral DNA polymerase and/or kinase genes cause emergence of drug-resistant strains and raise the need for alternative treatment options (Naesens and De Clercq, 2001).

While recent advances in antiherpesviral research lead to development of a plethora of treatment options *in vitro*, *in vivo* data is lacking for most of the compounds (Biron, 2007). One promising candidate for antiherpesviral drug therapy is artesunate and its related chemical derivatives (Qian et al., 1982; Efferth et al., 2008). Artesunate derivatives were found to be highly effective against cytomegalovirus (CMV) replication *in vitro* and *in vivo* (Hutterer et al., 2015; Hahn et al., 2018; Frohlich et al., 2018; Sonntag et al., 2019). Among these, the trimeric derivative TF27 (Figure S1) exhibits a remarkably strong antiviral activity against Marek's disease virus (MDV) replication *in vitro* by impinging on the nuclear factor kappa B (NF-κB) pathway with EC_{50} values in the low nanomolar range (Hahn et al., 2018; Sonntag et al., 2019). The mean TF27 EC_{50} value in conventional plaque assays was 0.21 μM (Hahn et al., 2018). Detection of viral copies by quantitative PCR (qPCR) lead to an even lower EC_{50} of 0.08 μM ± 0.01 μM (Sonntag et al., 2019). Furthermore, artesunate, and especially TF27 cell toxicity has been assessed *in vitro* and excluded for several cell types here (Figure S1 and S2), and in previous studies (Efferth et al., 2002, 2008; Kaptein et al., 2006; Hutterer et al., 2015; Sonntag et al., 2019).

MDV is a lymphotropic and oncogenic avian alphaherpesvirus and MDV infections can cause immunosuppression, generalized nerve

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inflammation, and rapid T cell lymphoma development in chickens (Davison, 2002, 2010). MDV causes worldwide annual losses in poultry industry in the range of 1–2 billion US$ (Morrow and Fehler, 2004) and leads to mortality rates of up to 100% in unvaccinated susceptible chickens. Until now, only live attenuated vaccines can protect chickens from MDV, while no drugs are available that can protect against this deadly disease (Bublot and Sharma, 2004). While MDV vaccines successfully prevent disease and tumor formation, they fail to provide sterilizing immunity and allow MDV field strains to infect vaccinated animals, transmit and evolve towards higher virulence in vaccinated flocks (Read et al., 2015). Beyond that, MDV infections of chickens are also commonly used as a small-animal model to investigate herpesvirus-induced pathogenesis and tumor development (Schat, 1987; Weiss, 1998, Osterrieder et al., 2006).

In this study, we used this animal model to assess the protective effects of the experimental antiviral drug TF27 and the nucleoside analogue VGCV. To assess the effects of both drugs *in vitro* and *in vivo*, we used the very virulent MDV RB-1B wild type virus (GenBank accession no. EF523390.1). The virus was propagated in passaged chicken embryo cells (CEC), stocks were frozen in liquid nitrogen and titrated on CEC as previously described (Bertzbach et al., 2018). TF27 and VGCV were obtained from Vichem Chemie Research (Budapest, Hungary) and Sigma Aldrich (St. Louis, MO, USA) respectively. Stocks were prepared in 5% Transcutol P (Gattefossé; Saint-Priest, France) dissolved in ddH_20 and stored at −20 °C.

First, we determined the 50% cytotoxic concentration (CC_{50}) of 30.18 ± 0.12 µM (4 days post-treatment) and 19.69 ± 0.13 µM (6 days post-treatment) by propidium iodide staining and subsequent FACS analyses on passaged CEC with increasing TF27 concentrations ranging from 0.1 µM to 111 µM (Figure S2).

To assess the antiviral activity of TF27 and VGCV against MDV *in vitro*, we then evaluated replication and cell-to-cell spread by multi-step growth kinetics and plaque size assays as described elsewhere (Eschke et al., 2018; Kheimar and Kaufer, 2018; Previdelli et al., 2019). We could demonstrate that both drugs strongly reduced MDV plaque sizes and their numbers (Fig. 1A and B) upon infection with 100 plaque-forming units (pfu). Growth kinetics confirmed the impact of the drugs on MDV replication (Fig. 1C). Collectively, these results validate that TF27 and VGCV are potent antivirals against MDV.

Finally, to assess the effect of both drugs on virus replication and pathogenesis *in vivo*, we infected three groups of 3-day-old VALO specific pathogen free chickens (VALO BioMedia GmbH; Osterholz-Scharmbeck, Germany) subcutaneously with 5000 pfu of the very virulent RB-1B MDV strain. Animal work was approved by the responsible governmental agency, the *Landesamt für Gesundheit und Soziales in Berlin* (LAGeSo; approval number G0294/17, approval date 2018-01-16). Eight hours post-infection, all animals of each group were injected intraperitoneally with either the solvent (mock, 5% Transcutol P, n = 10), TF27 (1 mg/kg body weight, n = 11) or VGCV (10 mg/kg body weight, n = 11). The treatment was repeated at day 2, 4, 6 and 8 post-infection. The treatment dosages for TF27 and VGCV were calculated based on the observed *in vitro* efficacies and a recent mouse experiment on TF27 (Sonntag et al., 2019). Notably, artesunate has been previously used at 10 mg/kg (and up to 50 mg/kg) in chickens with no toxicity reported (Kumnuan et al., 2013; Sohsuebngarm et al., 2014; Pruck-Ngern et al., 2015). Since the trimeric derivative TF27 is about 50 times more potent than the parental reference compound artesunate (Hahn et al., 2018), we reduced the dosage to 1 mg/kg in our experiment to minimize the risk of toxicity. All animals were weighted weekly and monitored for clinical Marek's disease (MD) signs twice a day. Chickens were humanely euthanized and thoroughly examined for MD lesions either when clinical signs were apparent or after termination of the animal experiment. To analyze the effect of TF27 and VGCV on lytic MDV replication in peripheral blood mononuclear cells (PBMCs) of infected chickens, 40 µl whole blood samples were taken from the brachial vein of all chickens at different time points post-infection and virus genome copies were determined by qPCR as previously described (Bertzbach et al., 2018). Virus load in the blood was reduced in animals treated with TF27 and VGCV at six time points post-infection (Fig. 2A), while replication in mock treated chickens was comparable to previous studies (Engel et al., 2012; Greco et al., 2014, Kheimar et al., 2018). Beyond that, the tumor incidence was reduced in the TF27 and VCGV groups (Fig. 2B, $p > 0.05$), with a protective index of 45 and 36 respectively - indices comparable to one of the commercially used vaccines (Sonoda et al., 2000). Moreover, tumor dissemination in MDV-infected animals was reduced in both treatment groups compared to the mock treated group as indicated by the numbers of organs with gross tumors (Fig. 2C, $p > 0.05$). The tumor lesions were mainly detected in visceral organs such as spleen, kidneys and liver as well as in breast muscles of infected chickens. Regarding the location of MDV tumors, we did not observe notable differences between the TF27 and VGCV groups and the mock-treated group. Interestingly, it has been proposed that NF-κB is a central player in MDV-induced tumorigenesis (Kumar et al., 2012). Our data using the NF-κB inhibitor TF27 provides evidence that this pathway might indeed play a role in MDV-induced tumor development.

Importantly, treatment with both compounds had no apparent compound-related adverse effects. Treatment of infected chickens with TF27 and VGCV even resulted in a mean bodyweight increase of 13 and 20% respectively (Fig. 2D). This observation could be explained by the reduced virus titers and reduced clinical symptoms upon antiviral treatment (Gimeno et al., 2011; Su et al., 2016).

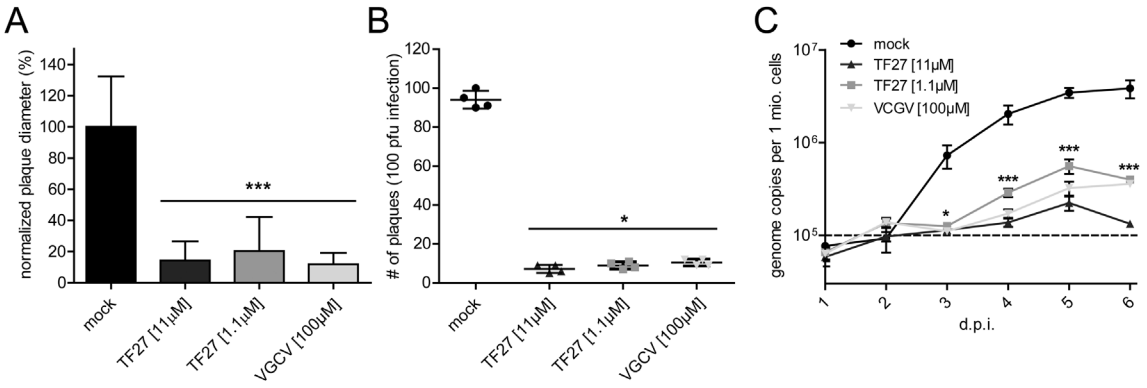

Fig. 1. *In vitro* characterization of TF27 and VGCV anti-Marek's disease virus (MDV) activity. (**A**) Plaque size assays under different treatment conditions. Plaque sizes are presented as bar graphs with standard deviation of three independent experiments (***$p < 0.001$; one-way ANOVA, n = 60). (**B**) Plaque reduction test of 100 plaque-forming units-infections with indicated treatments. Number of plaques from four independent experiments were counted at 6 days post-infection (*$p < 0.05$; one-way ANOVA). (**C**) Multistep growth kinetics with different treatment conditions. The average genome copies are shown as means with standard deviations (error bars) of three independent experiments (*$p < 0.05$ and ***$p < 0.001$; Kruskal–Wallis test).

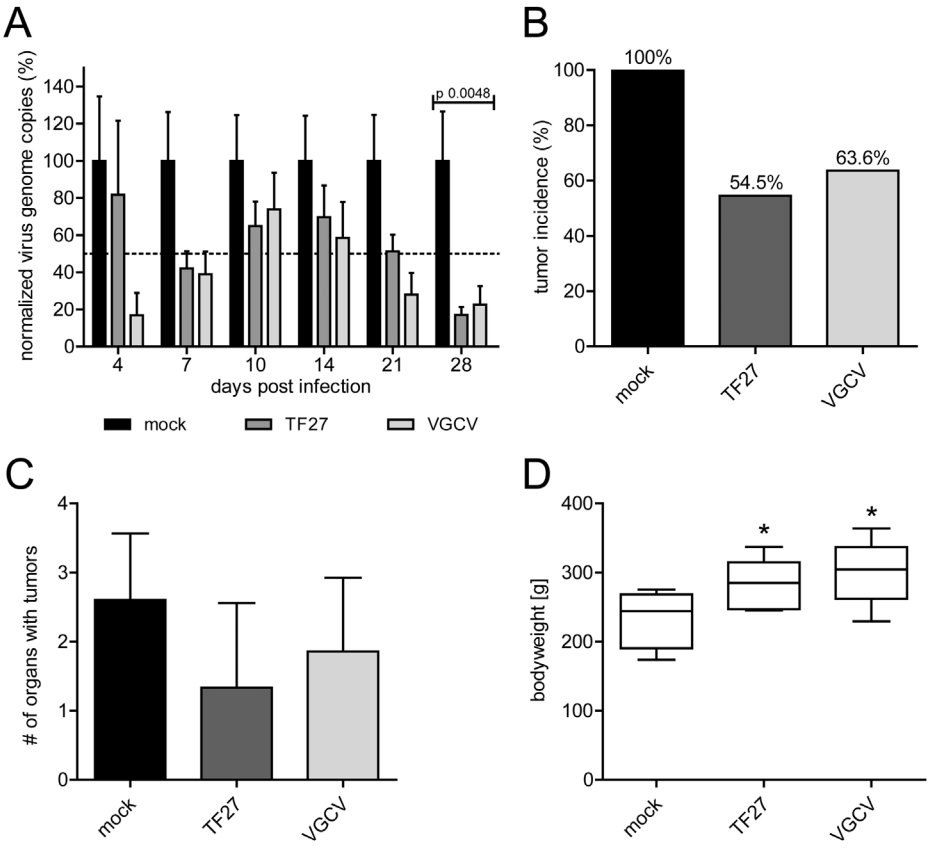

Fig. 2. *In vivo* characterization of TF27 and VGCV anti-Marek's disease virus (MDV) activity. (**A**) qPCR analysis of MDV genome copies in chicken whole blood samples infected with RB-1B and treated with either the vehicle control (mock, n = 10), TF27 (n = 11), or VGCV (n = 11). Normalized MDV genome copies (+standard deviation) of all chickens are displayed for the indicated time points post-infection and analyzed by Kruskal-Wallis test (*p < 0.05). (**B**) Tumor incidence of infected animals at termination of the experiment. Results are presented as the percentage of affected animals per group ($p > 0.05$, Fisher's exact test). (**C**) Mean number of affected organs with tumors per animal with standard deviation (error bars) ($p > 0.05$, Kruskal-Wallis test). (**D**) Changes in body weights in the different treatment groups on day 21 post-infection. Results are shown as means with boxes extending from the 25th to 75th percentiles and 5 and 95 percentiles (whiskers) (*p < 0.05, Kruskal-Wallis test).

In conclusion, we could demonstrate that TF27 and VGCV suppress MDV replication *in vitro* and *in vivo* and thereby provide valuable information on their antiviral efficacies and lack of pronounced side effects in an avian species. Moreover, our data show that the artesunate derivative TF27 is a very promising drug candidate that exhibits antiherpesviral activity in this model for herpesvirus-induced tumor formation.

Author contributions

L.D.B. and A.M.C. conducted the experiments; L.D.B., F.H., M.M. and B.B.K. designed the experiments; F.H., M.W. and M.M. characterized TF27 for human antiherpesviral analysis in parallel and provided *in vivo* active drug formulations and optimized protocols; L.D.B. drafted the manuscript and all authors edited and approved the final version of the paper.

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Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104606.

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