The University of South Bohemia in České Budějovice
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Potential antiviral effect of α-Spinasterol against tick-borne encephalitis virus

Bachelor's thesis

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Annotation:
Tick-borne encephalitis virus (TBEV) is endemic in many European countries. Apart from prevention by vaccination, no specific treatments are available for the TBE disease. Here, we assess the antiviral potential of α-Spinasterol (ASP) against TBEV. ASP is a phytosterol that has demonstrated a wide pharmacological profile. It reportedly exhibits anticancer, antibacterial, and antifungal properties and has shown antiviral effects against SARS-CoV 2. The examination of antiviral properties was preceded by the evaluation of the compound's cytotoxicity on a human medulloblastoma cell line. The experimental design was laid out to compare the effects of preventive and inhibiting ASP administration to evaluate the mechanism of action and potential interference with viral entry and viral replication. No conclusive results were obtained regarding the antiviral effects; however, additional testing is encouraged.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 09.05.2024

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Jana Koblmiller

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List of Abbreviations

Abbreviation	Definition
ASP	α-Spinasterol
ASP+TBEV	α-Spinasterol addition simultaneously to virus
ASP/TBEV	α-Spinasterol addition one day prior to virus
Asn154	Asparagine 154
BBB	Blood-brain barrier
C	Capsid
CNS	Central nervous system
CTHD	C-terminal hydrophobic chain
DI-DIV	Domain I-IV
DNA	Deoxyribonucleic acid
dpi	Days post-infection
ER	Endoplasmic reticulum
GAGs	Glycosaminoglycans
Ig	Immunoglobulin
MOI	Multiplicity of Infection
MTase	Methyltransferase
N	Nucleocapsid
NC	Negative Control
NS1-5	Non-structural Protein 1-5
NS	Non-structural Protein
PC	Positive Control
PCR	Polymerase Chain Reaction
PFU	Plaque-forming Units
prM	Precursor M
RNA	Ribonucleic acid
SP	Structural proteins
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
UPR	Unfolded protein response
+ssRNA	Positive single-strand RNA

Table of Contents

1 Introduction

1.1 Tick-Borne Encephalitis Virus

Orthoflavivirus encephalitidis, formerly known as Tick-borne encephalitis virus (TBEV) is an arthropod-borne viral human pathogen that causes tick-borne encephalitis (TBE), a disease primarily affecting the central nervous system (CNS) after transmission through the bite of infected ticks, particularly of the *Ixodes* species, or the consumption of unpasteurised dairy products (Kříž, Beneš, and Daniel 2009; Bogovic 2015). It is an enveloped, positive-stranded RNA virus of the *Flaviviridae* family and the *Orthoflavivirus* genus (Postler et al. 2023). Its geographical distribution is predominantly restricted to forested areas of Europe and northeastern Asia and is subclassified into five subtypes (Ruzek et al. 2019a). This classification differentiates between the European subtype (TBEV-Eu), which includes several strains from European countries, like the Hypr strain identified in Czechia as well as the prototype strain Neudörfl; the Far-Eastern subtype (TBEV-FE) with its Sofjin prototype and further strains found in east Russia, China, Japan, and other eastern European countries; and the Siberian subtype (TBEV-Sib) with the Lesopark-11, Aina, and Vasilchenko prototypes (Ecker et al. 1999; Demina et al. 2010). More recently identified subtypes include the Himalayan (TBEV-Him) and the Baikal subtypes (TBEV-Bkl) (Dai et al. 2018; Kozlova et al. 2018). TBE is endemic in central European countries, Siberia, far-eastern Russia, northern China, and Japan. Figure 1 shows the so-called TBE-belt representative of the geographic distribution. It has been suggested that climate change might affect vector biology and pathogen transmission, thereby playing a role in the increasing public health concern (Gray et al. 2009).

Figure 1. Geographic distribution of TBE in Europe and Asia. Map created by the Center for Disease Control and Prevention.

1.1.1 Structural and Genetic Characteristics

The spherical TBE particles have a diameter of about 50 nm and are composed of the three structural proteins (SP) capsid (C), envelope (E), and either membrane protein (M) or its precursor, prM, in immature virions, and seven non-structural proteins (NS) NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, with varying functions (King et al. 2012; Bamford and Zuckerman 2021). The ~11 kb-long positive-sense RNA (+ssRNA) genome is in the nucleocapsid (N) of the virion, enclosed by a lipid membrane derived from the host cell that also carries proteins E and M (Donoso-Mantke and Ruzek 2011).
The TBEV genome features one open reading frame bordered by the 5' and 3' untranslated regions. A cap is present at the 5' end, but the 3' end lacks a polyA tail (King et al. 2012). The single polyprotein composed of 3414 amino acids encoded by the ssRNA is processed into the SPs and NS via co- and post-translational cleavage by cellular proteases and the viral NS2B-NS3 serine protease (Donoso-Mantke and Ruzek 2011; King et al. 2012).

1.1.1.1 Structural Proteins

The C protein constructs the nucleocapsid (N) through its interaction with viral RNA. In its precursor state, it includes a C-terminal hydrophobic domain (CTHD) made up of 20 amino acids. This domain is commonly removed by a serine protease during the polyprotein processing stage, resulting in the formation of a brief CTHD polypeptide (Yamshchikov and Compans 1993). This domain has been attributed to a role in virion assembly (Kofler et al. 2003). The E protein constitutes a major component of the mature TBE virion and is an antiparallel dimer oriented horizontally to the viral membrane. It is a glycoprotein consisting of 496 residues and comprising four domains (DI, DII, DIII, and DIV) (Rey et al. 1995). Recent findings by Füzik et al. have provided detailed insights into the structure of the E protein, some of which will be outlined in the following passage: The ectodomain, which projects into the extracellular space and envelops the particle's surface, is constituted by the three N-terminal domains, all of which are primarily composed of β-strands. The β-barrel structure of the centrally located DI contains three segments with two loops, which form DII (Rey et al. 1995). DII is characterised by β-strands connected through loops and helices, thereby forming an extended structure (Füzik et al. 2018; Pulkkinen, Butcher, and Anastasina 2018). This elongated domain includes the only glycosylation site (Asn154) of the mature virus, which has been found to play a role in the secretion of virus-like particles from

mammalian cells (Goto et al. 2005; Füzik et al. 2018). It also contains a highly conserved fusion loop, which is fundamental to the fusion process with the host membrane during TBEV entry (Rey et al. 1995). DIII resembles the fold of immunoglobulin (Ig) and has been attributed to binding to host receptors. The C-terminal DIV attaches the E protein to the membrane. It has a stem region comprised of three peripheral membrane helices and a transmembrane region containing two helices (Füzik et al. 2018). As is characteristic of orthoflaviviruses, the E and M proteins slightly distort the lipid envelope. The M protein consists of 75 residues and is a remnant of prM, which consists of 162 residues (Pulkkinen, Butcher, and Anastasina 2018). M protein is composed of an N-terminal loop and three helices, one of which is perimembrane, and two are transmembrane. The N-terminal loop region interacts with E proteins in a E-M-M-E heterotetramer (Füzik et al. 2018).

1.1.1.2 Non-structural Proteins

NS1 is a conserved glycoprotein involved in viral RNA replication and translation, and immune invasion (Camarão et al. 2023). Orthoflaviviral NS1 has a molecular weight of about 48-55 kDa and a length of 352 amino acids (Rastogi, Sharma, and Singh, 2016). As a monomer, it is comprised of 2 to 3 glycosylation sites as well as 12 cysteines that form six disulfide bonds (Akey et al. 2014). NS1 exists in infected cells before the intracellular dimeric NS1 is confined in the lumen of the ER following post-translational modification in the initial stages of infection. As the infection progresses, NS1 particles are transported to the cell surface and subsequently secreted into the extracellular space, where they interact with the complement immune system, as proteolipid hexamers. A link between NS1 and the activation of Toll-like receptors has furthermore been reported (Kindberg et al. 2011). NS1 serves as an antigenic marker for early diagnosis of TBEV due to high expression levels during infection (Avirutnan et al. 2011). The orthoflaviviral NS1 protein is composed of three primary domains, the structure of which has been reported on by Akey et al. (2014). The β-roll dimerisation domain comprises amino acids 1-29 and is made of two β-hairpins that connect to form a four-stranded β-sheet. The wing-like domain spans amino acids 30-180 and contains two glycosylation sites, an internal disulphide, as well as two subdomains (Rastogi, Sharma, and Singh, 2016). The third domain, the β-ladder, is among the most well-established features of NS1 and comprises amino acids 181-352. It is a β-sheet extending the length of the dimer, and each monomer contributes nine strands to the antiparallel domain. The β-ladder forms a plane that divides the NS1 dimer, with the remaining domains and additional structural

elements on either side (Akey et al. 2014). NS1 interacts with other NS proteins to form a replication complex in the ER (Rastogi, Sharma, and Singh 2016). NS2A is a transmembrane protein that presents a pivotal element in RNA replication and the assembly of virions. Mutagenesis studies have shown that various subsets of NS2A, each situated in specific cellular areas, are utilised differently in these processes (Xie et al. 2015). NS2B is a membrane-associated protein composed of 130 amino acids and takes on the role of a cofactor for the NS3 protease (Falgout, Miller, and Lai 1993). It is essential to the replication process, especially through its contribution to the proteolytic processing of the viral polyprotein (Kurz et al. 2012). NS3 performs a function in viral replication and presents a primary target for the immune response following TBEV infection (Kubinski et al. 2024). NS3 has furthermore been found to boost adenosine triphosphatase (ATPase) activity, thereby facilitating viral replication and aiding in the evasion of type I interferon response (Silveira et al. 2015). Proteins NS4A and NS4B of orthoflaviviruses are known to contribute to viral replication, affect the host's immune response, and assist in virus assembly. NS4A is central to the suppression of the host interferon response by effectively disrupting the activation of IFN-stimulated genes (Q. Yang et al. 2020). In Bluetongue virus, which is comparable to TBEV with regards to transmission modes and some structural aspects, it enhances viral replication by interacting with Wilms' tumour 1-associated protein (Fablet et al. 2022). It has further been suggested that NS4 proteins in orthoflaviviruses engage in the virion assembly by mediating the recruitment of prM-E heterodimers and through interactions with additional viral proteins (Tang et al. 2023). NS5 is a highly conserved protein with a length of 900 amino acids (Belikov et al. 2014). It is a multifunctional protein contributing to RNA capping and viral RNA replication. As such, it features an N-terminal methyltransferase (MTase) domain responsible for RNA capping, and a C-terminal RNA-dependent RNA polymerase domain that is fundamental to viral replication (Zhao et al. 2017). The MTase domain of the NS5 protein has also been suggested to affect neural development by inhibiting neurite outgrowth induced by nerve growth factor in PC12 cells (Wigerius et al., 2010).

1.1.1.3 Replication and Life Cycle

The replication and assembly of TBEV particles undergo several maturation steps. The complete replication cycle is visualised in Figure 2. In the initial step of the cell entry pathway, the E glycoprotein attaches to the surface of a susceptible host cell, followed by internalisation. The role of heparan sulphate in virus attachment has been discussed in the literature, and it has

furthermore been suggested that orthoflaviviral virions likely target a ubiquitous cell surface molecule or use several receptors to enter cells, which is further supported by the recent identification of numerous attachment factors (Kroschewski et al. 2003; Perera-Lecoin et al. 2013; Van Den Elsen, Quek, and Luo 2021) This strategy allows the virus to infect a wide range of host cells. Multiple orthoflaviviruses reportedly utilise glycosaminoglycans (GAGs) as low-affinity attachment factors (Lee and Lobigs 2000). GAGs are negatively charged, highly sulphated polysaccharides abundantly expressed on many cell types. The interactions with GAGs are facilitated by DIII of protein E and aid in concentrating the virus on the cell surface. Receptor-mediated endocytosis regulates the transport of the virions into pre-lysosomal endocytic compartments of the host cell via clathrin-coated pits. The acidic pH endosomal environment has been demonstrated to induce an irreversible reorganisation of the envelope protein E homodimers into homotrimers (Allison et al. 1995). The conformational change leads to a fusion event of the viral and endosomal membranes, followed by the release of the N into the host cell cytosol (Smit et al. 2011). The N degrades, uncoating the viral RNA, which serves as a template for translation into a single polyprotein at the endoplasmic reticulum (ER) and subsequent processing into NS and SPs. The replication complex formed by the NS replicates a temporary negative-strand RNA using the genomic RNA template. The newly synthesised negative strand is further utilised as a template for the synthesis of a new positive-strand RNA genome. The assembly of immature virions is then initiated. C protein forms the spherical core with the viral RNA, the membrane-associated proteins prM and E in a heterodimeric complex. The virions are then secreted in vesicles and are transported through the Golgi apparatus, where they move through acidic compartments (Allison et al. 1995). The virions undergo conformational changes and maturation, which includes the cleavage of precursor proteins by cellular enzymes (Stadler et al. 1997). Mature virions are released from the host cell by exocytosis or cell lysis.

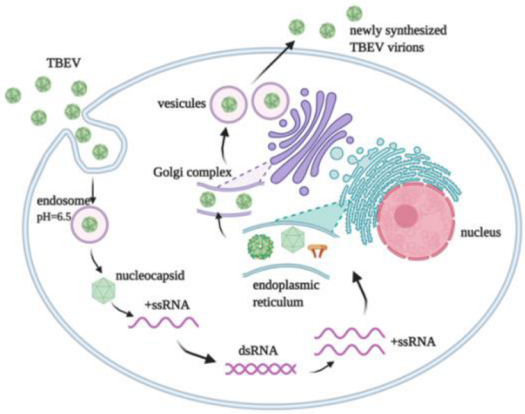

Figure 2. TBEV Replication Cycle. Figure created by H. Mašková using BioRender.com.

1.1.2 Transmission and Epidemiology

TBEV transmission entails a series of complex interactions between the virus and its hosts, as well as environmental factors. Transmission to humans occurs primarily through the bites of infected ticks, which function as both vectors (transmitters) and reservoirs (hosts) for the virus. The major tick vectors in Europe and Asia are *I. ricinus* and *I. persulcatus*. While small mammals are considered important reservoir hosts in the maintenance of the enzootic cycle of TBEV because they typically do not show signs of infection, humans are considered dead-end and mostly accidental hosts for TBEV that do not contribute to the transmission cycle (Kwasnik, Rola, and Rozek 2023). Transmission occurs horizontally and vertically. In the latter case, TBEV is passed from an infected female tick to the offspring. The transovarial mode of transmission ensures the virus' persistence without the need for an infected host. While vertical transmission between generations in the host has also been reported in rodents through the placenta to embryos (Bakhvalova et al. 2009), this mode has not been shown in humans (Divé et al. 2020). During horizontal transmission, ticks at larval, nymphal, and adult stages become infected with TBEV by feeding on the blood of vertebrates carrying the virus, such as small rodents, birds, or reptiles. Post-ingestion, the virus multiplies within the tick's gastrointestinal tract before dispersing further to various tissues, such as the salivary glands (Šimo et al. 2017). TBEV is capable of transstadial transmission, allowing the virus to persist in the tick through its developmental transitions from larva to nymph and finally to adult (Karbowiak et al. 2016). This allows for the preservation of the virus within the tick population over time. Adding to the transmission modes, and challenging the existing understanding of such, the cellular mechanism of co-feeding was explored in the 1990s when TBEV transmission between ticks occurred despite the absence of viraemia in the host (Labuda et al. 1993). This was further confirmed by findings showing virus transmission between infected and uninfected ticks feeding on the same host promoted by immune rodents despite the presence of neutralising antibodies (Labuda et al. 1997). During co-feeding, leukocytes migrate between feeding sites, thereby transporting virions between the ticks (Labuda et al. 1997; Randolph 2011). Systemic transmission to a mammalian host occurs during an infected tick's blood meal, during which the virus is released from the salivary glands into the host's bloodstream, while blood is ingested through the same channel (Kemp, Stone, and Binnington 1982; Šimo et al. 2017). There are several barriers the virus must surpass to cause disease in the host. However, saliva contains a wide variety of pharmacologically active proteinaceous and non-proteinaceous molecules and compounds that are secreted throughout the feeding

process, including anticoagulants and immunomodulatory components that are beneficial in transmission as they alter the skin barrier and compromise the immune responses (Hart et al. 2020). Mechanisms such as the interference with mast cells to suppress itching and pain, as well as modulating the secretion of inflammatory cytokines, and the restriction of wound healing by binding growth factors help ticks stay undetected while staying attached to the host for several days or weeks, depending on the tick's life stage (Šimo et al. 2017).

TBEV has been demonstrated to cause morphological changes in human intestinal Caco-2-cell monolayers, suggesting transmission through transcytosis and paracellular pathways (Yu et al. 2014). These findings potentially imply an alternative route for viral entry into the human host apart from vector-borne transmission. The alimentary route of transmission has been supported by a growing body of evidence (Kříž, Beneš, and Daniel 2009; Buczek et al. 2022; Nagańska et al. 2023). Additionally, a recent review emphasised the potential of breast milk as a transmission route (Dabas et al. 2023). TBE has been a notifiable disease in the EU since 2012, in an effort to update and refine the Union's surveillance and control of communicable diseases among its member states (Commission Decision No. 2012/492/EU). According to an annual report by the European Centre for Disease Prevention and Control (ECDC), epidemiological data for 2020 is available for 24 European countries, with the highest number of incidents per 100 000 reported in Lithuania, Slovenia, and Czechia. However, between 2012 and 2020, Czechia reported the most cases (Van Heuverswyn et al. 2023). The EU/EEA notification rate of 0.9 cases per 100 000 population shows an increase by 0.2 points since 2019, and by 0.3 points since 2016-2018. In 2020, 16 out of 3734 confirmed cases were fatal. Immunisation status was available for 64.1 % of confirmed cases, of which 94.7 % were reportedly unvaccinated. TBE predominantly occurs between April and November, corresponding to the seasonality of tick activity, with infections peaking in July (ECDC 2022; Van Heuverswyn et al. 2023). The increase in cases reported in 2020 was hypothesised to be subject to the Covid-19 pandemic which entailed an increase in outdoor activities (Van Heuverswyn et al. 2023), although environmental factors mentioned in Chapt. 1.1.4 may contribute to this dynamic. In addition to an increased number of cases, a north-west bound spread of endemicity was reported (Hills et al. 2023). Systematic TBE surveillance may be insufficient due to discrepancies in case definition and reporting among European countries, leading to incomplete databases and a potential underestimation of TBE as a public health threat (Erber, Schmitt, and Janković 2023). Additionally, vaccination rates are low in European countries and only a few countries recommend the vaccine for the general population (Erber, Schmitt, and Janković 2023). Austria presented a nationwide TBE vaccination campaign in

1981, resulting in a significant decline in reported yearly cases from 300-700 to 109 cases in 2023, as reported by the Austrian government, and a vaccination rate of above 80 % (Bundesministerium Soziales, Gesundheit, Pflege und Konsumentenschutz 2024).

1.1.3 Pathogenicity and Clinical Manifestation

The development of neuropathology in TBE is influenced by its ability to infiltrate the CNS following peripheral inoculation, known as "neuroinvasiveness", and its capability to multiply and induce harm within the CNS referred to as "neurovirulence" (Christian W. Mandl 2005). The infection cycle is outlined in Figure 3. TBEV infection initiation at the skin unfolds through a complexity of interconnected events at the host-tick interface. Langerhans cells of the epidermis are believed to transport saliva antigens to the draining lymph nodes (Nithiuthai and Allen 1985; Manuscript in preparation, Mašková et al. 2024), leading to the infection of lymphoid compartments. The virus triggers an immune response before it can disseminate into the body and lead to primary viremia. Hematogenous spread affects organs of the reticulo-endothelial system like bone marrow, spleen, and liver, where the virus replicates (Haglund and Günther 2003). Following the initial spread in the peripheral systems, TBEV can manifest neurologically after passing the blood-brain barrier (BBB) during secondary viremia after cytokine release (Sudhindra 2018; Stefania et al. 2023). The BBB is formed by endothelial cells lining cerebral microvessels and serves to protect the brain from toxins and pathogens while allowing nutrients to pass. Various methods have been suggested for the potential BBB crossing of TBEV. Although a correlation between a compromised BBB and TBEV infection has been established, the breakdown seems to be a consequence of the disease progression, implying the virus' capability of crossing an intact BBB (Růžek et al. 2011). This is further supported by evidence showing that the virus can cross the BBB within the endothelial cells without disrupting the barrier, thus maintaining the structural integrity of the BBB (Palus et al. 2017). Another possible method is a cytokine-mediated entry due to the initial systemic inflammatory response to TBEV infection. TNF-α and IL-6 may lead to increased endothelial cell permeability, creating gaps that allow the virus to pass. This discussion is based on reviewed findings in related viruses (Dörrbecker et al. 2010). Alternatively, the Trojan Horse mechanism, during which the virus surpasses the BBB while hidden within infected monocytes or macrophages, has been considered (Palus et al. 2017). Although not directly

applicable to TBEV, Zika virus has been shown to migrate across the BBB via transcytosis, during which the virus is transported within endothelial cells in vesicles (Chiu et al. 2020).

TBE is primarily asymptomatic in 70-98 % of cases. In symptomatic cases, it typically manifests as a monophasic or biphasic disease, following an incubation period of 4 to 28 days with a median of 8 days (Kaiser 1999) or less in the case of foodborne infection (Hudopisk et al. 2013). The initial stage of illness lasts between 2 and 10 days and is referred to as the abortive or febrile form. It entails non-specific symptoms like mild to moderate fever, fatigue and general malaise, headache, and body pain. Additionally, although less frequently, leukopenia, thrombocytopenia, and mildly elevated serum transaminases may occur (Kaiser 1999; Lindquist and Vapalahti 2008). Diagnosis follows from virus detection via reverse-transcription PCR. In about one-third of infected patients, the virus progresses to a second stage (biphasic TBE), during which it manifests neurologically (Dumpis et al. 1990). The second phase follows amelioration or a symptom-free period of 1-21 days, and the clinical spectrum includes meningitis and focal forms such as encephalitis, meningoencephalomyelitis as well as palsies of the cranial nerves that can lead to dysphagia and face paralysis. Further manifestation includes infection of the anterior horn cells of the spinal cord. Meningitis presents with symptoms such as high fever, headaches, nausea, and vomiting; while encephalitis may entail impaired consciousness ranging from drowsiness to stupor, and rarely, coma.

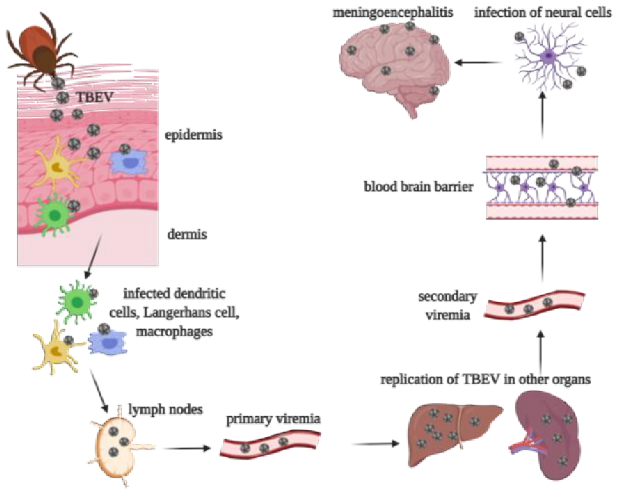

Figure 3. Virus Infection Cycle. Figure created by H. Mašková using BioRender.com.

Flaccid paralysis, a hallmark of meningoencephalomyelitis, often occurs after the febrile stage, and normally emerges following pain in the impacted muscle areas, with the arms more

frequently affected than the legs, and proximal parts of the limbs more susceptible than distal ones. Viral RNA is rarely present in cerebrospinal fluid in the second phase, and diagnosis is thus obtained serologically via enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay, and immunoblotting, through which specific IgM and IgG antibodies are detected (Bogovic 2015; Ergunay et al. 2016). Apart from the Far Eastern subtype, reports on the occurrence of the meningeal form of TBE exceed those of meningoencephalitis across all endemic regions (Ruzek et al. 2019b). Consequently, 50 % of patients experience meningitis, as opposed to 40 % suffering from meningoencephalitis and 10 % from meningoencephalomyelitis (Kaiser 2008). More than half of patients diagnosed with the biphasic disease experience long-term sequelae, with CNS damage potentially lasting for several years (Bogovic 2015; Siemieniako-Werszko et al. 2022). Cases of chronic (progressive) illness, during which patients experience continuing disease have been reported in Russia and may be associated with genetic mutations (Lindquist and Vapalahti 2008; Ruzek et al. 2019b). Thus, a condition termed post-TBE syndrome has been discussed in multiple studies, with symptoms prevailing after recovery, and some degree of neurological impairment following TBE (Lindquist and Vapalahti 2008; Siemieniako-Werszko et al. 2022). Additionally, discussions revolving around circadian disorders emerging as a potential long-term sequelae have been reviewed, and sleep-wake and circadian disorders reportedly persist for more than a year in up to 55 % of paediatric patients and 15 % of adults (Chiffi et al. 2022)[1]. However, the challenge of clinically differentiating between recuperation from acute symptoms and the emergence of long-term consequences needs to be emphasised, and more thorough experimental research models need to be developed. Recently, the TBE antibody index has been suggested as an effective predictive marker of sequelae development (Siemieniako-Werszko et al. 2022). The occurrence of TBE as well as the severity of diseases vary between the subtypes and the respective geographic region. TBEV-Eu infection usually leads to a biphasic disease and mild progression. In contrast, infections caused by TBEV-FE typically result in a higher incidence of severe neurological complications, with a mortality rate of 20 % or higher (Bogovic 2015; Ruzek et al. 2019b). Clinical progression data for TBEV-Sib is scarce, a fatality rate of 2-3 % has been reported (Poponnikova 2006). It has also been suggested that the variance in initial proinflammatory responses triggered by NS1 in

[1] Numbers rounded: As reported in the respective publication, paediatric cases ranged between 37.5 and 54.5 %, adult cases averaged at 14.8 %.

different TBEV subtypes might be one of the genetic factors affecting the severity of TBE as well as the potential development of long-term sequelae (Starodubova et al. 2023).

1.1.4 Treatment and Prevention

There are currently no specific therapeutic options available for the treatment of TBE beyond supportive and symptomatic management, including intensive care and assisted ventilation. Active immunisation effectively protects against TBE, thus endemicity control is limited to vaccination. The two approved vaccines in Europe target the Neudörfl (FSME-IMMUN) and K23 (Encepur) strains and have been modified multiple times since their introduction in 1976 (FSME-IMMUN) and 1991 (Encepur). The initial production of the first vaccine in 1971 was based on the cultivation of viral antigens in chicken embryo cells, followed by filtration, deactivation, and subsequent purification via hydroxyapatite chromatography. By means of stability enhancement, human albumin was utilised, and aluminium hydroxide served as an adjuvant (Barrett, Schober-Bendixen, and Ehrlich, 2003). Despite the availability of vaccines in endemic regions, low coverage and breakthrough infections underline the necessity for suitable therapeutic agents. Potential treatment options have been discussed in an extensive review by Ruzek et al (2019b). Moreover, the authors of this publication suggest that immunotherapy in the form of specific-anti-TBEV immunoglobulins and non-specific immunoglobulins presents a potential approach in the treatment of TBE patients (Ruzek et al. 2019b). Specific anti-TBE immunoglobulins are currently used in Russia and Kazakhstan, however, are currently no longer recommended in Europe. In contrast, high-dose intravenous immunoglobulins have been researched, and could potentially find use in post-exposure prophylaxis or therapy of severe TBE cases (Elsterova et al. 2017). TBEV infection has been shown to activate two major unfolded protein response (UPR) signalling pathways, namely inositol-requiring enzyme 1, and activating transcription factor 6 (Yu, Achazi, and Niedrig 2013). UPR is related to ER stress, which is involved in the maintenance of cellular homeostasis and the pathogenesis of various diseases, such as viral infections. These findings suggest a link between UPR pathways and successful viral replication, which could be relevant in future treatment efforts. In further endeavours, NS4A's role in TBEV's immune escape has been pointed out, implicating its potential as a target for therapeutic intervention (Yang et al. 2020). In testing the effectiveness of nucleoside analogues against TBEV, specific modifications that inhibit viral replication have been identified, the study of which could provide a basis for potential antiviral therapeutics (Eyer et al. 2016). These findings emphasise

the complexity of the structure-activity relationship due to the limited modifications showing effectiveness, thereby underscoring the difficulty of developing suitable drugs that target viral entry, replication, and assembly; and can cross the BBB to exert their therapeutic effect on the virus within the brain. NS1 has been identified as a promising component in future vaccine development (Beicht et al. 2023). In addition, a novel ELISA diagnostic tool for the detection of NS1-specific IgG antibodies has recently been reported on, stressing the protein's relevance in the accurate diagnosis process and future treatment endeavors (Girl et al. 2020). The urgency for treatment options is further manifested by the dynamic endemicity that is subject to climate change. Increasing temperatures and mild conditions are favourable for tick populations and can lead to an expansion of endemic areas (Heinz et al. 2015; Semenza and Suk 2018). Lower temperatures and different precipitation patterns reportedly lead to changes in latitudinal and altitudinal tick distribution (Voyiatzaki et al. 2022). Apart from socioeconomic and ecological factors, tick vector activity and transmission patterns are expected to be impacted significantly by global warming, resulting in an increased transmission potential, extended tick seasons, and a higher number of susceptible ticks (Nah et al. 2020). These shifting dynamics call for an increased effort to monitor tick-borne infection behaviour and adjust vaccine and disease-prevention strategies (Kaaijk and Luytjes 2018).

1.2 α-Spinasterol

Spinasterols are phytosterols, which are bioactive components of plant cells that are similar to cholesterol in their structure. As is common for phytosterols, spinasterols are found in seeds, leaves, and fruit of plants, as well as marine organisms. α-Spinasterol (ASP), also known as bessisterol or hitodesterol, was initially isolated from Alfalfa and spinach leaves (Hart and Heyl 1932; Fernholz and Moore 1939; Armarego, Goad, and Goodwin 1973; Adler and Salt 1983). It naturally occurs in spinach leaves, cucumber, pumpkin, and melon seeds, seed oils and argan oil, among others.

1.2.1 Chemical Structure and Properties

ASP is a steroid derived from a hydride of stigmastane, which is a saturated sterol without double bonds in its ring structure. The chemical structure is displayed in Figure 4. ASP is comprised of a complex ring structure with multiple chiral centres, as is common for steroidal

compounds. The steroid nucleus is a carbon framework composed of three cyclohexane rings and a cyclopentane ring. The hydroxyl group attached to the first ring indicates its function in membranes, as is the case for cholesterol. The methyl groups attached to the rings are involved in the molecule's hydrophobicity and might affect its position within lipid bilayers. The double bond in the second ring is typical for unsaturated sterols and can impact the molecule's rigidity. The hydrocarbon side chain attached to the ring and ending in an ethyl group is another structural detail characteristic of sterols and is important for the interaction with lipid bilayers.

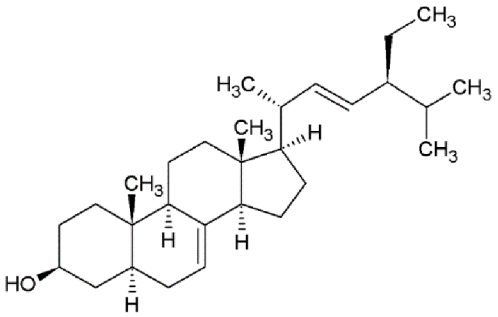

Figure 4. Chemical structure of ASP. Figure created by H. Mašková in ChemSketch Free Version.

1.2.2 Pharmacology of α-Spinasterol

In mammals, cholesterol plays an important role in the maintenance of the structural integrity and fluidity of cell membranes by inserting itself between phospholipid molecules in the membrane, which results in a modulation of its permeability and fluidity. Apart from the formation of lipid rafts, it is also involved in cell signalling. In plants and fungi, multiple compounds that structurally resemble cholesterol contribute similarly to membrane properties (Haralampiev et al. 2017). Due to this mimicking effect, ASP could potentially facilitate lowering cholesterol levels and thereby contribute to cardiovascular health (Piironen et al. 2000). Plant sterols have thus been implied to have a positive impact on health (Berger, Jones, and Abumweis 2004; Kopylov et al. 2021), and research interest has been developed to investigate ASP's potential pharmacological profile, which includes cytotoxicity, anti-inflammatory, antimicrobial, and antioxidant activity (Coballase-Urrutia et al. 2010; Borges et al. 2014; X. Yang et al. 2017; Sedky et al. 2018). ASP was identified as a novel antagonist of the TRPV1 receptor and has been reported to display antioedematogenic and antinociceptive characteristics, decreasing pain and inflammation without impacting body temperature or locomotor activity in animal models (Trevisan et al. 2012). Reinforcing the role of TRPV1 receptor antagonism in the treatment of mental health disorders, ASP has

additionally been shown to exert antidepressant-like properties without exhibiting significant anxiolytic-like features (Socała and Wlaź 2016). Highlighting potential BBB permeability, it was further suggested that ASP displays anticonvulsant qualities and may elevate seizure thresholds (Socała et al. 2015). According to more recent research, glucose uptake in skeleton muscle cells and insulin secretion in pancreatic β-cells can be affected by ASP (Lee et al. 2022), supporting claims suggesting ASP as a potential anti-diabetic agent (Jeong et al. 2004). Additionally, an inhibitory effect against the cholinesterase enzyme was reported, which, in addition to diabetes mellitus, is relevant in research focusing on developing therapies for Alzheimer's disease (Lawal et al. 2020). A review of the pharmacological findings by Majeed et al. (2022) has recently highlighted ASP's nutraceutical potential for effectively managing various health concerns given the wide range of pharmacological indications.

1.2.2.1 Antibacterial Effects and Cytotoxicity

ASP extracted from *Cucumis dipsaceus* was tested for antibacterial activity and was found to impact the bacterial growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes* (Assefa et al. 2024). ASP has also been studied as an antibacterial agent in combination with the antibiotic Ceftiofur and showed increased efficacy against four pathogenic strains, namely *Salmonella pullorum* CVCC 533, *Str. pneumoniae* CAU0070, *E. coli*, and *Staphylococcus aureus* (Yang et al. 2017). These findings align with results demonstrating Spinasterol's antibacterial and bactericidal activity against *Helicobacter pylori* (Wang et al. 2011). A study assessing ASP isolated from *Impatiens glandulifera* roots furthermore concluded that ASP was cytotoxic to blood cells after a 2-day incubation period, with a reduction in metabolic activity of about 50 %; however, it did not exhibit significant cytotoxicity on hepatic cells (Vrchotová et al. 2024). These results highlight the compound's potential for antimicrobial application, especially concerning pathogens affecting the liver.

1.2.2.2 Spinasterols as Potential Antiviral Agents

An insufficient amount of research has been published about ASP as an antiviral agent. However, despite the limited available research, some findings allow for cautious optimism about its potential. ASP is among the phytoconstituents recently investigated for their possible antiviral activity in a study focusing on interactions with SARS-CoV-2 proteins. Spinasterol was also effective in targeting the SARS-CoV-2 main protease in a recently published pre-

print (Palanisamy et al. 2022). ASP demonstrated significant binding affinity with two viral proteins, suggesting its potential in future antiviral research efforts (Siddiqui et al. 2022). Additionally, Spinasterol was shown to bind an enzyme critical for coronavirus replication in research endeavours identifying protease inhibitors (Zubair et al. 2021). While little research exists on the antiviral effects of ASP, several plant extracts containing the compound have shown relevant biological activity. For example, extracts of *Bupleurum marginatum* were shown to have antitrypanosomal, antimicrobial, and antiviral activity against the hepatitis A virus at certain concentrations (Ashour et al. 2014). Another plant extract from *Platycodon grandiflorum* demonstrated antiviral activity against hepatitis C virus in mice and inhibition of its RNA replication (Kim et al. 2012). In addition to the above-mentioned results regarding SARS-CoV-2, hepatitis A and C, Spinasterol was among some compounds demonstrating anti-HIV activity in H9 lymphocytes (Hsieh et al. 2004). No research has been conducted on TBEV, and although hardly conclusive, the above outlined results, specifically regarding RNA viruses, offer insights relevant to future research on the biological effects of ASP and its role as an inhibitor of viruses.

2 Aims and Objectives

- Determination of cytotoxic concentrations of α-Spinasterol for a DAOY-HTB 186 medulloblastoma cell line.
- Determination of the inhibitory concentration of α-Spinasterol during TBEV infection.
- Examination of the effects of α-Spinasterol on TBEV replication and the production of newly created virions using a selected concentration of α-Spinasterol.

3 Materials and Methods

3.1 Cells and Viruses

Medulloblastoma DAOY (ATCC HTB-186) and A549 (human lung adenocarcinoma cells, kindly gifted by R. Randall, University of St. Andrews, UK) cell lines were cultivated in low glucose Dulbecco's Modified Eagle's Medium (D-MEM) medium with stable glutamine and sodium pyruvate (Biosera) supplemented with 10 % fetal bovine serum (FBS, Cytiva), 1 % antibiotics (ATB, Penicillin-streptomycin solution 100x (10^7 U/l), Biosera) (complete DMEM medium) at 37 °C with 5 % CO_2, and passaged twice per week. DAOY cell line was chosen as an appropriate type of cell since TBEV infects the central nervous system. A549 cells were used for plaque assay experiments. TBEV-Eu strains Hypr and Neudörfl were used for the infection of DAOY cells and the examination of the potential antiviral effect of ASP. TBEV Hypr and TBEV Neudörfl used in the experiments were passaged once in Vero E6 cells (ATCC CRL-1586). The stock concentrations used were $3*10^7$ PFU/mL and $3.73*10^6$ PFU/mL for TBEV Hypr and TBEV Neudörfl, respectively.

3.2 Cell Seeding and Cytotoxicity

DAOY cells were seeded at a density of 10^4 cells/well for 96-well plates. The cells were let to adhere for 4-5 hours prior to ASP-addition. Purified ASP extract from *I. glandulifera* was obtained from Prof. Ing. Jan Tříska, CSc's group. ASP extract was prepared according to the published protocol (Vrchotová et al. 2024). ASP powder was dissolved in DMSO to get a stock concentration of 10 mg/mL. The following examined dilutions were prepared: 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1 µg/mL. All these dilutions contained 1 % DMSO and a complete DMEM medium.

After the cells adhered to the wells, the medium was removed, and media with different examined concentrations were added. The control cells with 1 % DMSO were also prepared. Cells were incubated at 37 °C with 5 % CO_2 atmosphere until the measurement day. Cytotoxicity was measured on days one, two, and four after the addition of ASP using the alamarBlue reagent (Thermo Fisher Scientific). On selected days, the medium was removed from each well, and fresh medium with alamarBlue reagent (10:1, v/v) was added. After a 1.5 -hour incubation period at 37°C in a 5 % CO_2 atmosphere, the fluorescence was measured using a Tecan Infinite M200 spectrophotometer, with excitation set to 550 nm and emission

set to 590 nm. The blank value was subtracted from each measurement and the relative viability of cells with ASP was calculated in comparison to the control cells. The control cells represent the 100 % level of relative viability. The whole experiment was performed in technical and biological triplicates.

3.3 Viral Infection and Sample Collection

Two variants of ASP administration were chosen to examine the antiviral effect on TBEV. One group received ASP on the same day as TBEV infection (ASP+TBEV), and the second group (ASP/TBEV) received ASP one day before TBEV infection, and again after infection to preserve the ASP effect, to examine the prophylactic effect of ASP. First, the inhibitory effect of ASP on TBEV was examined. Cells were seeded in a 96-well plate as described in 3.1. ASP was added according to the examined variants. TBEV infection using Hypr and Neudörfl strains was performed at a multiplicity of infection (MOI) 1, on cells containing prophylactic ASP (ASP/TBEV) or plain medium (ASP+TBEV). Positive control (PC) infected with TBEV and containing 1 % DMSO, and negative control (NC) containing mock (medium from non-infected Vero E6 cells) and 1 % DMSO were also prepared. The virus was removed after an incubation period of 2 hours at 37 °C with 5 % CO_2, washed with Phosphate Buffered Saline (PBS), and replaced with medium containing 1 % DMSO (PC, NC) or ASP (ASP+TBEV, ASP/TBEV). Cell viability was measured as described previously in Chapter 3.2, 2 days post-infection (dpi) for samples infected with Hypr, and 4 dpi for samples infected with Neudörfl. Based on the results, the most suitable concentration showing the highest viability ratio to PC was selected for each strain, and the experiments were repeated in 12-well plates to obtain a sufficient number of cells for RNA isolation and media for plaque assay. The used concentration of the cells for 12-well plates was $5*10^5$ cells/well.

3.4 RNA Isolation and LiCl-Precipitation

On collection days, medium was collected from the cells in 12-well plates for the plaque assay. The cells were washed with PBS. For the collection, 200 µL PBS was added, and cells were scraped off the wells, resuspended in PBS, and collected in tubes. RNA isolation was performed using a B-200 Nucleic Acid Extraction Kit (Zybio) in EXM3000 Nucleic Isolation System (Zybio) according to the manufacturer's manual.

Lithium chloride precipitation was performed on all samples to remove DNA (Barlow et al. 1963; Gautam 2022), according to the protocol provided by Invitrogen. Solutions of 2.5 M LiCl in RNA were prepared and stored at -20 °C overnight. The samples were centrifuged at 17 000 x g for 60 minutes. The resulting pellets were washed with ice-cold 70 % ethanol (prepared with DEPC-treated water) and centrifuged for 20 minutes. The pellets were resuspended in nuclease-free water, and RNA concentration measurements were conducted using a Nanophotometer (Implen).

3.5 NS1 and HPRT qRT-PCR Assay

For qRT-PCR, the housekeeping gene Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) and the viral NS1 were chosen. The RNA samples processed in chapter 3.3 were used for qRT-PCR. The detection of NS1 was performed using KAPA PROBE FAST One-Step qRT-PCR Master Mix (Kapa Biosystems) with the composition for one reaction mentioned in Table 1 and with the qRT-PCR parameters showed in Table 2. The primers (TBE F: TggAYTTYAgACAggAAYCAACACA; and TBE R: TCCAgAgACTYTgRTCDgTgTggA) and hydrolysis minor groove binder (MGB) probe (FAM-CCCATCACTCCWgTgTCAC-MGB-NFQ for TBEV detection were used according to a previously established protocol (Achazi et al. 2011). HPRT detection was performed with the KAPA SYBR FAST Universal One-Step qRT-PCR Kit (Kapa Biosystems). The sequences of used HPRT primers were HPRT F: TGACACTGGCAAAACAATGCA and HPRT R: GGTCCTTTTCACCAGCAAGCT. The composition of one reaction is listed in Table 3, and the qRT-PCR programme is shown in Table 4. qRT-PCR reactions were performed in the thermocycler CFX96 Touch Real-Time PCR System (Bio-Rad). The threshold cycles (Ct) were exported using CFX Maestro software (Bio-Rad).

Table 1: Composition for one reaction (NS1)

Component	Volume
2x Kapa Mastermix	7.5 µL
ddH2O	1.1 µL
F Primer 20 µM	0.9 µL
R Primer 20 µM	0.3 µL
50 x Kapa RT mix	0.3 µL
RNA template (20 ng/µL)	4 µL

Table 2: qRT-PCR parameters (NS1)

Program	°C	t
Reverse transcription	42	10 min
Enzyme inactivation	95	5 min
Denaturation	95	5 sec
Primers annealing	60	34 sec
45 two-step cycles (denaturation & annealing)		

Table 3: Composition for one reaction (HPRT)

Component	Volume
2x Kapa Mastermix	7.5 µL
ddH2O	2.3 µL
F Primer 20 µM	0.45 µL
R Primer 20 µM	0.45 µL
50 x RT mix	0.3 µL
RNA template (20 ng/µL)	4 µL

Table 4: qRT-PCR parameters (HPRT)

Program	°C	t
Reverse transcription	42	10 min
Enzyme inactivation	95	5 min
Denaturation	95	5 sec
Primers annealing	60	30 sec
40 two-step cycles (denaturation & annealing)		

3.6 Plaque Assay

A suspension of A549 cells was prepared at a concentration of $5*10^5$ cells/mL. A decimal dilution of the samples (20 µL) collected in chapter 3.3 was created in 24-well plates with 180 µL of complete DMEM medium. The cell suspension was added (300 µL), and cells were incubated for 4 hours at 37 °C with 5 % CO_2. A solution containing 3 % carboxymethylcellulose with 2x concentrated DMEM medium with 20 % FBS, 2 % ATB, and 2 % L-glutamine (1:1) was prepared and added to each well (400 µL). Prior to staining, the cells were incubated for 5 days at 37 °C with 5 % CO_2. The cells were rinsed with a saline solution (0.9 % NaCl) and stained in Naphthalene black solution (1 g naphthalene black, 62.5 mL glacial acetic acid, 13.6 g sodium acetate, filled to 1 L with distilled water) for 60 minutes to visualise the plaques. After drying, the plaques were counted, and the viral titres were determined (PFU/mL).

3.6 Statisical Analysis

Relative viability, ΔΔCt and viral titres were calculated using Microsoft Office Excel 365. Statistical analyses and graphs were created in the GraphPad Prism software (version 10.2.2., GraphPad Software, San Diego, CA, US) using two-way analyses of variance (ANOVA) with Fisher's LSD post-hoc test. Statistical significance levels were determined as * $p<0.05$, ** $p<0.01$. The calculation of half maximal inhibitory concentration IC_{50} was performed using Nonlinear Regression: Dose-response-inhibition with Variable slope in the GraphPad Prism software.

4 Results

4.1 α-Spinasterol Cytotoxicity

Determining the studied substance's cytotoxicity is an important initial step when considering its potential as an antiviral agent to ensure its safety for the cells it will interact with. By understanding the cytotoxicity, a maximum concentration usable to inhibit viral activity without causing unproportionable damage to the cells should be determined. Cytotoxicity is assessed by measuring the cell viability of samples treated with alamarBlue, which indicates cellular metabolic activity. AlamarBlue contains the non-fluorescent and non-toxic blue dye resazurin, which is reduced to the pink and highly fluorescent substance resorufin by metabolically active cells. Based on the degree of this reduction after several hours of incubation, the viability of the cells can be determined by measuring the fluorescence. Figure 5 presents the relative cell viability changes post-ASP treatment. The spectrophotometric measurements were conducted at three time points: On the first (blue crosses), second (orange circles) and fourth (grey triangles) day following the treatment with ASP. The 24-hour measurement shows an immediate effect of the substance on the cells, while the 48-hour interval allows for the detection of intermediate responses caused by slower mechanisms of action. The third time interval allows for the observation of long-term effects. This selection of incubation periods has been suggested to be sufficient for ASP to develop its biological effects (Vrchotová et al. 2024). The viability percentages are normalised to a control group treated with 1 % DMSO (dotted green line) instead of ASP, allowing a direct comparison of ASP's effect on cell viability. The horizontal solid red line indicates a threshold of 80 % viability and serves as a reference for substantial viability reduction. On the first day post-treatment, only the highest tested concentration of 100 µg/mL falls below the threshold (average 51 %). On the second day, cell viability of the groups containing 25, 50, and 100 µg/mL ASP fell below 80 % (67 %, 27 %, and 21 %, respectively). On day four, the concentration of 50 and 100 µg/mL undercut the 80 % threshold significantly at 23 % and 28 %, respectively. These initial experiments aimed to determine two concentrations that balance effectiveness and risk of cytotoxicity. The results in Figure 4 show that ASP addition at concentrations above 25 µg/mL may be cytotoxic and, therefore, deemed unsuitable for subsequent experiments. The concentrations of 25 and 10 µg/mL were determined as potential candidates for future testing as antiviral agents in TBEV-infected cells.

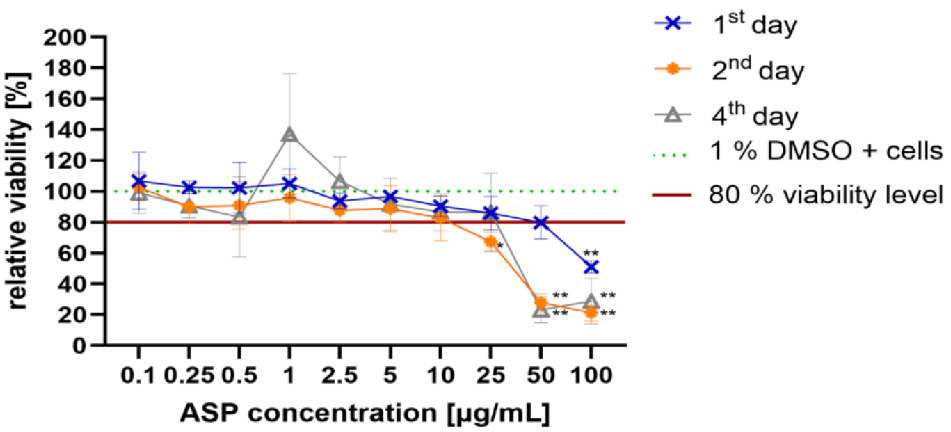

Figure 5. Development of Relative Cell Viability Following ASP Treatment. Viability was measured on the 1st (blue crosses), 2nd (orange circles), and 4th (grey triangles) day post-ASP addition at varying concentrations ranging from 0.1 to 100 µg/mL. The red solid line indicates an 80 % viability threshold. The dotted green line represents the viability of the control group treated with 1 % DMSO. Statistical analysis was performed using two-way ANOVA with Fisher LSD post-hoc test in GraphPad Prism (version 10.2.2). * $p < 0.05$, ** $p < 0.01$

Moreover, half maximal inhibitory concentration IC_{50}, showing the concentration where cell viability is reduced by 50 %, was calculated. On the first day, the highest tested ASP concentration did not decrease cell viability below 50 %, meaning short-term exposure to the substance did not entail immediate cytotoxic effects. On the second day following the ASP addition, the IC_{50} was determined to be 30 µg/mL. By the fourth and final day of measurements, IC_{50} was slightly lower, at 27.24 µg/mL. The average IC_{50} for ASP cytotoxicity was calculated to be 28.6 µg/mL from data on the second and fourth day following ASP treatment.

4.2 Inhibitory Effects of ASP on TBEV

Two closely related European TBEV strains with varying virulence were chosen to examine the effect on ASP-treated cells using two variants of ASP addition. Hypr has been characterised as highly virulent with higher neuro-invasiveness in mice than the prototypic low-virulence strain Neudörfl (Wallner et al. 1996; Mandl et al. 1997). Testing two strains allows for a comparison when evaluating the effectiveness of the substance applied on a low- versus a high-virulence strain and enhances the relevance of the results. All ASP concentrations were examined to determine the most appropriate concentration for further analysis. A delayed onset of cytotoxic effects caused by the virus has been shown, thus the 24-hour interval is not measured following TBEV-infection (Selinger et al. 2019; Brzuska et al. 2020).

Hypr Strain

The relative viability of Hypr-infected cells under two conditions is shown in Figure 6: One group received a prophylactic ASP treatment (ASP/TBEV Hypr, depicted in blue) and the second group was infected without preceding prophylaxis and solely received ASP at the time of infection (ASP+TBEV Hypr, depicted in red). Viability percentages of both groups are benchmarked to a positive control group (PC, dotted green line), representing TBEV-infected cells with 1 % DMSO added to the medium that did not receive any ASP. The prophylactic group shows a trend exceeding the 100 % benchmark. At ASP concentrations of 0.1 and 25 µg/mL (marked by red asterisks), a statistically significant increase in cell viability (150 % and 135 %, respectively) can be observed for the prophylactic group compared to the PC. Moreover, the results from ASP concentrations of 5 and 10 µg/mL showed trends of increased viability (147 % and 151 %, respectively); however, with no statistical significance, this is caused by wide standard deviation among samples. In contrast, the group treated with ASP at the time of infection demonstrates reduced cell viability, especially at higher ASP concentrations, as indicated by the blue asterisks. The results were compared to PC instead of NC to show the differences between the infected cells and treated cells.

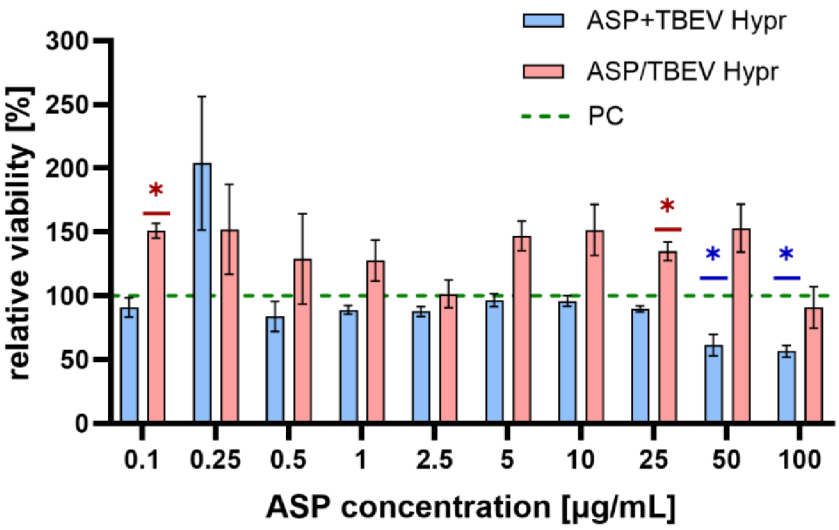

Figure 6. Relative Viability of Hypr-Infected Cells under Different ASP Treatment Conditions. The bar graph compares the relative viability of cells infected with TBEV Hypr under two conditions: Prophylactic ASP treatment before infection (red, ASP/TBEV Hypr) and ASP treatment simultaneously with the infection (blue, ASP+TBEV Hypr). Viability percentages were normalised to a positive control (dotted green line). Significant deviations from the control were indicated by colour-matched asterisks (*) represented in the respective colour of the group. Statistical analysis was performed using two-way ANOVA with Fisher LSD post-hoc test in GraphPad Prism (version 10.2.2). * $p < 0.05$

Neudörfl Strain

Figure 7 outlines the results of the measured relative cell viability following infection with the Neudörfl strain under two distinct conditions: The prophylactic group (ASP/TBEV Neu, depicted in grey) received ASP prior to and following viral infection. The second group (ASP+TBEV Neu, depicted in yellow) was treated with ASP at the time of infection without prior treatment. The prophylactic group shows increased viability and surpasses the 100 % benchmark compared to PC throughout most tested concentrations. The differences are not statistically significant due to the wide standard deviation of the three independent measurements, however, the concentrations 5 and 10 µg/mL are particularly notable as they almost doubled in relative viability, more concretely 196 % and 198 %, respectively. The group treated at the time of infection shows a contrast in viability compared to the prophylactic group, with all tested concentrations falling below the baseline established by the positive control group, with an average of 51 % viability. As highlighted by the asterisks, the concentrations 0.5, 1, 50, and 100 µg/mL ASP deviate significantly from the positive control and fall below an average of 35%.

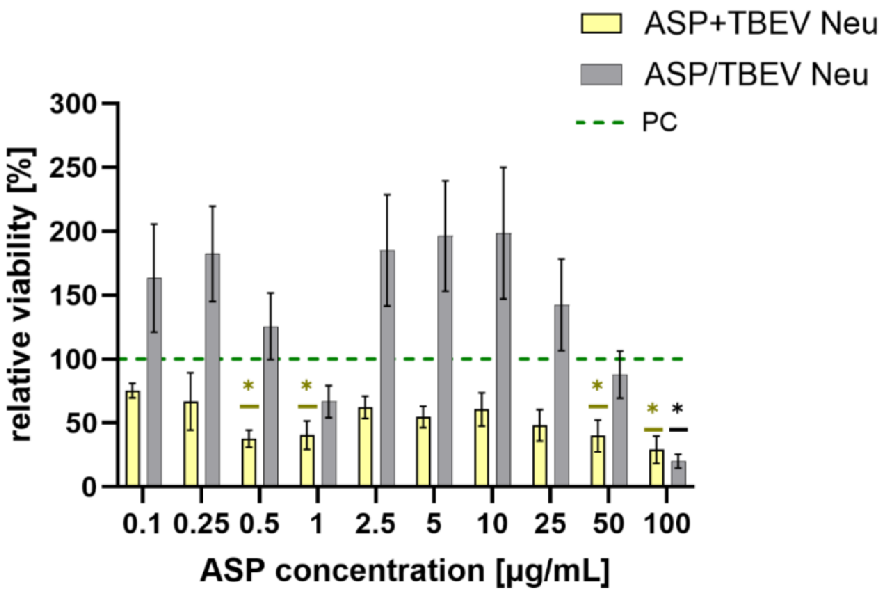

Figure 7. Relative Viability of Neudörfl-Infected Cells under Different ASP Treatment Conditions. Cell viability of Neudörfl-infected cells under varying conditions is compared. The prophylactic group (ASP/TBEV Neu, in grey) received ASP treatment prior to and post-infection, while the simultaneous treatment group (ASP+TBEV Neu, in yellow) only received ASP at the same time as the infection. The positive control group (green dotted line) represents infected cells that received no ASP treatment. Significant differences from the control group are indicated with colour-matched asterisks.

Based on the outlined results, the ASP concentrations of 10 and 25 µg/mL were initially considered for further testing (Chapt 4.1). However, since the latter was approaching the IC_{50} average value (28.6 µg/mL), only the concentration of 10 µg/mL was finally selected since it seemed to remain well above the threshold that exhibits significant loss in cell viability over the monitored time period.

4.2 qRT-PCR

To determine the antiviral effect of ASP, the level of viral RNA present in the examined cells was detected via real-time quantitative reverse transcription PCR (qRT-PCR), which measures the relative RNA concentration fold change of the TBEV NS1 gene. Results were evaluated using the ΔΔCt method, where the expression of the gene of interest (NS1 gene) in tested samples and controls was normalised to the expression of the reference housekeeping gene (HPRT). The NC samples were also examined with no detection of the TBEV NS1 gene. The red dashed line represents the PC baseline. The ASP+TBEV Hypr group shows a high relative RNA amount fold change with an average of 3.35, although considerable variability is indicated by the respective error bar. The relative RNA amount fold change is lower in the prophylactic group (ASP/TBEV Hypr) with an average value of 1.11. At an average of 1.80, the RNA amount fold change in the ASP+TBEV group infected with the Neudörfl strain is much lower compared to the respective Hypr group and almost doubles compared to PC. Exhibiting a similar level, the prophylactic group (ASP/TBEV Neu) shows the lowest relative RNA fold change among all tested conditions, with its average level of 0.88 just below the baseline (Figure 8).

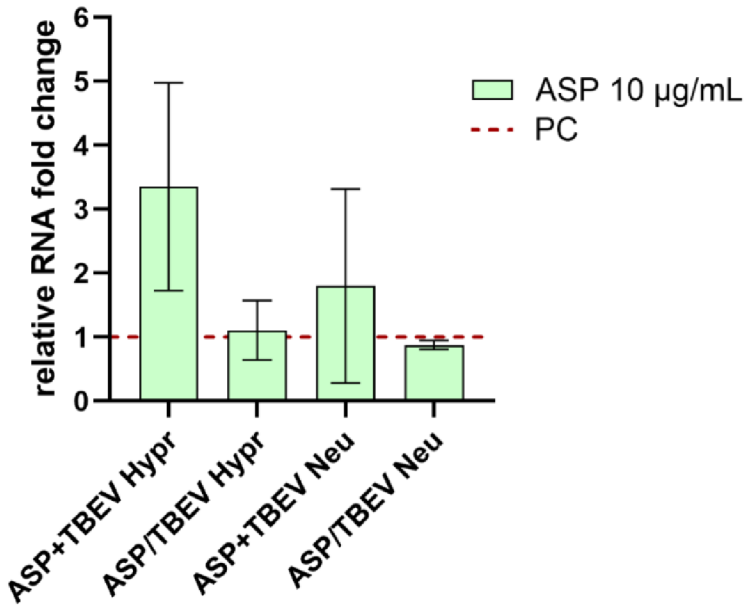

Figure 8. Representation of the Relative RNA Fold Change of the NS1 Gene of TBEV via qRT-PCR. The quantified NS1 gene expression levels were normalised to the housekeeping gene HPRT using the ΔΔCt method. The results are presented relative to the PC, displayed as a red dashed line, which reflects the baseline level of viral replication without ASP addition. The green bars represent the different experimental conditions: ASP-addition at the time of Hypr or Neudörfl infection (ASP+TBEV Hypr, ASP+TBEV Neu), and prophylactic ASP addition (ASP/TBEV Hypr, ASP/TBEV Neu). The black error bars denote the standard deviation of the mean for each tested condition, which indicates the variability of the replicates.

4.3 Plaque Assay

A combination of techniques enables a more informed interpretation, thus in addition to tracking changes in viral RNA levels, plaque assays were performed to assess virion production. The titre is a measure of the infectious viral particle concentration in the examined samples. Figure 9 presents the viral titres in terms of plaque-forming units per millilitre (PFU/mL), which assesses the impact of ASP addition on viral replication. No virions were detected in the NC samples prepared during the experiment. Figure 9 shows the viral titre of ASP-treated samples, displayed in blue, to PC, in red. With an average value of 6.65 logPFU/mL, the prophylactic Neudörfl group (ASP/TBEV Neu) showed a lower average logPFU/mL compared to the group that received ASP simultaneously with the Neudörfl strain (ASP+TBEV Neu), with an average of 6.8 logPFU/mL. This compares to an average of 6.59 and 6.84 logPFU/mL in the respective positive control groups. The averages for the analysed Hypr groups are higher in ASP+TBEV Hypr and ASP/TBEV Hypr than the Neudörfl groups, with average values of 7.05 and 6.99 logPFU/mL, respectively. The positive control groups average 7.09 and 7.17 logPFU/mL, respectively. The results show no substantial difference

between the prophylactic and simultaneous treatment within the Hypr groups, and statistically irrelevant differences between the ASP-treated versus the control groups.

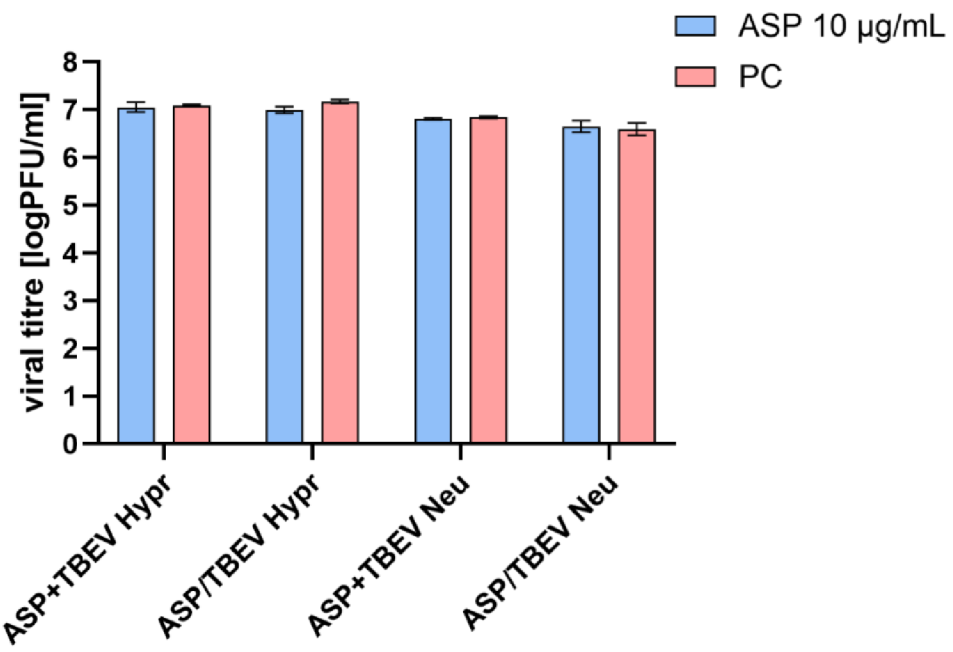

Figure 9. Comparative Analysis of Viral Titre. The graph represents viral titres results showing the average number of plaque-forming units formed across different experimental conditions (in blue), compared to the positive control displayed in red.

5 Discussion

TBEV is an orthoflavivirus which causes TBE, an infection of the central nervous system. It exhibits widespread geographic distribution and is endemic in various European countries (ECDC 2022). TBE is often asymptomatic and is preventable through vaccination, however, there is an urgent need to develop antiviral therapeutics that target TBEV. This relevance is further underscored by the apparent effect of climate change on the endemic tick and tick-borne disease dynamics (Voyiatzaki et al. 2022). In this quest, the assessment of phytochemicals exhibiting antiviral activity as therapies could aid in discovering novel treatments (Naithani et al. 2010). Some progress has been made in the past years, and several agents of plant origin have been shown to be effective in inhibiting TBEV and other viruses (Talactac et al. 2016; Leonova et al. 2020; Khasnatinov et al. 2021; Mammari et al. 2021).
ASP is a compound of a great therapeutic potential, and although scarce literature evaluating its antiviral activity is available, and no findings regarding the compound's ability to interfere with TBEV have been published, its pharmacological profile has been vastly reported on (Majeed et al. 2022). Therefore, hypothesising about ASP's potential antiviral properties requires a speculative approach based on existing knowledge of its mechanism of action, as well as the established characteristics of TBEV and other viruses. First, sterols are essential components of eukaryotic cell membranes and play a role in the governance of membrane fluidity and permeability (Hartmann 1998; Piironen et al. 2000; Rogowska and Szakiel 2020). ASP may incorporate into the viral envelope or host cell membrane, thereby interfering with the entry or attachment of the virus into the host cell by modifying the composition of the cell membrane. Second, ASP may be a protease inhibitor (Palanisamy et al. 2022). If ASP can penetrate infected host cells, it could potentially show an inhibitory effect against the TBEV NS2B-NS3 protease complex. Third, ASP exhibits anti-inflammatory properties (Jeong et al. 2010; Lee et al. 2012; Borges et al. 2014). This effect could lead to a modulation of the host's immune response to viral infection. Fourth, ASP has demonstrated antioxidant properties (Jeong et al. 2004; Lawal et al. 2020). These might facilitate the mitigation of oxidative stress in infected host cells, which may affect the life cycle of TBEV. More research is needed to establish hypotheses about the mechanisms of action as well as prospective biological applications of ASP.

Assessing the cytotoxicity is considered an essential step preceding experiments testing a compound's biological effects (Di Nunzio et al. 2017). This can further help in subsequent research on the mechanism of action, where it is important to distinguish between

the antiviral activity of a substance and its cytotoxic effects when interpreting results. Furthermore, this is a common initial step in protocols studying antiviral effects against TBEV (Krylova et al. 2018; Leonova et al. 2020; Tang et al. 2023). The performed cytotoxicity assay compared ASP's continuous effects on cell viability over a period of 4 days. The viability was compared to a baseline of 80 %. This is based on characterisation guidelines of the International Organization for Standardization, which deem values below 80 % indicative of cytotoxicity (ISO 2009). The IC_{50} calculations yielded an average of 28.6 µg/mL and for the higher concentrations, a substantial decrease in cell viability was observed on the second and fourth day. This suggests that the mechanism of action causing cytotoxicity might not occur immediately. The calculated IC_{50} values are lower than previous findings, where hexane was used instead of DMSO as a solvent (Vrchotová et al. 2024). These differences may be attributed to the properties of the solvents used. DMSO could enhance cellular penetration of ASP across cell membranes (Brayton 1986) and is commonly used as a cryopreservant at 10 % (Whaley et al. 2021), while hexane may result in a reduced interaction of ASP with the cells due to its poorer solubility in the polar environment of cellular interiors (Corby and Elworthy 2011).

Various experimental schemes have been applied in the study of novel antiviral compounds, some of which include a comparison between prophylactic and post-infection treatment (Tang et al. 2023; Leonova et al. 2020; Talactac et al. 2016; Krylova et al. 2018). Although unrelated to TBEV research, the "time-of-addition" method has been established to narrow down the mechanism of action of a novel compound by assessing the time of administration as a crucial factor in interfering with the viral replication process (Daelemans et al. 2011). Additionally, in an assessment of the antiviral activity of a compound from the *Zosteraceae* family, potential preventive, and inhibitory effects against TBEV were analysed to study the mechanism of action (Krylova et al. 2018).

Thus, a similar time-dependent approach was chosen for this project and two experimental conditions were set for the assessment of ASP as an antiviral agent, thereby potentially targeting early viral entry, as well as interfering with viral replication at a later stage of infection. The first group, depicted in red bars in Figure 6 (ASP/TBEV Hypr) received prophylactic ASP. This condition represents ASP treatment as a preventive measure to assess whether administration before viral exposure can prevent or reduce the infection. The second group, displayed as blue bars (ASP+TBEV) in Figure 6, represents an acute treatment scenario. Here, the aim is to evaluate ASP's ability to combat the virus once the infection has

proceeded. This experimental design gives insights into ASP's ability to interfere with the early stages of the infection and its effectiveness after the virus has started replicating.

In Figure 6, the development of cell viability following Hypr-infection is visualised. The general trend suggests a higher viability of the prophylactic group (ASP/TBEV Hypr) compared to the group receiving ASP at the time of viral infection (ASP+TBEV Hypr). This aligns with previous findings applying an experimental scheme that included the assessment of a compound's preventive potential (Leonova et al. 2020).

Most viability values in the prophylactic group exceed 100 %, suggesting not just cell survival but proliferation. This might mean that ASP treatment potentially promotes cell growth when added before and again after infection. In contrast, the main reason for a decrease in cell viability as highlighted by blue asterisks in Figure 6 may be the toxic effect of TBEV. The result suggests that, unless countered by ASP, the presence of the virus reduces the cell's ability to survive. When comparing the two examined conditions, it is evident that prophylactic treatment seems generally more effective at maintaining cell viability across most tested concentrations compared to the treatment administered simultaneously with infection (ASP+TBEV Hypr). This may suggest that ASP potentially provides some protection when added prior to viral exposure. The statistical significance noted in some of the higher concentrations means that the differences observed are likely not due to chance and there might be a causal effect. This suggests a concentration-dependent effect of ASP when administered as a prophylactic treatment.

While this partially holds true in the case of the Neudörfl-infected cells, the results presented in Figure 7 are less consistent. The ASP+TBEV Neu group generally shows low viability compared to PC, while the prophylactic ASP/TBEV Neu group exceeds the viability threshold of 100 % in most concentrations. Notably, the addition of ASP at concentrations of 50 µg/mL and higher, combined with viral infection causes a stark decrease in viability, probably due to ASP's cytotoxic potential and TBEV activity. As is the case with the Hypr-infected cells, the prophylactic Neudörfl group shows a trend in increased viability compared to those cells receiving ASP concurrently with viral infection. In comparison, the viability trends of Neudörfl-infected and Hypr-infected cells correspond to the virulence of the strains (Wallner et al. 1996). In Figure 6, viability values are generally closer to the PC, while in Figure 7, there is a higher contrast in values across concentrations and groups. From this, it can be inferred that the more invasive Hypr strain damages the cells more severely compared to Neudörfl in the ASP/TBEV group. This coincides with findings of a previously published comparison between these TBEV strains, according to which the viability of Hypr-infected cells was

affected more gravely than those infected with Neudörfl (Selinger et al. 2019). Based on the assessment of a balance between minimal cytotoxicity and maximum potential antiviral effects, the ASP concentration of 10 µg/mL was deemed suitable for further testing, although testing 25 µg/mL is highly recommended for future research.

In the interpretation of RT-qPCR results, levels rising above the baseline level of viral replication represented by the PC indicate an increase in NS1 gene expression, while levels below the baseline suggest reduced expression. Relative quantification and normalisation using the ΔΔCt method has been described in a recent publication studying TBEV (Selinger et al. 2019). The average RNA fold changes of 3.35 and 1.80 for the ASP+TBEV Hypr and ASP+TBEV Neu groups, respectively, are higher than the baseline, suggesting an increase in viral replication or in NS1 gene expression in the groups that received ASP simultaneously with viral infection. However, a lack of significance is indicated by wide error bars. Significant viral RNA production was observed despite ASP addition, indicating little antiviral effectiveness of ASP, especially against Hypr. Notably, the wide standard deviations imply variability. In contrast, the average RNA fold changes of 1.11 for the ASP/TBEV Hypr group, and 0.88 for the ASP/TBEV Neu group could indicate an inhibitory effect of a prophylactic ASP administration, although this effect would predominantly apply to the cells infected with the Neudörfl strain, which are the sole group exhibiting levels below the PC with a small standard deviation. The reduced levels displayed by the Neudörfl groups compared to Hypr are likely representative of the strain's lower virulence, and possibly imply a strain-dependence of ASP's effectiveness. As was discussed previously, ASP may affect virion assembly or protein production and hence the production of infectious virions (Siddiqui et al. 2022). Thus, the determination of viral titres by plaque assay in addition to quantitative RNA measurements were important to gain more conclusive insights. The results from the performed plaque assay do not indicate a significant inhibitory effect of ASP across all experimental conditions. The average of 7.05 logPFU/mL of the ASP+TBEV Hypr group indicates a negligible reduction of viral titre compared to 7.09 logPFU/mL in the PC. The effect of ASP is similarly weak in the prophylactic Hypr group, with an average of 6.99 logPFU/mL compared to the 7.17 logPFU/mL in the PC. In the ASP+TBEV Neu group, the average of 6.81 logPFU/mL is almost identical to the average of 6.84 logPFU/mL in the PC and differs only slightly from the PC in the prophylactic group (6.65 logPFU/mL in ASP/TBEV Neu versus 6.59 logPFU/mL in PC). Interestingly, the titre is higher in the ASP-treated group than in the PC, possibly due to the positively affected cell viability by ASP.

ASP has not been thoroughly studied as a potential antiviral agent, although the following observations may be taken into consideration: ASP was among several active phytocomponents analysed and screened for their ligand-protein interactions in a comparison of antiviral drugs used against SARS-CoV-2 proteins, as well as for their interaction with the SARS-CoV-2 main protease (Palanisamy et al. 2022; Siddiqui et al. 2022). ASP demonstrated a complementary fit in the studied viral protein binding pockets (Siddiqui et al. 2022). ASP reportedly shows relevant binding affinities with the N-terminal RNA-binding domain and the Papain-like protease, which are involved in viral assembly, as well as processing viral proteins, thus in viral replication (Siddiqui et al. 2022). It also exhibited strong binding affinity to the SARS-CoV-2 main protease, suggesting the ASP could inhibit the protease activity required for viral replication (Palanisamy et al. 2022).

Like TBEV, SARS-CoV-2 is a +ssRNA virus, and although proteases are different in coronaviruses and orthoflaviviruses, some of the findings may have the potential to translate to TBEV. No direct comparison can be drawn between our results and the cited publications due to different methodological approaches, however, molecular docking studies and studying the molecular dynamics might reveal insights into the binding affinities of ASP to TBEV proteins.

In summary, we have shown that ASP at higher concentrations demonstrated cytotoxic effects on medulloblastoma cells, which reinforces findings suggesting ASP's antitumour effects (Sedky et al. 2018). Regarding the assessed antiviral potential, our results, as outlined above, do not allow for a definite conclusion concerning the mechanism of action. Further testing was not possible due to time constraints and the deficiency of ASP stock that depends on the growing season of the source plant *I. glandulifera*. However, additional experiments using higher ASP concentrations (25 μg/mL) may be conducted in future research endeavours to assess the inhibitory concentration and possible interference with viral replication. Additionally, the protocol can be repeated using primary cells at a subsequent experimental stage.

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