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Research Article

Determination of non-cytotoxic antiviral concentrations of purine and indole derivatives *in vitro*

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Abstract

Mammalian cells from lines derived from bovine embryonic trachea (EBTr) were used in the present study. After the formation of semi-confluent monolayers, one subpopulation of cells was inoculated with the vaccine avian poxvirus strain FK (fowl) and the other with the vaccine avian poxvirus strain Dessau (pigeon) (*Poxviridae* family). Twenty-four hours after viral inoculation, individual subsets of infected cells were treated with the purine derivative aminophylline and the remaining subsets with the indole derivative ergotamine tartrate, respectively. The effects of the analogues thus administered on the cells were recorded at the 24th hour and 48th hour after treatment with each substance, respectively. In cells inoculated with the FK viral strain and treated with both aminophylline and ergotamine tartrate, decreased cell viability was observed at all dilutions at the 48th hour post-treatment compared to the 24th hour. In aminophylline-treated cells, these differences were not statistically significant, unlike in ergotamine tartrate-treated cells, where they were statistically significant. In the same cells infected with the Dessau strain and treated with aminophylline, although decreased cell viability was found at the 48th hour of treatment compared to the 24th hour, in most cases no statistically significant differences were found. In cells infected with the same strain but treated with ergotamine tartrate, despite the lack of statistically significant differences, increased cell viability was seen at the 48th hour post-treatment compared to the 24th hour, specifically at the higher concentrations of 10^{-3} and 10^{-4} M/mL. These results suggest a lower cytotoxicity of aminophylline compared to ergotamine tartrate, but on the other hand, a higher anti-viral activity of ergotamine tartrate against the Dessau virus strain compared to aminophylline in *in vitro* conditions. Further studies need to be conducted in this regard.

Keywords

mammalian embryonic cells, vaccine avipoxviral strains, purine and indole derivatives

Introduction

In recent years, the key role of nucleic acid base modifications in gene regulation has been demonstrated (Rausch et al. 2021; Zhang et al. 2021). These modifications can affect the stability of the DNA double helix (e.g., by methylation of a cytosine residue). Of particular interest is the impact of modifications in nucleotides and nucleosides in the structure of RNA molecules (Dulin et al. 2017; Gonzales-Van Horn and Sarnow 2019; Williams et al. 2019; Imam et al. 2020; Zhang et al. 2020; Johnson and Dangerfield 2021; Lee et al. 2021; Burgess et al. 2022). Evidence from a number of studies reveals their roles in mRNA functions and some transporter RNA (tRNA) molecules, thus influencing a number of physiological and/or pathological processes (Bao et al. 2023; Väre et al. 2017). In the interaction of indole compounds with pyrimidine bases, antitumor and antimicrobial effects have been demonstrated (Mohamed et al. 2014). The role of the N6-methyladenosine (m^6A) modification has been demonstrated in a number of physiological processes, including malignancies and viral infections (Dang et al. 2019; Williams et al. 2019; Zhang et al. 2021; Zhang et al. 2020; Zhang et al. 2021). Chemical modification of nucleic acids has emerged as a novel mechanism of epigenetic regulation in cells, playing a key role in a variety of biological processes (Zhang et al. 2020), as well as in the regulation of the life cycle of viruses (Manners et al. 2019). These modifications shed new light on elucidating the pathogenesis of viral diseases (Wu et al. 2020), as well as on pathologies that affect the cellular genome (Zhang et al. 2021), including mechanisms of innate and adaptive immunity (Gonzales-Van Horn and Sarnow 2019; Williams et al. 2019). In this regard, there are opportunities to develop novel therapeutic strategies against a range of diseases, including infectious (Mahmoud et al. 2018; Mirza 2019; Chen et al. 2021; Kataev and Garifullin 2021) and malignant ones (Berdis 2022).

New therapeutic approaches have been developed, as well as new substances belonging to the nucleotide and nucleoside analogue groups (Fung et al. 2011). The ability of these analogues to induce changes in heterocyclic bases and in carbohydrate residues as well as combined changes has been demonstrated (Agrawal and Gait 2019). For example, replacing an oxygen atom with an atom of another element can affect both the spatial structure and biological properties of nucleosides (Väre et al. 2017). Most often, the oxygen atom in a carbohydrate residue is replaced by an atom of carbon, nitrogen, sulfur, and/or phosphorus. The terminal compounds are generally called carbocyclic nucleosides: azanucleosides, thionucleosides, and phosphonucleosides, respectively. According to a number of studies, the influence of these compounds on RNA and/or DNA replication mechanisms underlies their antiviral and antitumor effects (Hill et al. 1998; Holý et al. 1999; Parker 2009; McGuigan et al. 2016; Dulin et al. 2017; Baroud et al. 2021; Johnson and Dangerfield 2021; Ramesh et al. 2021). The inhibitory effect of nucleotide and nucleoside analogues by influencing the enzymes DNA-dependent DNA polymerase and reverse transcriptase (RT) results in reduced production of infectious viral particles (Seigneres et al. 2002). Other targets for these substances are the enzymes viral DNA polymerase, AdoHcy hydrolase, CTP synthetase, dTMP synthetase, OMP decarboxylase (De Clercq 1987; Chien et al. 2020; Sepúlveda et al. 2022), and mitochondrial DNA of the host cell (Lee and Kool 2005). The antimicrobial and antitumor effects of some purine analogues have been linked to kinase inhibition (Kawana et al. 1987; Kucukdumlu et al. 2017; Sidwell et al. 1968). These effects can be enhanced when nucleotide and nucleoside analogues are combined with appropriate viral vectors and/or specific antiviral antibodies (Ungerechts et al. 2007). According to another study, the delivery of nucleoside and nucleotide analogues by nanoparticles significantly reduces their cytotoxic effects on cells (Baroud et al. 2021).

Therapeutic (synthetic) derivatives of natural nucleotide and nucleoside analogues show involvement in the same metabolic pathways as endogenous nucleosides and nucleotides. Therefore, another mode of action in this regard is by substituting natural purine and/or pyrimidine bases with suitable structural analogues (Geraghty et al. 2021; Ju et al. 2020; Lee et al. 2003). Conversion of the purine ring to the indole ring has also been demonstrated (Dimicoli and Hélène 1973).

In the present study, the antiviral activity of chemical analogues of purine bases was investigated for the first time. An experimental model of *in vitro*-incubated mammalian embryo cells was used. The main objective was aimed at determining the concentrations of two derivatives, containing purine and indole rings, respectively, exhibiting antiviral effects yet being non-toxic to the cells.

Materials and methods

In this study, mammalian cells from the embryonic bovine trachea (EBTr) cell line were used (at an initial volume of 3×10^4 on 1 mL of cultural fluid). The cultivation medium was a combination of Parker-E199 (Sigma) and Iskov's modification of Dulbecco's medium (IMDM - Sigma), in ratio 1:1. The growth incubation medium was supplemented with 25 mM HEPES buffer (Sigma), 5% normal bovine serum (NBS - Sigma), and the respective antibiotics (100 IU/mL Penicillin - Sigma and 100 µg/mL Streptomycin - Sigma). All cell cultures were incubated in a humidified 5% CO_2/95% air incubator at 37 ^{0}C.

After formation of semi-confluent monolayers in 24-well plates (24 Nunclon; Space Sever Flow Lab.; Linbro), sub-populations of the *in vitro*-incubated mammalian cells were inoculated with suspension of the avian DNA vaccine avipoxviral strain FK (fowl) and other sub-populations with suspension of vaccine avipoxviral strain Dessau (pigeon) (*Avipoxvirus* genus, *Poxviridae* family). After absorption for 45 minutes at room temperature, the monolayers were washed three times with 1 mL per

a well-phosphate buffered solution (PBS, pH 7.2). Subsequently, the PBS was turned off, and 1 mL per well of the cultivation medium was added. A non-inoculated control culture of the same cells was also prepared. Different dilutions of aminophylline and ergotamine tartrate, containing purine and indole rings, respectively (Fig. 1), were applied.

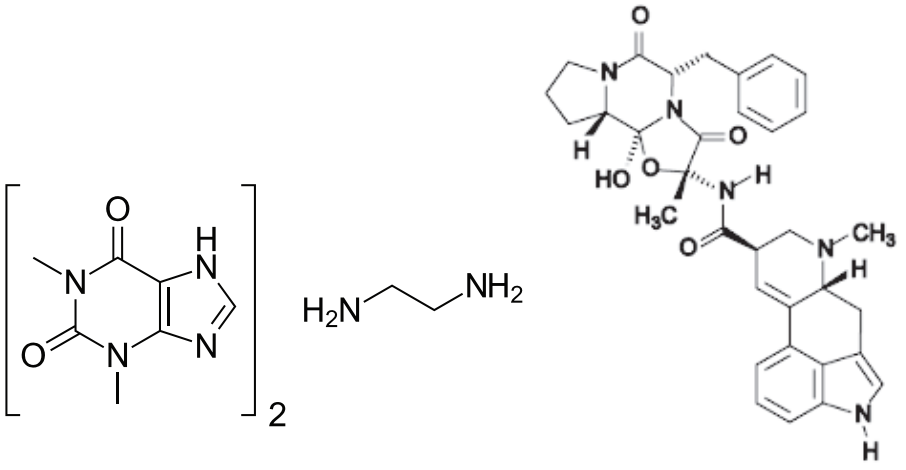
Sub-populations of cells infected with each of the two vaccine avipoxviral strains were treated with each one of the two compounds. The treatment was performed 24 hours post viral inoculation with the respective viral strain. The results were assessed at the 24^{-th} hour of the treatment with each substance and at the 48^{-th} hour of it. The prepared *in vitro*-cell cultures were separated into several sub-groups: inoculated with vaccine virus strain FK, but not treated with any chemical substance; inoculated with vaccine virus strain Dessau but not treated with any chemical substance; treated with aminophylline 24 hours after inoculation with vaccine avipoxviral strain FK; treated with ergotamine tartrate 24 hours after inoculation with vaccine viral strain FK; treated with aminophylline 24 hours after inoculation with vaccine viral strain Dessau; treated with ergotamine tartrate 24 hours after inoculation with vaccine avipoxvirus Dessau; and non-virus-inoculated, non-treated control cultures. The cell cultures were treated with previously prepared gradual dilutions of each one of the tested substances. The values were expressed in M/mL of cultural fluid. Cells were observed using an inverted microscope Televal at 24-hour intervals. Cellular viability was assessed using the Trypan Blue Dye Exclusion Test after previous trypsinization and resuspendation in PBS. The test is based on the capability of the intact membranes of the viable cells to exclude the dye, unlike the unviable (dead) cells (Strober 2001):

% cell viability = (total number of viable cells per 1 mL cell suspension/total number of cells per 1 mL cell suspension) × 100

In all cases, the values were expressed as mean ± standard deviation (SD), and a Student's *t*-test was applied. The differences were determined as statistically significant at $p < 0.01$.

Results

At the 48th hour after treatment of cells infected with the FK vaccine virus strain with aminophylline, cell viability was reduced compared to the 24th hour, but the differences were not statistically significant (Fig. 2A). With respect to cells infected with the same strain and treated with ergotamine tartrate, decreased viability was also found at the 48th hour compared to the 24th hour, and here the differences were statistically significant (Fig. 2B). Only cells treated with a 10^{-4} M/mL dilution of ergotamine tartrate at the 24th hour had cell viability (Fig. 2B) approximately equal to that of cells treated with the same dilution of aminophylline for the same time period (Fig. 2A). At all other dilutions of the two substances, there was higher viability in cells treated with aminophylline at the 24th hour compared to the viability of those treated with ergotamine tartrate for the same time period (Fig. 2). The viability of cells infected with the FK strain was significantly higher at dilutions of aminophylline 10^{-8}, 10^{-7}, and 10^{-6} M/mL at both the 24th and 48th hours (Fig. 2A) compared to that of cells infected with the same strain and treated with the same dilutions of ergotamine tartrate for the same time periods (Fig. 2B). At dilutions of 10^{-5} and 10^{-4} M/mL at



A ($C_{16}H_{24}N_{10}O_4$) **B** ($C_{70}H_{76}N_{10}O_{16}$)

Figure 1. Structural and chemical formulas of each one of the two tested purine and indole derivatives: (**A**) purine derivative aminophylline (1H-Purine-2,6-dione, 3,7-dihydro-1,3-dimethyl-, compd. with 1,2-ethanediamine) ; (**B**) indole derivative ergotamine tartrate ((6*aR*,9*R*)-*N*-[(1*S*,2*S*,4*R*,7*S*)-7-benzyl-2-hydroxy-4-methyl-5,8-dioxo-3-oxa-6,9-diazatricyclo[7.3.0.02,6]dodecan-4-yl]-7-methyl-6,6*a*,8,9-tetrahydro-4*H*-indolo[4,3-fg]quinoline-9-carboxamide;(2*R*,3*R*)-2,3-dihdroxybutanedioic acid).

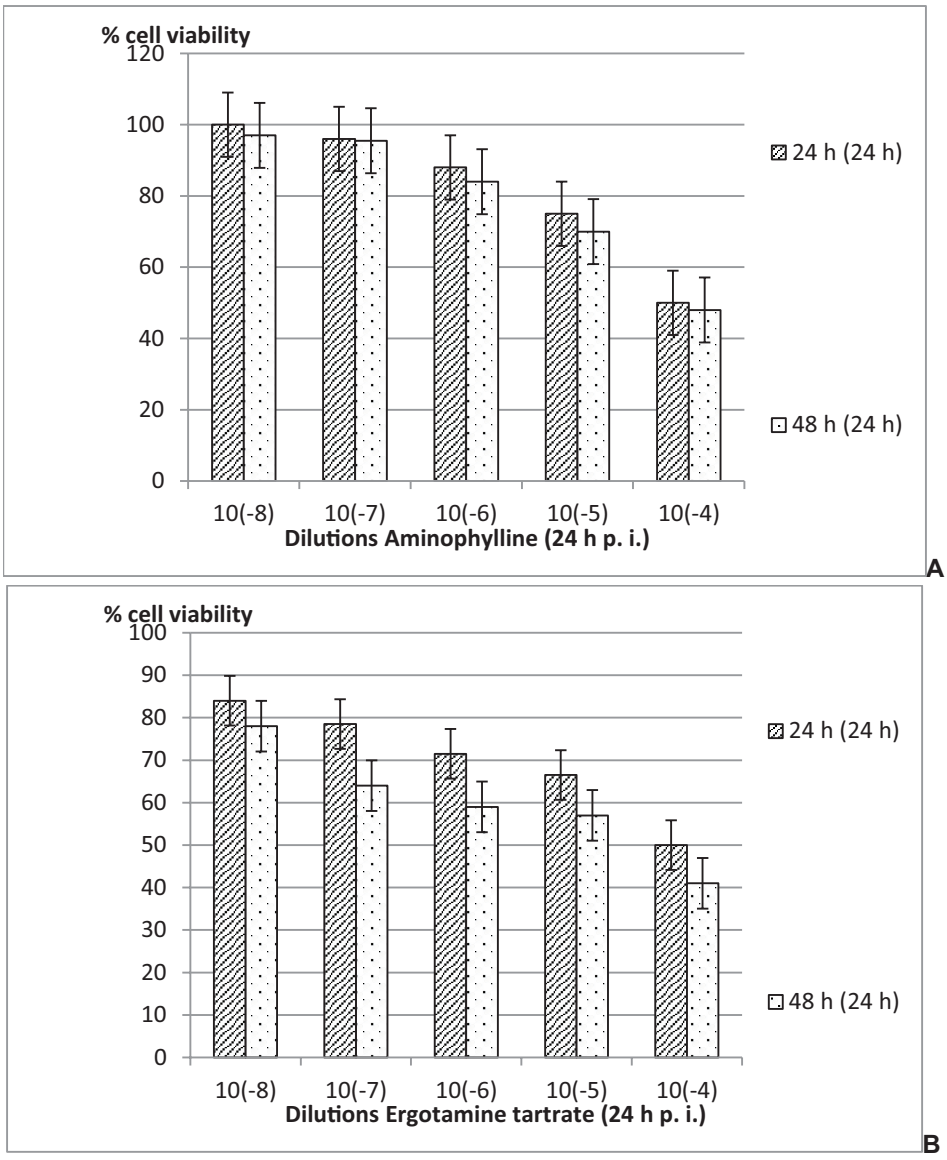


Figure 2. Cytotoxic and anti-viral activity of the tested chemical derivatives at the 24-th and 48-th hours of the treatment of cell cultures from embryonic mammalian cells EBTr, inoculated with vaccine fowl pox viral strain FK: **A**. Treated with the purine analogue aminophylline; **B**. Treated with the indole analogue ergotamine tartrate.

the 24th hour, approximately equal values were observed in the viability of cells treated with either substance. By the 48th hour, the viability of cells treated with the same dilutions of ergotamine tartrate was significantly lower (Fig. 2B) compared to that of cells treated with these dilutions of aminophylline over the same time period (Fig. 2A). The lack of statistically significant differences in cell viability at the 24th and 48th hour of treatment with all dilutions of aminophylline (Fig. 2A) may be explained by the lower cytotoxicity of this compound compared to ergotamine tartrate.

For cells infected with the vaccine virus strain Dessau and treated with aminophylline, there was a slight decrease in cell viability at the 48th hour compared to the 24th hour (Fig. 3A). In contrast, cells infected with the same strain but treated with 10^{-7}, 10^{-4}, and 10^{-3} M/mL dilutions of ergotamine tartrate showed a marginal increase in cell viability at the 48th hour compared to the 24th hour (Fig. 3B). In most cases, similar cell viability was maintained in cells infected with the vaccine Dessau virus strain and subsequently treated with both aminophylline (Fig. 3A) and ergotamine tartrate (Fig. 3B) at the 24th and 48th hours. Significantly lower viability of cells treated with a 10^{-3} M/mL dilution of aminophylline was only found at the 48th hour (Fig. 3A) compared to the same cells treated with the same dilution of ergotamine tartrate for the same time period (Fig. 3B). At the same dilution of ergotamine tartrate, as well as at dilutions of 10^{-4} and 10^{-7} M/mL, increased cell viability was observed at the 48th hour compared to the 24th hour, although the differences were not statistically significant (Fig. 3B). In contrast, at dilutions of ergotamine tartrate 10^{-5} and 10^{-6} M/mL, decreased cell viability was found at the 48th hour compared to the 24th hour, but again no statistically significant differences were observed.

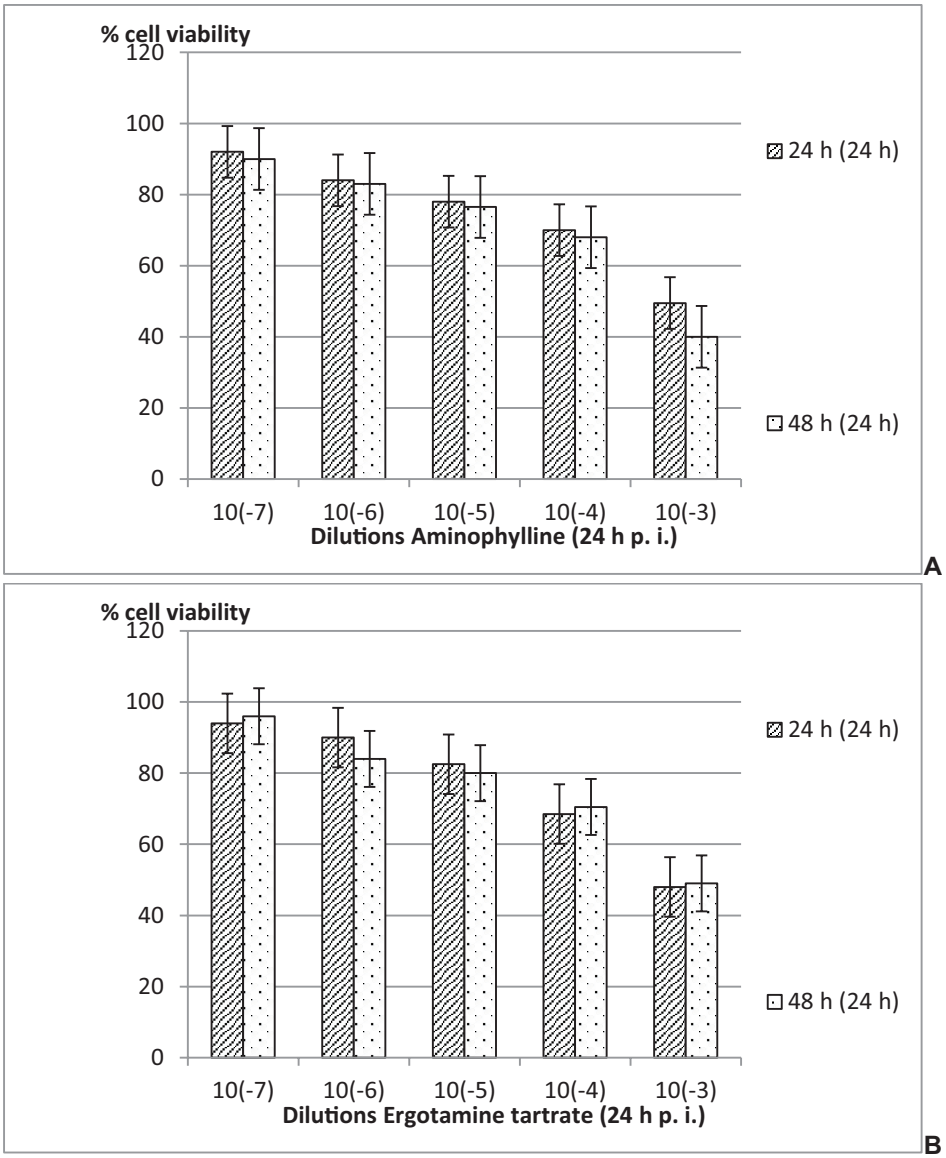


Figure 3. Cytotoxic and anti-viral activity of the tested chemical derivatives at the 24-th and 48-th hours of the treatment of cell cultures from embryonic mammalian cells EBTr, inoculated with the vaccine pigeon pox viral strain Dessau: **A**. Treated with the purine derivative aminophylline; **B**. Treated with the indole derivative ergotamine tartrate.

These data suggest a more pronounced anti-viral activity of ergotamine tartrate against the Dessau strain in *in vitro* conditions compared to that of aminophylline. At the same time, the presented results indicate lower *in vitro* cytotoxicity of the purine analogue aminophylline compared to the indole analogue ergotamine tartrate.

Discussion

The present results are in agreement with the literature regarding the antiviral impact of substances that increase the frequency of mutations in viral populations incompatible with their survival (Perales et al. 2019). Similar effects in *in vivo* conditions have been reported on the influence of 6-Mercaptopurine on variola virus (genus *Orthopoxvirus*, *Poxviridae* family) in infected *Macaccus rhesus* monkeys, in which case modulation of the immune response was also affected (Janssen et al. 1962). In immunodeficient SCID mice infected with the *Vaccinia* virus (genus *Orthopoxvirus*, *Poxviridae* family), analogous effects were reported on the influence of the compound 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine (Neyts et al. 2001). Previous studies on the same compound have shown its inhibitory effect on the replication of some herpesviruses (*Herpesviridae* family) both *in vitro* (Neyts et al. 1998) and *in vivo* (Neyts et al. 1995). Suggestions have also been made regarding enhanced anti-herpesvirus activity of the synthetic purine analogue hydroxyurea, which is expressed by competition with natural nucleotides at the level of viral DNA polymerase (Neyts et al. 1999). In the treatment of human immunodeficiency virus (HIV) (*Retroviridae* family) with azidothymidine, of key importance appears to be the azido group at the 3rd

position in the molecule (Sirivolu et al. 2013). Recently, similar studies have been conducted on the effects of nucleoside analogues, including the anti-SARS-CoV-2 drugs remdesivir and molnupiravir, in *in vitro* conditions on virus-infected human respiratory cells (Schultz et al. 2022). A suppressive effect on the replication of the RNA genome of viruses belonging to the *Coronaviridae* family, including SARS-CoV-2, has been shown (Burgess et al. 2022). On the other hand, the compound 6-methylmercaptopurine (6-MMPr) showed an inhibitory effect on the West Nile Fever virus (*Flaviviridae* family) in cell cultures but not in *in vivo* conditions (Lim et al. 2011). Although the authors did not find marked viremia and/or significantly elevated viral titers in peripheral tissues, elevated titers were found in the brains of virus-infected mice. These features suggest tissue-dependent activity of this virus. The antiviral influence of a number of nucleoside analogues is reflected in the inhibition of intracellular nucleoside synthesis by suppressing the action of the enzymes flavivirus RNA-dependent RNA polymerase, methyltransferase, and helicase/NTPase (Eyer et al. 2018). Other nucleotide and nucleoside derivatives inhibit the activity of other enzymes such as kinases (Kawana et al. 1987; Kucukdumlu et al. 2017; Sidwell et al. 1968), synthetases, and decarboxylases (De Clercq 1987; Chien et al. 2020; Sepúlveda et al. 2022). Under the influence of these analogues, base substitutions in the genomes of viruses such as Zika (ZIKV) and Usutu (USUV) (*Flaviviridae* family) have also been found to cause lethal mutations for these viruses (Bassi et al. 2018). Reports have also been obtained on the effect of purine analogues against Lamivudine-resistant mutants of human hepatitis B virus (hHBV) and duck hepatitis B virus (DHBV) (*Hepadnaviridae* family) incubated *in vitro* on liver cells from the HepG2 line (Seigneres et al. 2002). Antiviral effects of the acyclic nucleoside phosphonates HPMPC (sidofovir), PMEA (adefovir), and PMPA (tenofovir) have been demonstrated in *in vitro*- and *in vivo*-conditions (de Clercq 2003; Snoeck et al. 2001). Mechanisms of influence on intermolecular interactions of viruses with both RNA- and DNA-genomes have been suggested. This also includes interactions of viral RNA (representing the viral genome or viral RNA transcript/mRNA) with various cellular proteins (Imam et al. 2020; Lee et al. 2021). Some purine analogues, such as acyclovir and its derivatives (ganciclovir, valacyclovir, famciclovir, etc.), have been found to affect DNA polymerase enzyme activity as competing enzyme inhibitors. Thus, irreversible binding of these compounds to the broken DNA strand often leads to its premature termination.

Unlike most of the literature data, which address the antiviral effects of nucleotide and nucleoside analogues through different mechanisms of action on viral DNA and/or RNA molecules as well as on different intermolecular interactions, the present publication is the first to investigate analogous mechanisms of antiviral effects of chemical analogues of purine bases. In this regard, concentrations of the base analogues used are sought that are non-cytotoxic yet exhibit antiviral activity. Our previous studies have shown similar values of non-cytotoxic concentrations of the two substances on uninfected embryonic cells from the EBTr line with the values of non-cytotoxic concentrations on cells from the 3T3 line of mouse embryos (Sainova et al. 2024). Future studies are planned on the determination of non-cytotoxic concentrations of the two compounds exhibiting antiviral effects on virus-infected cells from mouse embryonic fibroblasts. In our studies, we focused on these cell types given the analogous features in terms of genetic and transcriptional characteristics between bovine, mouse, and human that have been demonstrated in the literature (Breschi et al. 2017; Yao et al. 2022).

Conclusion

In the present study, non-cytotoxic antiviral concentrations of the purine analogue aminophylline and the indole analogue ergotamine-tartrate were investigated in *in vitro* conditions. Laboratory-incubated cells from the embryonic bovine trachea (EBTr) line were infected with heterologous avian poxvirus vaccine strains. One sub-population of the cells was infected with the FK fowl strain and the other sub-population with the Dessau pigeon strain, respectively. Twenty-four hours after viral inoculation of the cells with each of the two vaccine virus strains, individual sub-populations were treated with aminophylline and the remaining sub-populations with ergotamine tartrate for 24 and 48 hours, respectively. There was a decreased viability of cells inoculated with the FK viral strain at the 48th hour after treatment with both aminophylline and ergotamine tartrate compared to the 24th hour. In ergotamine tartrate-treated cells, these differences were statistically significant, unlike in the same cells treated with aminophylline. With respect to the cells infected with the Dessau virus strain and treated with ergotamine tartrate, a higher viability value was observed at the 48th hour of treatment compared to the 24th hour at low dilutions of this substance, despite the absence of statistically significant differences. The results obtained indicate a less pronounced cytotoxicity of aminophylline but a more pronounced anti-viral activity of ergotamine tartrate towards the Dessau virus strain in *in vitro* conditions. Further studies in this direction are needed.

Authors' contributions

I.S.: Performance of the experiments and participation in the writing of the manuscript; V.K.: Statistical assay; I.I.: Formal design; R.N.: Performance of the experiments and participation in the writing of the manuscript; A.P.: Participation in the analysis of the experimental data; R.H.: Providing of the necessary sources about the experiments, described in the manuscript; D.D.: Providing of the necessary sources and participation in the writing of the manuscript; Tz.M.: Performance of the experiments and participation in the writing of the manuscript.

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