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Synergistic activity of an RNA polymerase PA-PB1 interaction inhibitor with oseltamivir against human and avian influenza viruses in cell culture and *in ovo*

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A R T I C L E I N F O

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A B S T R A C T

In search of novel therapeutic options to treat influenza virus (IV) infections, we previously identified a series of inhibitors that act by disrupting the interactions between the PA and PB1 subunits of the viral RNA polymerase. These compounds showed broad-spectrum antiviral activity against human influenza A and B viruses and a high barrier to the induction of drug resistance *in vitro*. In this short communication, we investigated the effects of combinations of the PA-PB1 interaction inhibitor **54** with oseltamivir carboxylate (OSC), zanamivir (ZA), favipiravir (FPV), and baloxavir marboxil (BXM) on the inhibition of influenza A and B virus replication *in vitro*. We observed a synergistic effect of the **54**/OSC and **54**/ZA combinations and an antagonistic effect when **54** was combined with either FPV or BXM. Moreover, we demonstrated the efficacy of **54** against highly pathogenic avian influenza viruses (HPAIVs) both in cell culture and in the embryonated chicken eggs model. Finally, we observed that **54** enhances OSC protective effect against HPAIV replication in the embryonated eggs model. Our findings represent an advance in the development of alternative therapeutic strategies against both human and avian IV infections.

Influenza viruses (IV) are listed among the most important threats for world Public Health, given their pandemic potential due to the high transmissibility, spillover events, and antigenic variability (Webster, 2023). Current anti-influenza (flu) therapy relies on drugs targeting different steps of the viral replication cycle (Kumari et al., 2023). Among the approved drugs, oseltamivir carboxylate (OSC), zanamivir (ZA), and peramivir inhibit the neuraminidase (NA) enzymatic activity and virus release, while baloxavir marboxil (BXM) and favipiravir (FPV) both inhibit the viral RNA-dependent RNA polymerase (RdRp) complex, by blocking the endonuclease activity of PA subunit (Noshi et al., 2018) or the catalytic activity of PB1 subunit (Furuta et al., 2013), respectively. All these drugs have been associated with the emergence of resistance or reduced sensitivity during clinical therapy (Li et al., 2015; Omoto et al., 2018; Imai et al., 2020). In addition, FPV has been associated with severe side effects, including teratogenicity and embryotoxicity (Lagocka et al., 2021). Thus, there is an urgent need to develop new antiviral strategies to counteract the emergence of both new IV strains of animal origin with pandemic potential and drug-resistant viruses (Hou et al., 2022). A possible solution to combat drug resistance might be combination therapy, effectively applied for the treatment of viral infections by different pathogens, such as human immunodeficiency virus and hepatitis C virus (Bartlett et al., 2001; Kohli et al., 2014). In this regard,

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Abbreviations

BXM	baloxavir marboxil
CEFs	chicken embryo fibroblasts
CI	combination index
DRI	dose reduction index
flu	influenza
FPV	favipiravir
HPAIV	highly pathogenic avian influenza virus
IV	influenza virus
NA	neuraminidase
OSC	oseltamivir carboxylate
RdRp	RNA-dependent RNA polymerase
ZA	zanamivir

several studies evaluated the synergistic effect of OSC and different approved and investigational drugs (Smee et al., 2010; Nguyen et al., 2012; Checkmahomed et al., 2020; Mu et al., 2023; Gaisina et al., 2024). Combination therapy is also applied for the treatment of flu in critically ill patients not responsive to first-line therapy, or in cases of infection with IV strains resistant to licensed anti-flu drugs (Koszalka et al., 2022; Batool et al., 2023).

We focused our previous work on the development of IV inhibitors that act by an innovative mechanism, i.e., the disruption of the interaction between the PA and PB1 subunits of viral RdRp (Muratore et al., 2012a, 2012b; Desantis et al., 2017; Massari et al., 2017, 2021; Nannetti et al., 2019; Pismataro et al., 2021). Some of the inhibitors developed so far showed broad-spectrum antiviral activity against both human influenza A and B viruses (IAV and IBV). In particular, the cycloheptathiophene derivative **54** (Fig. S1) (Desantis et al., 2017) showed effective concentrations at half-maximal response (EC_{50}) ranging from 0.08 to 0.46 μM against a panel of IAV and IBV strains, including clinical isolates and an OSC-resistant strain (Nannetti et al., 2019 and Table S1). Moreover, **54** was able to disrupt PA-PB1 interaction *in vitro*, to inhibit the activity of the IAV RdRp complex in cells, and showed a high barrier to the induction of drug resistance *in vitro* (Nannetti et al., 2019).

Here, we report on the investigation of the effects of combining compound **54** with different approved anti-flu drugs, and on its efficacy against different avian IVs both in cell culture and in the embryonated chicken egg model.

First, we evaluated the efficacy in inhibiting IV replication in cell culture of a double combination antiviral drug regimen consisting of compound **54**, which blocks viral RNA synthesis by interfering with the assembly of RdRp complex, and anti-flu drugs acting by other molecular mechanisms, i.e., OSC, ZA, BXM, and FPV. Plaque reduction assays (PRAs) with influenza virus A/PR/8/34 (PR8, H1N1) strain in infected MDCK cells were performed with equipotent combinations of **54** with OSC, ZA, BXM, or FPV. The antiviral effects of the drug combinations were determined by applying the Chou & Talalay method as previously reported (Chou, 2010; Mercorelli et al., 2020, 2021). The combination of drugs that act by different mechanisms during the virus life cycle might result in synergism or at least in an enhancement of the effectiveness of the approved drug, which could be administered at lower concentrations thus limiting toxicity and drug-resistance issues. As reported in Table 1, we observed a synergistic effect on the inhibition of IAV replication with the **54**/OSC combination at all the concentrations tested (Combination Index, CI < 0.7), even when the two drugs were combined at 0.125-fold of their respective EC_{50}s (Table S1). A similar synergistic activity was observed for **54** in combination with ZA, another FDA-approved inhibitor of NA (Table S2).

On the contrary, we observed an antagonistic effect when **54** was combined with either FPV or BXM at almost all concentrations tested (Table 1). This result is in keeping with the observation that when two drugs share the same target (in this case the viral RdRp complex), they might interfere with each other's antiviral activity (Dunning et al., 2014; Ison, 2017). Importantly, we did not observe cytotoxicity in uninfected cells treated even with the highest drug combinations by MTT assays performed as described (Muratore et al., 2012a; Mercorelli et al., 2016) (Fig. S2), thus the synergistic effects of **54**/OSC and **54**/ZA were most likely the result of combining two drugs with different targets and mechanisms of action, rather than of increased toxicity.

To confirm the synergism between **54** and OSC, we investigated the effects of combining 0.125-fold of their EC_{50}s, hereafter referred as the *lowest dose combination*, on the production of infectious viral progeny by virus yield reduction assays and on the expression of different viral proteins by Western blot (for experimental details please refer to Table S3 and Trevisan et al., 2024). As shown in Fig. 1, the titer of released viral progeny significantly decreased and viral protein expression was almost abolished when the two drugs were combined at concentrations that block virus replication only weakly when used as single agents. To assess whether a synergistic effect could also be observed against IBV, which like IAV is cause of seasonal epidemics in human population, we tested different equipotent combinations of **54**/OSC against the replication of an IBV strain (B/Malaysia/2506/2004, Victoria lineage) by PRAs in MDCK cells. As reported in Table S4, we confirmed the synergistic effect of combining **54** and OSC also for IBV, since we observed a CI < 0.9 for all the drug combinations tested.

We then wished to investigate the activity of **54** both alone and in combination with OSC against IVs of avian origin, since these could be responsible of epidemics in both animals and humans and have a high pandemic potential (Nguyen et al., 2023; Charostad et al., 2023; Shi

Table 1
Analysis of the effects of the combination of **54** and approved antivirals against IAV.

54/drug combination at equipotent ratio (fold of EC_{50}) [a]	CI[b] 54 + OSC	Drug combination effect[c]	CI 54 + FPV	Drug combination effect	CI 54 + BXM	Drug combination effect
0.125	0.6 ± 0.3	*Synergism*	1.7 ± 0.4	*Antagonism*	1.5 ± 0.9	*Antagonism*
0.25	0.6 ± 0.2	*Synergism*	1.5 ± 0.5	*Antagonism*	2.0 ± 0.7	*Antagonism*
0.5	0.6 ± 0.3	*Synergism*	1.4 ± 0.3	*Antagonism*	1.2 ± 0.5	*Antagonism*
1	0.4 ± 0.1	*Synergism*	1.3 ± 0.2	*Antagonism*	1.1 ± 0.4	*Additive effect*
2	0.3 ± 0.1	*Strong synergism*	0.5 ± 0.2	*Synergism*	0.7 ± 0.4	*Moderate synergism*
4	0.20 ± 0.05	*Strong synergism*	0.8 ± 0.1	*Moderate synergism*	0.6 ± 0.3	*Synergism*

[a] Fold of 50% Effective Concentration, i.e., the effective concentration at half-maximal response, for **54**/drug combination yielding an equipotent concentration ratio between the two combined drugs. EC_{50} values were determined by plaque reduction assays (PRA) against IAV/PR8 strain in MDCK cells for each drug either alone or in combination at concentrations starting from 4-fold to 0.125-fold the equipotent ratio of the drugs. EC_{50} values considered for each drug were the following: for **54**, EC_{50} = 0.46 μM; for OSC, EC_{50} = 0.01 μM; for FPV, EC_{50} = 7.7 μM; and for BXM, EC_{50} = 0.0005 μM (please also refer to Table S1).

[b] Combination Index, obtained by computational analysis with Calcusyn software. Reported values represent means ± SD of data derived from n = 3 independent experiments in duplicate.

[c] Drug combination effect defined as: strong synergism for 0.1 < CI < 0.3; synergism for 0.3 < CI < 0.7; moderate synergism for 0.7 < CI < 0.9; additive effect for 0.9 < CI < 1.1; antagonism for CI > 1.1, according to Chou (2010).

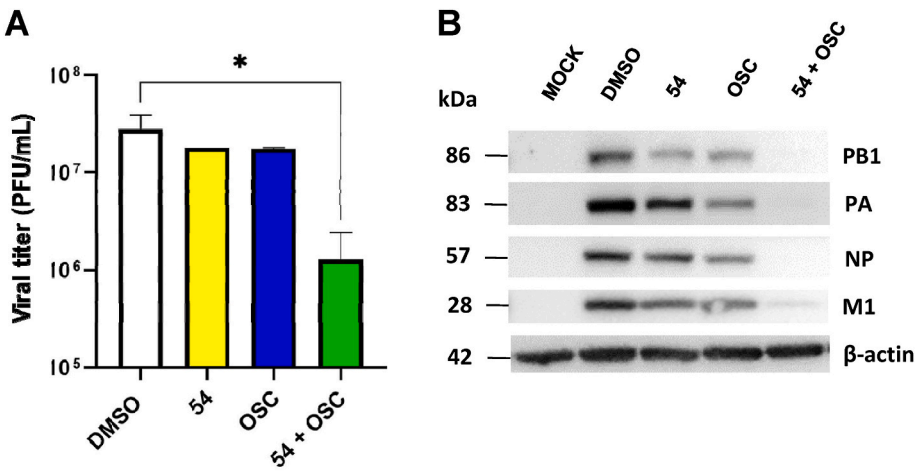

Fig. 1. Effects of the lowest dose combination of 54/OSC on infectious viral progeny production and viral proteins expression. (A) MDCK cells were infected with IAV/PR8 and were treated with 0.057 μM **54** and 0.001 μM OSC, either alone or in combination (at 0.125-fold, i.e. the *lowest dose combination*), or 0.1% DMSO as a control. At 24 h post-infection (hpi), viral progeny released in the supernatants was titrated on fresh MDCK cells by plaque assays. Data shown represent the mean ± SD of n = 4 independent experiments performed in duplicate. Data were analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test. *p < 0.05 *versus* calibrator sample (infected, DMSO-treated cells). (B) Whole cells lysates from mock-infected (Mock), infected DMSO-treated, and drug-treated MDCK cells were collected at 24 hpi and analyzed by Western blot with antibodies recognizing viral PB1, PA, NP, and M1 proteins (Table S3). Cellular β-actin was used as a loading control. Molecular masses in kDa are indicated on the left. A representative image is shown.

et al., 2023). First, we tested **54** and OSC by cytopathic effect (CPE)--based antiviral assays in infected chicken embryo fibroblasts (CEFs) against the replication of two different highly pathogenic avian IVs (HPAIV), i.e., A/Goose/Guangdong/SH7/2013 (H5N1) and A/Goose/-Jiangsu/1306/2014 (H5N8) (Ju et al., 2021). In parallel, cytotoxicity was assessed in uninfected CEFs by CCK-8 assays. As reported in Table S5, both **54** and OSC inhibited the replication of HPAIVs without showing toxicity in CEFs. The EC_{50} values that we observed for **54** and OSC were 81- and 390-fold higher, respectively, than those determined against human viruses (Table S1). The lower and variable potency of OSC and other anti-flu compounds against avian viruses as compared to human strains was previously reported (Zhang et al., 2018; Ju et al., 2021; Jia et al., 2021, 2023), while this is the first report on the antiviral activity of a PA-PB1 inhibitor against viruses of avian origin.

Next, the anti-flu efficacy of compound **54** as a single agent was assessed *in ovo* in a lethal model of influenza infection as previously described (Ju et al., 2021, 2022; Jia et al., 2023). To this aim, specific pathogen free chicken embryonated eggs were challenged with the two HPAIVs (H5N1 and H5N8 subtypes) via allantoic route and treated with different concentrations of **54** or OSC, included as a control. Specific concentrations for **54** starting from 10 mM were selected based on preliminary studies and dose-response curves, which guided us in selecting concentrations that would provide a comprehensive understanding of both compounds' antiviral activity and potential side effects.

Table 2
Survival rates of chicken embryos challenged with highly pathogenic avian IAVs in the presence of different doses of **54** or OSC.

Virus strain	Compound	Concentration (mM)	Chick embryo survival rate (survived/dead)[a]	
			24 h	48 h
	no	without infection	100% (5/0)	100% (5/0)
H5N1[b]	no	without treatment	100% (5/0)	0% (0/5)
	54	10	100% (5/0)	100% (5/0)
		5	100% (5/0)	20% (1/4)
		2.5	100% (5/0)	0% (0/5)
		0.625	100% (5/0)	0% (0/5)
	OSC	10	100% (5/0)	100% (5/0)
		2.5	100% (5/0)	100% (5/0)
		0.625	100% (5/0)	0% (0/5)
		0.156	100% (5/0)	0% (0/5)
H5N8[c]	no	without treatment	100% (5/0)	0% (0/5)
	54	10	100% (5/0)	100% (5/0)
		5	100% (5/0)	20% (1/4)
		2.5	100% (5/0)	0% (0/5)
		0.625	100% (5/0)	0% (0/5)
	OSC	10	100% (5/0)	100% (5/0)
		2.5	100% (5/0)	100% (5/0)
		0.625	100% (5/0)	40% (2/3)
		0.156	100% (5/0)	0% (0/5)

[a] Virus solution (400 $TCID_{50}$) was mixed with an equal volume of the test compound at the indicated concentrations and then incubated for 1 h before chicken eggs inoculation.
[b] A/Goose/Guangdong/SH7/2013.
[c] A/Goose/Jiangsu/1306/2014.

Table 3
Survival rates of chick embryos challenged with a highly pathogenic avian IAV H5N1 strain in the presence of different doses of OSC alone or in combination with compound **54**.

Virus strain	Compound	Concentration (mM)	Chick embryo survival rate (survived/dead)[a]		
			24h	36h	48h
	no	without infection	100% (5/0)	100% (5/0)	100% (5/0)
H5N1[b]	no	without treatment	20% (1/4)	0% (0/5)	0% (0/5)
	54	5	70% (7/3)	30% (3/7)	10% (1/9)
	OSC	2.5	100% (10/0)	70% (7/3)	30% (3/7)
		0.625	90% (9/1)	60% (6/4)	0% (0/10)
		0.156	80% (8/2)	50% (5/5)	0% (0/10)
		0.039	50% (5/5)	20% (2/8)	0% (0/10)
		0.0097	30% (3/7)	0% (0/10)	0% (0/10)
	OSC + 54 (5 mM)	2.5	100% (10/0)	90% (9/1)	40% (4/6)
		0.625	100% (10/0)	70% (7/3)	10% (1/9)
		0.156	100% (10/0)	50% (5/5)	0% (0/10)
		0.039	80% (8/2)	30% (3/7)	0% (0/10)
		0.0097	50% (5/5)	20% (2/8)	0% (0/10)

[a] Virus solution (400 $TCID_{50}$) was mixed with an equal volume of the test compound at the indicated concentrations and then incubated for 1 h before chicken eggs inoculation.
[b] A/Goose/Guangdong/SH7/2013.

As reported in Table 2 and Fig. S3, at 48 h post-challenge, **54** showed a certain level of protection for embryonated chicken eggs against both avian IAV strains at 5 and 10 mM, but not at lower concentrations. On the other hand, OSC was effective at 48 h also at lower concentrations (Table 2), as previously reported (Ju et al., 2021, 2022; Jia et al., 2023). As expected from the lethal model setting, at 48 h post-challenge none of the vehicle-treated chicken embryos survived (Table 2). Although **54** needs further optimization to increase potency and pharmacological properties, this is to our knowledge the first report of an RdRp PA-PB1 interaction inhibitor showing efficacy against avian IVs both in cell culture and *in ovo*.

Finally, we tested the combination of a fixed dose of **54** (i.e., 5 mM) with different concentrations of OSC in the embryonated chicken eggs model against a H5N1 strain of IV to assess the possible synergistic effect of the two-drug combination also *in ovo*. As reported in Table 3, at 24 and 36 h post-challenge the survival rates were higher for the chicken embryos treated with the combinations of OSC/**54** than those of the chicken treated with OSC alone, which was also confirmed by HA titration of the virus recovered from allantoic fluid samples (Table S6). The two-drug combination was more effective in protecting the embryos than OSC alone, since the morphological changes in the OSC/**54** treated embryos were less evident than in the vehicle- or OSC-treated eggs (Fig. S4).

In conclusion, in this study we demonstrated that an RdRp PA-PB1 interaction inhibitor is able to act synergistically with a NA inhibitor both in cell culture and in infected eggs given their different mechanism of action. In contrast, it would be not convenient to couple **54** or other PA-PB1 inhibitors with drugs that share the same target despite acting by a different mechanism, such as BXM and FPV. Noteworthy, the simulated Dose Reduction Index (DRI) of OSC combined with **54** indicated that 95% inhibition of IAV replication may be obtained by reducing OSC dose by 14-fold when combined with **54** at their respective equipotent doses *in vitro*, compared to the concentration of OSC alone needed to achieve the same effect (EC_{95}, Table S7) (Chou, 2006). These findings pave the way to further preclinical evaluation of combination therapy protocols that could exploit PA-PB1 inhibitors to potentiate OSC efficacy. Importantly, this novel therapeutic strategy might allow decreasing OSC dosages and reducing the emergence of *de novo* resistance or the risks of potential loss of sensitivity to OSC during clinical therapy.

Moreover, we also provided a proof-of-concept of the efficacy of a PA-PB1 interaction inhibitor against avian IVs both *in vitro* and in a relevant biological animal model and demonstrated that the synergistic effect in cell culture is maintained also *in ovo*. Further chemical optimization of PA-PB1 interaction inhibitors is needed before proceeding with preclinical evaluation in more advanced models such as mice. Nonetheless, our study represents a significant advance in the evaluation of the translational potential of alternative anti-flu strategies, which is highly needed taking also into account the current outbreak of avian H5N1 IV in dairy cattle in the U.S. (Burrough et al., 2024).

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CRediT authorship contribution statement

Anna Bonomini: Writing – original draft, Methodology, Investigation. **Jiwei Zhang:** Writing – original draft, Methodology, Investigation. **Han Ju:** Writing – original draft, Methodology, Investigation. **Alessia Zago:** Investigation. **Martina Pacetti:** Investigation. **Oriana Tabarrini:** Writing – review & editing, Resources. **Serena Massari:** Writing – review & editing, Resources. **Xinyong Liu:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Beatrice Mercorelli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Peng Zhan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Arianna Loregian:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2024.105980.

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