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Short Communication

Combination of MEK inhibitors and oseltamivir leads to synergistic antiviral effects after influenza A virus infection *in vitro*

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ABSTRACT

MEK inhibitors are very potent and promising compounds in cancer therapy. Earlier investigations have demonstrated that they also possess antiviral properties against influenza virus. This is due to the fact that activation of the Raf/MEK/ERK signaling pathway is a prerequisite for influenza virus replication. As an alternative to vaccination, antiviral therapy is a means to control influenza. The appearance of influenza virus strains that are resistant to current treatment options demonstrates the need for new antiviral strategies. The aim of the presented study was to investigate whether the combination of MEK inhibitors with oseltamivir, an inhibitor of viral neuraminidase activity, would result in a synergistic antiviral effect against pandemic influenza A/Regensburg/D6/2009 (H1N1pdm09) virus. Here we show that four different MEK inhibitors, PD-0325901, AZD-6244, AZD-8330 and RDEA-119 that are orally available and at least in a phase I clinical trial against cancer demonstrate antiviral activity as single agents or in combination with oseltamivir. Combination treatment increased the antiviral activity of oseltamivir significantly and resulted in a synergistic antiviral effect as determined by the Chou–Talalay method. Taken together, the results demonstrate increased antiviral activity of oseltamivir after combination with MEK inhibitors. These data are promising for further preclinical *in vitro* and *in vivo* investigations on the way to developing new antiviral regimens against influenza.

The need for new antiviral strategies against influenza virus infection is a topic of intense discussion. In recent years the viral polymerase was identified as a promising target (Boltz et al., 2010; Furuta et al., 2009; Hayden, 2009). Inhibiting influenza virus polymerase function with favipiravir (T-705) shows a high antiviral potential. Moreover, a combination of this polymerase inhibitor with peramivir, targeting the viral neuraminidase, showed a synergistic antiviral effect (Tarbet et al., 2012). Beside these new strategies targeting the virus directly a new concept arose in the last decade to target the cellular factors required for influenza virus replication (Ludwig, 2009, 2011; Ludwig and Planz, 2008; Pleschka, 2008). In particular the Ras-dependent Raf/MEK/ERK mitogen-activated protein (MAP) kinase signaling pathway, which is activated during influenza virus infection was described as a target for antiviral strategies. The Raf/MEK/ERK signaling pathway is a major regulator of cell proliferation and survival. This pathway became of interest because it is most frequently dysregulated in human cancer where most if not all physiological changes leading to the development of cancer cells involve alteration of signal transduction pathways. Hyperactivation of the Raf/MEK/ERK pathway, which is associated with the development of human malignancies, is frequently observed as a result of mutations in *ras* or *raf* genes. Components of the Raf/MEK/ERK pathway are therefore viewed as attractive candidates for the development of targeted therapies of cancer (Sebolt-Leopold, 2004; Sebolt-Leopold and Herrera, 2004). The question now arises whether these compounds developed for cancer therapy are also suitable as antivirals against influenza?

In our earlier investigations we have used U0126 a potent inhibitor of both MEK1 and MEK2 isoforms (Pleschka et al., 2001; Droebner et al., 2011). Together with PD-98059 and RO-09-2210, U0126 belongs to the first generation of MEK inhibitors. These inhibitors were used in a large variety of preclinical studies demonstrating that blockade of the Raf/MEK/ERK pathway markedly restrains the proliferation of various carcinoma and leukemic cell lines by inducing cell cycle arrest and apoptosis. Unfortunately, because of pharmaceutical limitations none of these compounds were further investigated in clinical evaluation. Until now more than ten MEK inhibitors of the second and third generation with improved bioavailability successfully passed phase I in clinical trials. One of these, Zelboraf (Vemurafenib, PLX-4032), is the first FDA-approved compound for treatment of B-RAF mutation-positive metastatic melanoma. We therefore raised the question whether this new generation of MEK inhibitors would also show

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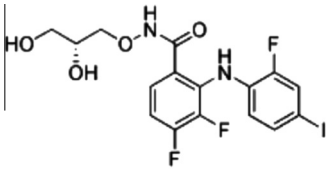
Table 1
Collection of MEK inhibitors.

MEK inhibitor	Company	Phase	Status
PD0325901	Pfizer	Phase I/II	Development stopped
AZD-6244	Array BioPharma/AstraZeneca	Phase II	In progress
AZD-8330	Array BioPharma/AstraZeneca	Phase I	In progress
RDEA-119	Ardea Biosciences/Bayer	Phase I/II	In progress

antiviral activity against influenza virus and therefore would confirm our earlier findings generated primarily with the U0126 MEK inhibitor (Droebner et al., 2011; Pinto et al., 2011; Planz et al., 2001; Pleschka et al., 2001). Therefore, we chose four orally available MEK inhibitors that successfully passed preclinical investigations and are currently in phase I/II clinical trials (Table 1). The antiviral potential of these inhibitors was tested as single agents and in combination with oseltamivir-carboxylate (OC).

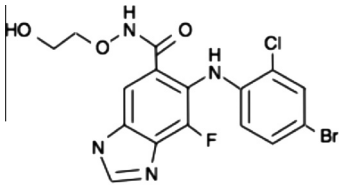
PD-0325901 is a second-generation MEK1/2 inhibitor with significantly improved potency, solubility and bioavailability compared to its structural analogue, CI-1040 (Barrett et al., 2008). Both inhibitors were already tested for their antiviral activity against H5N1 influenza virus in a pilot experiment (Droebner et al., 2011). In the present study, we chose to investigate the antiviral properties of PD-0325901 in more detail, because PD-0325901 has a very low inhibitory concentration 50% (IC_{50}) value of 1 nM against purified MEK1/MEK2, and inhibits the growth of some tumor cell lines at sub-nanomolar concentrations (Sebolt-Leopold and Herrera, 2004; Solit et al., 2006).



PD-0325901; MW 482.19

No toxic effects were found on A549 cells, when concentrations up to 100 μM were used, indicating a CC_{50} (cytotoxic concentration 50%) value of >100 μM (Fig. 1A). The EC_{50} (effective concentration 50%) value of PD-0325901 against H1N1pdm09 influenza virus on A549 cells was 5 nM (Fig. 1B). OC revealed an EC_{50} value of 9 nM (data not shown). Next, we tested whether combination with OC would have a synergistic effect against influenza virus compared to single treatment with these compounds. Only a 10 μM concentration of PD-0325901 showed an increased antiviral effect as indicated by a reduced viral titer when PD-0325901 was combined with different concentrations of OC (Fig. 2A). In order to investigate whether this effect was synergistic the combination index (CI) theorem of Chou–Talalay was used for the quantitative definition of additive effect ($CI = 1$), synergism ($CI < 1$), and antagonism ($CI > 1$) in drug combination (Chou, 2010). As indicated in Table 2 the 1:10 combination of OC and PD-0325901 leads to a very strong synergistic antiviral effect with very low *CI* values at different percentage of influenza virus inhibition. The synergistic effect was best demonstrated when virus infected cells were treated with suboptimal concentrations of OC (Fig. 3A).

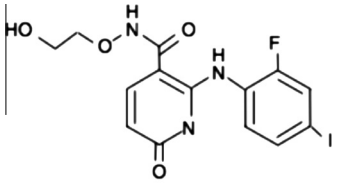
AZD-6244 a benzimidazole derivative is another second-generation potent inhibitor of MEK1/MEK2 with an IC_{50} of 14 nM. Based on promising pre-clinical data AZD-6244 was advanced into clinical development against melanoma and non-small cell lung cancer (Yeh et al., 2007).



AZD-6244; MW 457.68

AZD-6244 is well tolerated by A549 cells and when concentrations up to 100 μM were used no toxic effect was found (CC_{50} of >100 μM; Fig. 1C). The EC_{50} value was 750 nM indicating a lower antiviral potential compared to PD-0325901 (Fig. 1D). Combination of AZD-6244 with OC resulted in an increased antiviral activity even when concentrations of 1 μM AZD-6244 were used (Fig. 2B). The antiviral activity of AZD-6244 against H1N1pdm09 was not as potent as compared to other compounds. The combination with OC resulted in a strong and significant reduction of viral titer even at suboptimal concentrations of the single compounds alone. When 10 μM AZD-6244, which showed only a marginal effect against influenza virus (0.5 $\log_{10}$ pfu/ml virus titer reduction compared to untreated control), was combined with 0.1 μM OC, which is usually used at least 10-fold more in cell culture, a significant increase ($p < 0.05$) of viral titer reduction was found (2.0 $\log_{10}$ pfu/ml; Fig. 3B). The CI index demonstrated synergism at 1:10 (Table 2) and 1:100 (data not shown) combinations of AZD-6244 with OC. Thus, from these data one might conclude that the antiviral activity of AZD-6244 alone against H1N1pdm09 is limited but the antiviral activity of OC may be augmented even when this drug is given in suboptimal concentrations.

AZD-8330, another MEK inhibitor from Astra Zeneca, is highly specific against MEK1/MEK2 with an IC_{50} of 7 nM (Wallace et al., 2009). A phase I clinical trial with this compound to investigate the safety and tolerability in patients with advanced malignancies has recently been completed. The low IC_{50} value and the fact that it was more potent against cancer cells as PD-0325901 prompted us to determine the EC_{50} value against H1N1pan09.



AZD-8330; MW 461.23

Not surprisingly the EC_{50} value on A549 cells was in the nM range (40 nM; Fig. 1F). As already demonstrated with the other MEK inhibitors, A549 cells did not show any toxic effects when treated with concentrations up to 100 μM (CC_{50} of >100 μM; Fig. 1E). AZD-8330 demonstrated strong antiviral activity against H1N1pdm09 and increased the antiviral activity of OC after combination (Fig. 2C). Combination with OC in a concentration of 1 μM resulted in maximum antiviral effect. This antiviral activity could be increased even with low amounts such as 0.1 μM and significantly increased with 1 μM AZD-8330. When 10 μM AZD-8330 was used for combination with OC, almost no progeny virus was detectable (Fig. 3C white bars). The CI value indicated a synergistic effect for the combination of AZD-8330 with OC in a 1:10 ratio (Table 2) and also in 1:1 and 1:0.1 (data not shown). Thus, AZD-8330 might be considered for further preclinical development in order to investigate its antiviral potential in more detail. Moreover, the combination with OC demonstrated a potent synergistic effect.

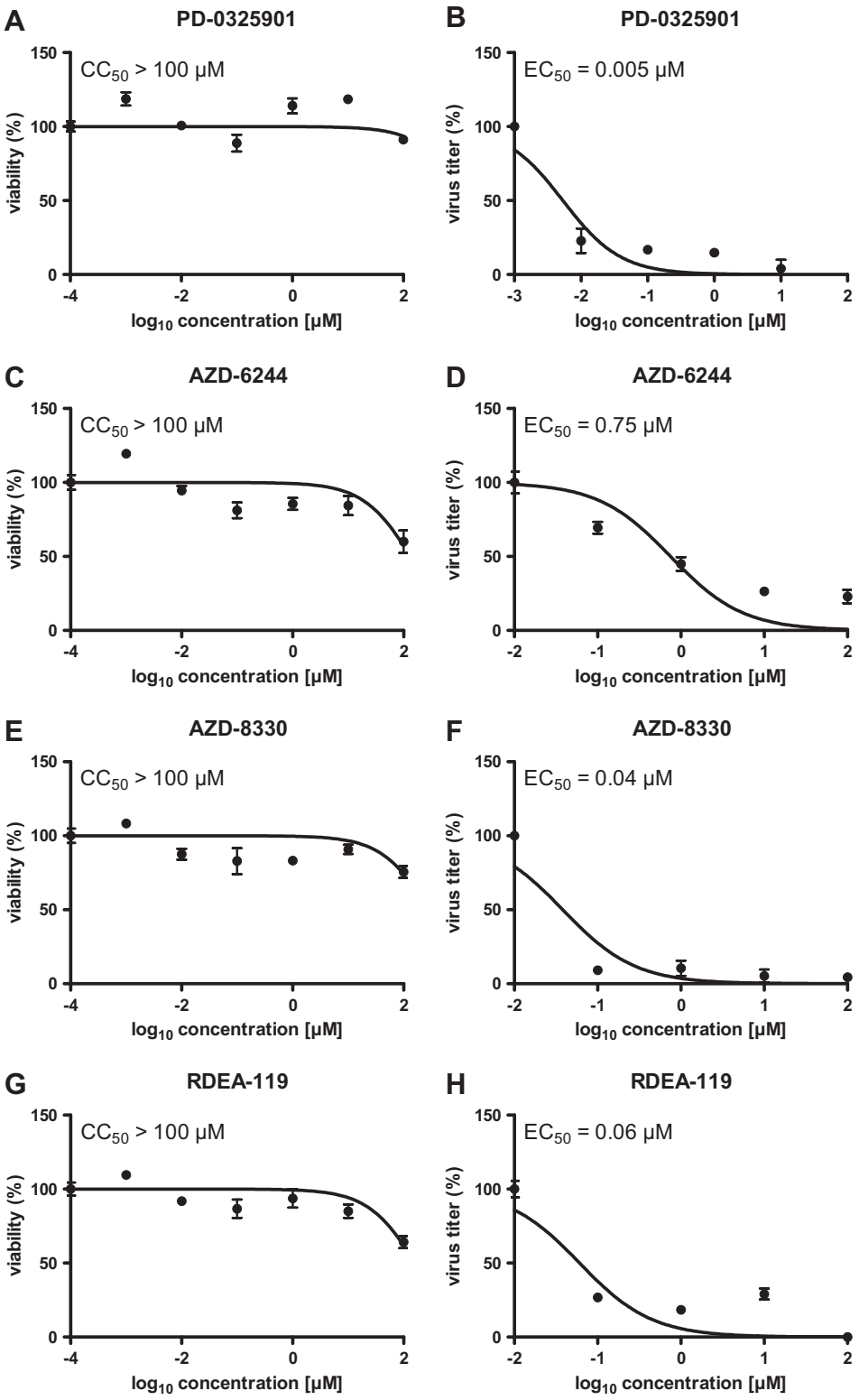


Fig. 1. CC_{50} and EC_{50} values of different MEK inhibitors *in vitro*. The cytotoxic concentration 50% (CC_{50}) of four different MEK inhibitors PD-0325901 (A) (Pfizer), AZD-6244 (C), AZD-8330 (E) (Array BioPharma/AstraZeneca) and RDEA-119 (G) (Ardea Biosciences/Bayer) was measured in human lung adenocarcinoma epithelial cells (A549). Cells were treated with different concentrations (0–100 µM) and after 24 h the cytotoxicity was measured by WST-1 assay (Roche Diagnostics). The effective concentration 50% (EC_{50}) was determined in influenza A virus (A/Regensburg/D6/09, H1N1pdm09)-infected A549 cells (moi = 0.001). After 24 h treatment with different concentrations (0–100 µM) the progeny virus in the supernatant was measured by plaque assay (PD-0325901 (B), AZD-6244 (D), AZD-8330 (F), RDEA-119 (H)) as described earlier (Haasbach et al., 2011). The CC_{50} and EC_{50} values were calculated with the GraphPad Prism 5 software.

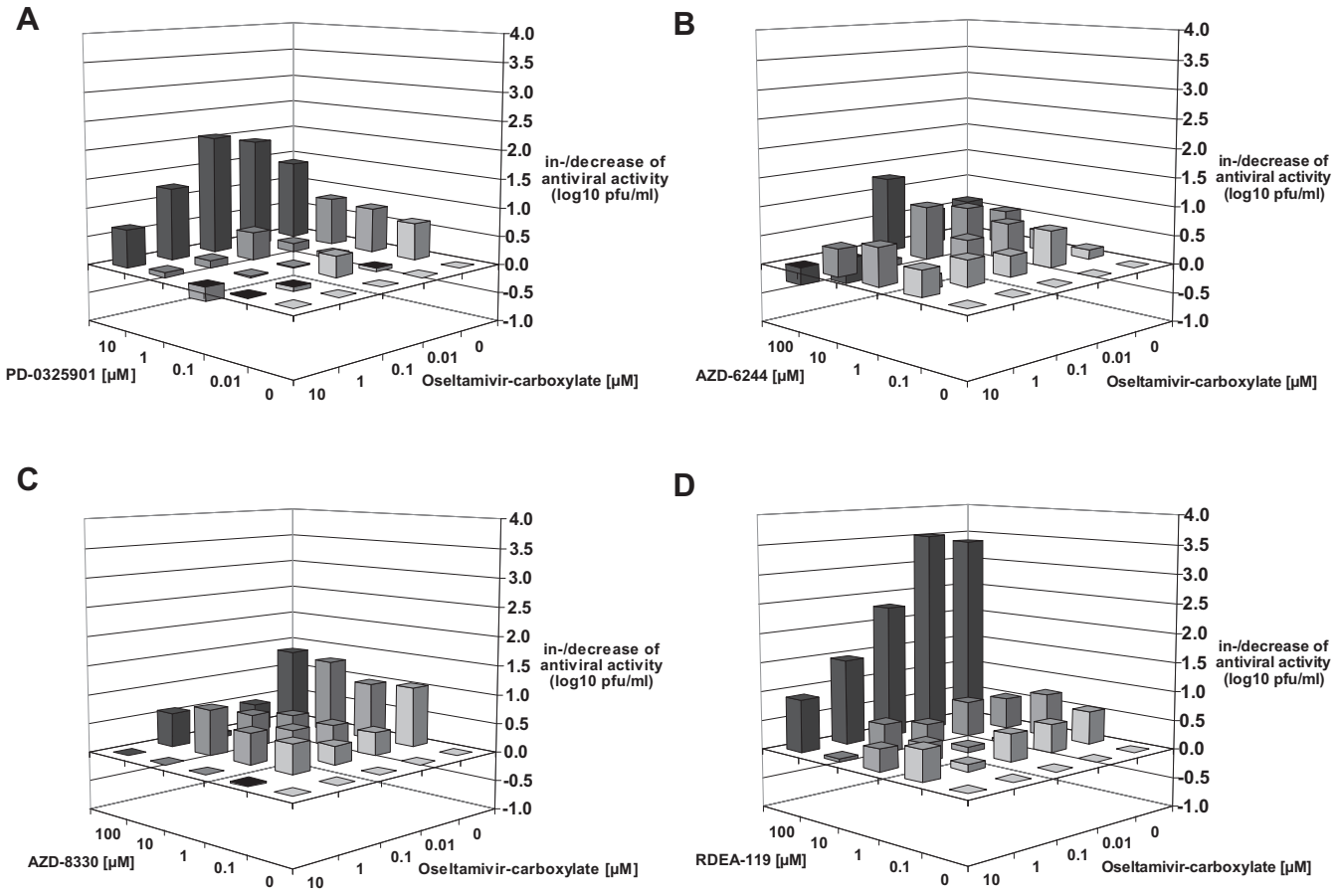


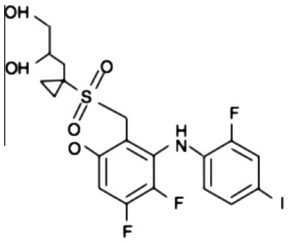
Fig. 2. Increased or reduced antiviral effect of different MEK inhibitors on the antiviral effect of OC. A549 cells were infected with A/Regensburg/D6/09, H1N1pdm09 (moi = 0.001) and treated with different concentrations of MEK inhibitors and oseltamivir-carboxylate (0–100 µM) in a chessboard pattern. After 24 h progeny virus titer was determined by plaque assay (PD-0325901 (A), AZD-6244 (B), AZD-8330 (C), RDEA-119 (D)). The plotted virus titer indicates the increased or reduced antiviral effect of oseltamivir-carboxylate combined with different concentrations of the MEK inhibitor. The values are normalized to the antiviral activity found with the respective concentrations of OC.

Table 2
CIs for two-drug combination regimens of oseltamivir-carboxlyate and various MEK inhibitors against influenza virus replication *in vitro*.

MEK inhibitor	Conc. (µM) of		CI at influenza virus inhibition of	
	Oseltamivir-carboxylyate	MEK inhibitor	90%	95%
PD-0325901	10, 1, 0.1, 0.01	100, 10, 1, 0.1	0.12	0.52
AZD-6244	10, 1, 0.1, 0.01	100, 10, 1, 0.1	0.30	0.38
AZD-8330	10, 1, 0.1, 0.01	100, 10, 1, 0.1	0.10	0.11
RDEA-119	7.5, 5, 2.5, 1, 0.5, 0.25	75, 50, 25, 10, 5, 2.5	0.47	0.47

For each agent and the combination the Chou-Talalay method for drug combination was used to calculate the median-effect equation. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. RDEA-119: best results were obtained with the presented concentrations.

RDEA-119 is another allosteric inhibitor of MEK1/2 that selectively inhibits MEK1 (IC_{50} of 19 nM) and MEK2 (IC_{50} of 47 nM) in a non-ATP competitive manner.



RDEA-119; MW 572.34

Moreover, RDEA-119 potently inhibits ERK1/2 phosphorylation (IC_{50} of 16 nM). Pharmacodynamic studies have revealed that the compound has low central nervous system penetration (Iverson et al., 2009). RDEA-119 is under evaluation as single agent in a phase I study against human malignancies, and in a phase I/II study in combination with sorafenib, a multikinase and Raf inhibitor (reviewed in Fremin and Meloche, 2010). RDEA-119 is very potent in inhibiting progeny influenza virus production with an EC_{50} of 60 nM in the assay system used (Fig. 1H). Again no toxic effects were observed with 100 µM or less indicating a CC_{50} of >100 µM (Fig. 1G). Moreover, RDEA-119 increased antiviral activity of OC in concentrations of 100, 10, 1 and 0.1 µM (Fig. 2D). When different concentrations of RDEA-119 were combined with 0.1 µM OC (a sub-optimal concentration) antiviral activity of OC was always increased but only the combination of 1 µM RDEA-119 with 0.1 µM

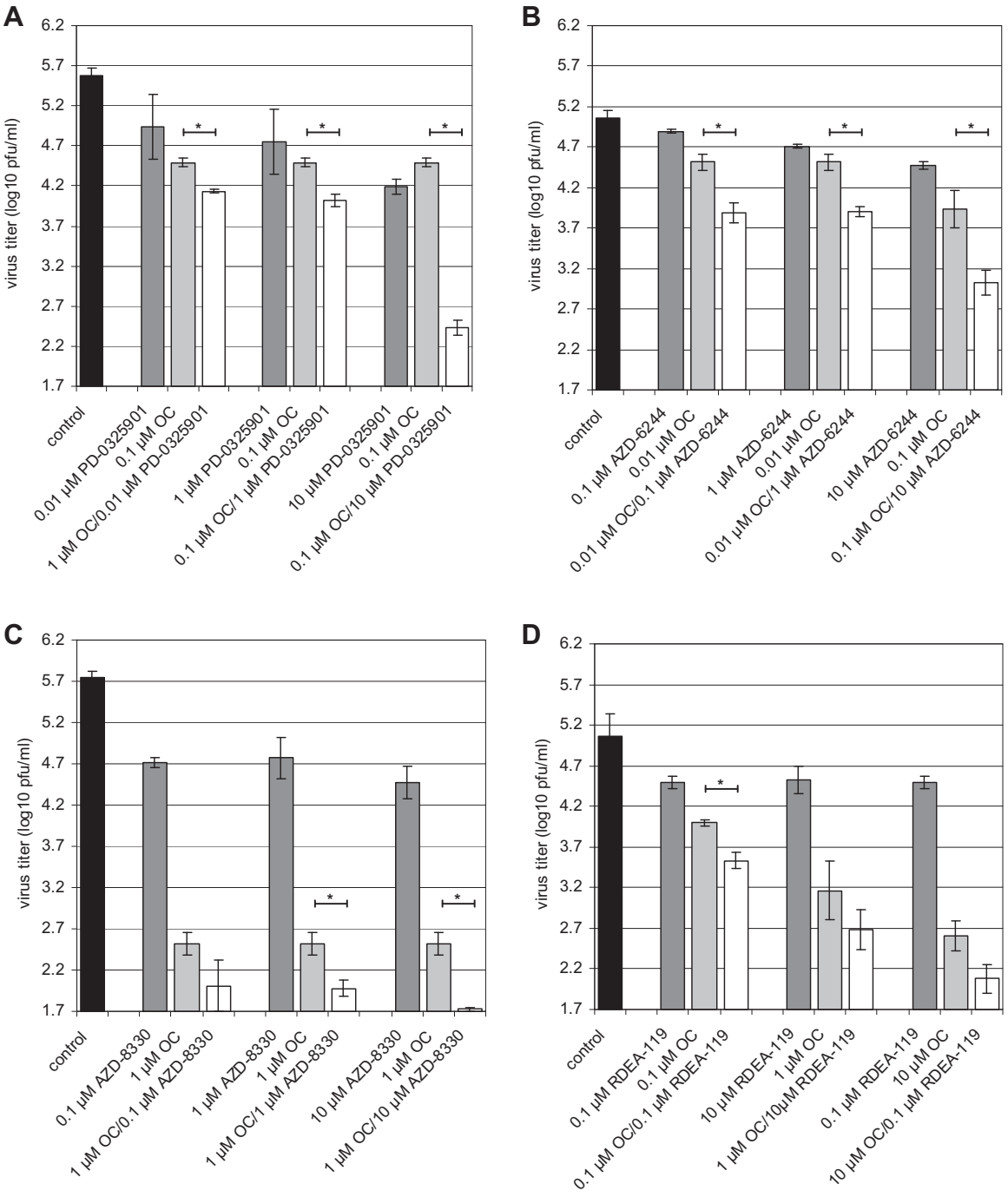


Fig. 3. Comparison of single and combination drug treatment. A/Regensburg/D6/09, H1N1pdm09 (moi = 0.001) infected A549 cells were either treated with MEK inhibitor or OC alone or in combination (PD-0325901 (A), AZD-6244 (B), AZD-8330 (C), RDEA-119 (D)). The progeny virus titer was determined 24 h post infection as described above. A *t*-test was used to determine significant virus titer reduction ($^{*}p < 0.05$).

OC lead to significant synergism compared to OC treatment alone (Fig. 3D; white bars).

In conclusion, combination of oseltamivir with orally available MEK inhibitors that already passed at least phase I clinical trial against cancer might be an interesting new strategy in the development for new drugs against influenza. Further *in vitro* investigations need to be performed in order to investigate the best compound combination before these will be tested in an *in vivo* model.

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