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LETTER TO THE EDITOR

Testing of MERS-CoV replication inhibitors for their ability to block viral entry

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As of 23 July 2014, 837 laboratory-confirmed cases of MERS-CoV infection, including 291 deaths, had been reported to WHO (http://www.who.int/csr/disease/coronavirus_infections/en/), raising concerns about its pandemic potential and calling for the development of vaccines and therapeutics against MERS-CoV infection.

We previously identified potent peptidic HIV-1 and SARS-CoV fusion inhibitors (5, 7), leading to the development of MERS-CoV spike (S) protein-mediated cell-cell fusion and six-helix bundle (6-HB) formation assays. Using these assays, we identified a peptide from the of MERS-CoV S protein HR2 region termed HR2P, which potently inhibited 6-HB formation, cell-cell fusion and MERS-CoV replication (8). To identify small molecule MERS-CoV fusion inhibitors, we used the cell-cell fusion assay to screen 1,280 compounds from an FDA-approved drug library obtained from MicroSource Discovery Systems, Inc. (Gaylordsville, CT), but none of the compounds at 10 μM could significantly inhibit MERS-CoV S-mediated membrane fusion.

Most recently, de Wilde *et al.* (2) and Dyall *et al.* (3) used the cytopathogenic effect (CPE) assay to screen several hundreds of compounds from FDA-approved drug libraries and identified a series of compounds inhibiting both MERS-CoV and SARS-CoV replication in the low micromolar range. Although their mechanisms of action have not been defined, both groups suggested that some of them, such as chlorpromazine (a clathrin-mediated endocytosis inhibitor), might block virus entry (2, 3).

Coronavirus enters into the target cell via endocytosis or plasma membrane fusion, while the latter is the main pathway for MERS-CoV entry (8). To determine whether

these reported MERS-CoV replication inhibitors also block virus entry via plasma membrane fusion, we tested 16 compounds with MERS-CoV replication-inhibiting activity available in the FDA-approved drug library from MicroSource, and ribavirin and mycophenolic acid (Sigma-Aldrich) that were reported to inhibit MERS-CoV replication (1), for their inhibitory activity on MERS-CoV S-mediated cell-cell fusion using HR2P as a control. Cell-cell fusion inhibition assay was performed as we described before (8). Briefly, Huh-7 cells were used as the target cells, and 293T cells that instantaneously express MERS-CoV S protein and EGFP (293T/MERS/EGFP), used as the effector cells. The 293T/MERS/EGFP cells were cocultured with HR2P or compounds at graded concentrations (initial concentration at 40 μM) for 30min, then added into Huh-7 cells at 37 °C for 2 to 4 hours (8). As expected, HR2P inhibited cell-cell fusion with IC_{50} (half maximal inhibitory concentration) value of ~ 1 μM and effectively blocked 6-HB formation. In contrast, most of these compounds at 40 μM exhibited no significant inhibitory activity, except the three neurotransmitter inhibitors (chlorpromazine, promethazine, and fluphenazine) showing moderate inhibitory activity with IC_{50} values of about 20, 20, and 29 μM, respectively, on cell-cell fusion (**Table 1**).

Subsequently, we determined the inhibitory ability of these compounds (40 μM) on 6-HB formed between HR1P and HR2P-FITC, using a fluorescence native polyacrylamide gel electrophoresis (FN-PAGE), adapted from the FN-PAGE assay for testing HIV fusion inhibitors (6). As expected, HR1P showed no band because it carries net positive charges, thus migrating up and off the gel under native electrophoresis condition, which is consistent with the results of HR1 peptides from HIV-1 (6) and SARS-CoV (7), while HR2P-FITC showed a band at a lower position. The mixture of

HR1P and HR2P-FITC showed a band at a higher position, suggesting the formation of an HR1P/HR2P-FITC complex, possibly the 6-HB band (**Fig. 1**). In the presence of HR2P, the upper band disappeared, while lower HR2P-FITC band displayed, suggesting that HR2P binds to HR1P and block the 6-HB formation between HR2P-FITC and HR1P. However, none of the MERS-CoV replication inhibitors at 40 μM could block the 6-HB formation by HR2P-FITC and HR1P (**Fig. 1** and **Table 1**).

The cytotoxicity of these MERS-CoV replication inhibitors to the Huh-7 cells, which were used as the target cells in cell-cell fusion assay, was determined using Cell Counting Kit-8(CCK-8, Dojindo, Kumamoto, Japan) as previously described (8). Except Emetine dihydrochloride, Triflupromazine hydrochloride, and Clomipramine hydrochloride with CC_{50} (the concentration of a compound causing 50% cytotoxicity) at 28.63, 33.58, and <5 μM, respectively, all other compounds exhibited no cytotoxicity at 40 μM (**Table 1**).

We then tested the inhibitory activity of the 16 MERS-CoV replication-inhibitors on MERS-CoV pseudovirus-based, clathrin-mediated endocytosis using an assay adapted from the method for testing SARS-CoV inhibitors as previously described (4). The pseudotyped MERS-CoV was constructed as describe before (8). Huh7 cells were incubated with chlorpromazine hydrochloride (as a positive control) and other MERS-CoV replication inhibitors at graded concentrations for 1 h and then infected with the MERS-CoV pseudovirus for an additional 12 h. After extensive washes with PBS to remove the virus and compounds, cells were further incubated for 48 h before the luciferase activities were determined as described previously (4). HR2P was included as a control. In addition to chlorpromazine, promethazine, and fluphenazine, all other neurotransmitter inhibitors also exhibited inhibitory activity against clathrin-mediated

endocytosis of MERS-CoV with IC50 values in a range of 3.23 ~ 8.79 μM. Unexpectedly, HR2P and Tamoxifen citrate, an estrogen receptor inhibitor, also displayed some inhibitory activity on clathrin-mediated endocytosis of MERS-CoV with IC_{50} valuea of 14.28 and 7.46 μM, respectively, while other MERS-CoV replication inhibitors had no significant inhibitory activity at the concentration of 40 μM (**Table 1**).

Poste *et al.* (10) reported that the three neurotransmitter inhibitors (chlorpromazine, promethazine, and fluphenazine) also inhibited herpes simplex virus-induced cell fusion without impairing virus replication, suggesting that their weak cell-cell fusion inhibitory activity may not contribute to their inhibition of MERS-CoV replication. Indeed, de Wilde et al. (2) demonstrated that chlorpromazine inhibited MERS-CoV replication at both an early and a post-entry stage, indicating that endocytosis is unlikely to be the sole antiviral mechanism. Why HR2P also exhibited inhibitory activity in the pseudovirus-based, clathrin-mediated endocytosis assay is possibly because the HR2P peptide on the cell surface may be engulfed by the plasma membrane into endosome where the peptide inhibits endosomal membrane fusion, in a similar way that HIV fusion inhibitor, enfuvirtide, inhibits HIV endocytosis (9).

In conclusion, some of the reported MERS-CoV replication inhibitors from the FDA-approved drug libraries could inhibit clathrin-mediated endocytosis, but most of them do not block MERS-CoV fusion with the target cell membrane (only three of these showed moderate inhibitory activity) and none of them inhibits 6-HB, suggesting that their mechanisms of action are different from the MERS-CoV fusion inhibitor HR2P. Therefore, the combinational use of HR2P with these reported MERS-CoV replication inhibitors may have synergistic effect against MERS-CoV infection.

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Figure legend

Figure 1. Inhibition of the HR2P peptide and compounds on the MERS-CoV S protein 6-HB formation. Inhibitory activity of the peptide and compounds on 6-HB formation between HR1P and HR2P-FITC was detected using an FN-PAGE assay. Briefly, HR1P (20 μM) was incubated with HR2P (40 μM) or each of the compound tested (40 μM) at 37 ◦C for 30 min before addition of HR2P-FITC (20 μM). Tris-glycine native sample buffer (Invitrogen, Carlsbad, CA) was then to add to the mixture at a ratio of 1:1. The samples were then loaded to a 10 cm × 1 cm precast gel (25 μl each well) and the gel electrophoresis was carried out with 125V constant voltage at room temperature for 2 h. The fluorescence bands in the gel were then imaged by the FluorChem 8800 Imaging System using a transillumination UV light source with excitation wavelength at 302 nm and a fluorescence filter with emission wavelength at 520 nm (6).

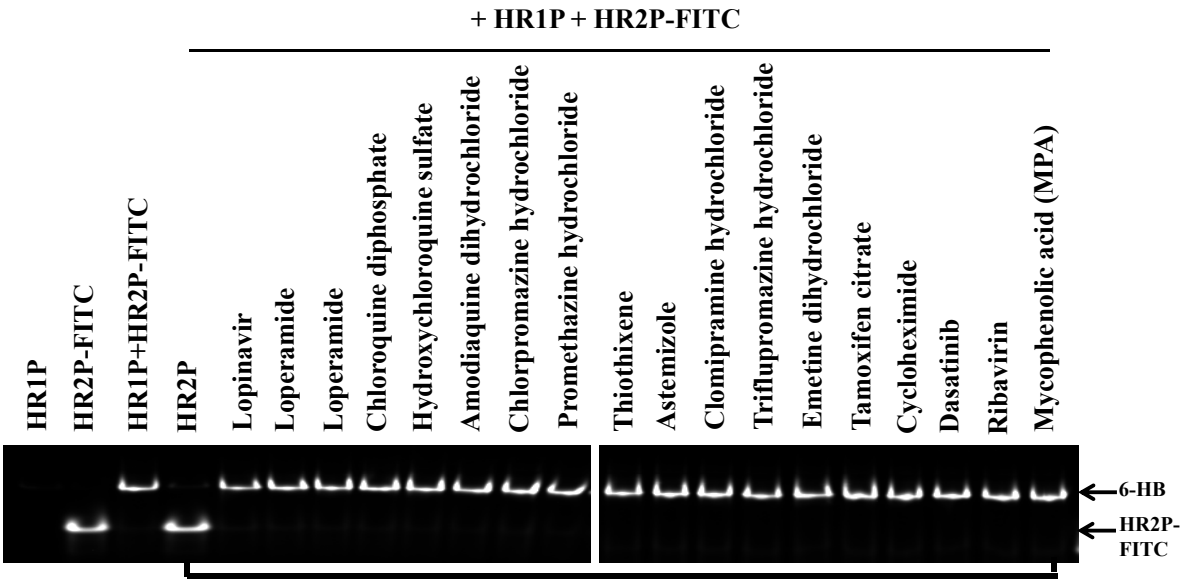
Table 1. Inhibitory activity of the reported MERS-CoV replication inhibitors available in the FDA-approved drug library from MicroSource and the control HR2P peptide on MERS-CoV S-mediated cell-cell fusion and 6-HB formation.

Drug name	Original drug function	IC_{50} (μM) for inhibiting MERS-CoV replication (ref)	IC_{50} (μM) for inhibiting cell-cell fusion*	IC_{50} (μM) for inhibiting clathrin-mediated endocytosis*	CC_{50} (μM) *†	Inhibiting 6-HB formation at 40 μM*§
Lopinavir	HIV protease inhibitor	17.10 (2)	>40	>40	>40	-
Loperamide	Opioid-receptor agonist	5.90 (2)	>40	>40	>40	-
Chloroquine diphosphate	Antiparasitic agent	4.10(2); 6.28 (3)	>40	>40	>40	-
Hydroxychloroquine sulfate	Antiparasitic agent	8.28 (3)	>40	>40	>40	-
Amodiaquine dihydrochloride	Antiparasitic agent	6.21 (3)	>40	>40	>40	-
Chlorpromazine hydrochloride	Neurotransmitter inhibitor	8.80 (2); 9.51(3)	23.33±2.89	7.24 ± 2.55	>40	-
Promethazine hydrochloride	Neurotransmitter inhibitor	11.80 (3)	16.67±7.22	7.48 ± 4.53	>40	-
Fluphenazine hydrochloride	Neurotransmitter inhibitor	5.86 (3)	15.00±4.33	3.23 ± 2.79	~40	-
Thiothixene	Neurotransmitter inhibitor	9.30 (3)	>40	5.74 ± 2.51	>40	-
Astemizole	Neurotransmitter inhibitor	4.88 (3)	>40	3.48± 1.34	28.63±1.94	-
Triflupromazine hydrochloride	Neurotransmitter inhibitor	5.76 (3)	>40	3.32 ± 1.51	33.58±2.37	-
Clomipramine hydrochloride	Neurotransmitter inhibitor	9.33 (3)	>40	8.79 ± 2.35	>40	-
Emetine dihydrochloride	Antibacterial agent	0.01(3)	>40	>5‡	<5	-
Tamoxifen citrate	Estrogen receptor inhibitor	10.12 (3)	>40	7.46 ± 2.74	>40	-
Cycloheximide	Protein-processing inhibitor	0.19 (3)	>40	>40	>40	-
Dasatinib	Kinase signaling inhibitor	5.47 (3)	>40	>40	>40	-
Ribavirin	Nucleoside analogue	9.99 (1)	>40	>40	>40	-
Mycophenolic acid (MPA)	Immunosuppressant agent	0.17 (1)	>40	>40	>40	-
HR2P	Fusion inhibitor	0.60 (8)	1.64 ± 0.75	14.28 ± 5.57	>40	+

*The samples were tested in triplicate and the experiment was repeated twice. The data are presented as mean ± SD.

If a compound at 40 μM had no more than 50% inhibition, its IC_{50} was recorded as > 40 μM. †If a compound at 40 μM had no more than 50% cytotoxicity, its CC_{50} was recorded as > 40 μM. § If a compound at 40 μM could or could not inhibit 6-HB formation, its activity was recorded as "+" or "-", respectively.

‡No inhibitory activity at its CC_{50} value (5 μM).



Inhibition of 6-HB formation by HR2P or compounds