Antiviral Compound Testing Platform – ACTP of DZIF

Currently, there is an unmet medical need for antiviral drugs that are active against highly variable RNA viruses which represent the vast majority of emerging pathogens. For most emerging virus infections, no vaccines or specific antiviral drugs are available, and symptomatic treatment in intensive care units is often the only possible medical intervention. However, since the virus variant or group that might cause an outbreak at some time in the future is difficult to predict, antiviral drugs with broad-spectrum activity are needed.

The evaluation and validation of candidate molecules as broad-spectrum antivirals is only possible by the coordinated effort of experts in different virus families, who possess the tools, expertise and infrastructure (e.g. BSL-3 and BSL-4 laboratories) to run adequate antiviral tests in a standardized fashion. For this reason, we have established the DZIF antiviral compound testing platform (ACTP), which represents the complete collection of viral systems provided by members of this project. Members of the ACTP cover a large panel of highly pathogenic viruses such as coronaviruses (SARS-CoV, MERS-CoV), filoviruses (Ebola), bunyaviruses (RVFV, CCHF), flaviviruses (ZIKV, DENV, WNV), influenzaviruses, arenaviruses (Lassa) and hanta viruses (see Table I). This unique collection contains 9 of the 10 viruses included in the WHO list of priority diseases (revised in 2018) and classified as “Priority diseases needing R&D actions”. The implementation of an Operating Protocol (see Figure 1 as example) enables us to standardize the testing of antiviral molecules, to minimize miscommunication and to allow cross-site testing for multiple drugs.

In order to expand the ACTP beyond the testing in routinely used immortalized cell lines, we are currently generating a collection of primary cell culture systems (see Table 2) and animal models (see Table 3) provided by members of the TTU Emerging Infections and their close collaborators. Engineered reporter viruses will constitute the “Fast Antiviral Screening Platform”, which is essential for rapid and simultaneous testing of large numbers of compounds as generated e.g. in hit-to-lead optimization campaigns. It is the mission of the ACTP to accelerate the discovery process of broad-spectrum antivirals suitable for the treatment of emerging infections.

Table 1. *Viruses and partner sites constituting the ACTP*

	Flaviviridae	*Picornaviridae*	*Coronaviridae*	*Orthomyxviridae*	*Paramyxoviridae*	*Filoviridae*	*Arenaviridae*	*Togaviridae*	*Bunyavirales*	HBV/HDV
Heidelberg	HCV DENV-1 DENV-2 DENV-3 DENV-4 WNV **ZIKV**	CVB3 Mengo-ZN (EMCV)		Influenza WSN	NDV-La Sota NDV-GK NDV-New Jersey				PUUV HTNV **RVFV** **UUKV** LACV	HBV/ HDV
Hamburg						**Ebola**	**Lassa**		**CCHFV** ANDV	
Lübeck		HAV EV-71 CVB3 PV	**SARS**							
Marburg			**MERS**		**Nipah**	**Ebola** **Marburg**	**Lassa**		**CCHFV**	
Bonn	YFV DENV **ZIKV**							SINV **CHIKV***		
Gießen	DENV WNV	Human rhino-virus PV	HCoV-229E **MERS**	Influenza A virus** H1N1 H2N2 H3N2 H5N1 H5N8 H7N1 H7N3 H7N7 H7N9 H9N2	HPV2 SendaiV MasernV			SFV SindbisV RubellaV **CHIKV**		HBV/ HDV
Berlin			**MERS** **SARS** HCoV-NL63							
München			HCoV-229E HCoV-NL63 **SARS** (replicon) **ZIKV**							

Bold: viruses included in the list of priority of the WHO

*: CHIKV was discussed during the WHO meeting and a number of experts stressed the risks it poses. Although not included in the priority list, there was agreement that CHIKV continues to warrant further research and development.

**: many as reverse genetic systems

Abbreviations: ANDV, Andes virus; CCHFV, Crimean-Congo haemorrhagic fever virus; CHIKV, Chikungunya virus; CVB3, Coxsackievirus B3; DENV, Dengue virus; EMCV, Encephalomyocarditisvirus; EV-71, Enterovirus 71; HAV, Hepatitis A virus; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HCoV-229E, Human coronavirus 229E; HCoV-NL63, Human coronavirus NL63; HDV, Hepatitis Delta virus; HTNV, Hantaan virus; HPV2, Human papillomavirus type 2; LACV, La Crosse encephalitis virus; MERS, Middle East respiratory syndrome coronavirus; NDV, Newcastle disease virus; PUUV, Puumala virus; PV, Polio virus; RVFV, Rift Valley fever virus; SARS, Severe acute respiratory syndrome virus; SFV, Semliki Forest virus; SINV, Sindbis virus; WNV, UUKV, Uukuniemi virus; West Nile virus; YFV, Yellow fever virus; ZIKV, ZIKA virus

Table 2. *Primary cell culture systems and partner sites constituting the ACTP*

	Flaviviridae	*Coronaviridae*	*Togaviridae*	*Bunyavirales*	HBV/HDV
Heidelberg	- Primary human hepatocytes (HCV) - Primary human monocytes (DENV) - Human Neuronal Progenitor cells **(ZIKV)**			- Podocytes - tubular epithelial cells (PUUV, HTNV)	- Primary human hepatocytes (HBV/ HDV)
Marburg		Mouse alveolar epithelial cells **(MERS)**	Guinea pig (macrophages, hepatocytes, abdominal cavity, monocytes); mouse neuronal cells (olfactory neurons, neuroglial mixed culture, olfactory ensheathing cells) **(Ebola, Marburg)**		
Bonn			- Mesenchymal stem cells - primary human skeletal muscle myoblasts (**CHIKV)*** **not available but could be established**		
Gießen		- Peripheral Blood Mononuclear Cells -primary airway epithelial cell system (HCoV-229E)			

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*: CHIKV was discussed during the WHO meeting and a number of experts stressed the risks it poses. Although not included in the priority list, there was agreement that CHIKV continues to warrant further research and development.

Abbreviations: CHIKV, Chikungunya virus; DENV, Dengue virus; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HCoV-229E, Human coronavirus 229E; HDV, Hepatitis Delta virus; HTNV, Hantaan virus; MERS, Middle East respiratory syndrome coronavirus; PUUV, Puumala virus; ZIKV, ZIKA virus

Table 3. *Animal models and partner sites constituting the ACTP*

	Flaviviridae	*Coronaviridae*	*Filoviridae*	*Arenaviridae*	*Togaviridae*	*Bunyaviridae*
Heidelberg	AG129 mice (DENV) via external collaboration					
Hamburg			Mouse with IFNAR backgound transplanted with WT mouse stem cells **(Ebola)**	Mouse with IFNAR backgound transplanted with WT mouse stem cells **(Lassa)**		Mouse with IFNAR backgound transplanted with WT mouse stem cells **(CCHFV)**
Marburg		Mice expressing hDPP4 receptor **(MERS)**	IFNAR -/- mice **(Ebola, Nipah);** Guinea pig (strain 2) **(Marburg)**			
Bonn					C57BL/6 mice via external collaboration **(CHIKV)***	
Gießen		C57BL/6 mice, mouse hepatitis virus (representative of SARS and MERS) via external collaboration				
München		Mouse hepatitis virus via external collaboration				

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*: CHIKV was discussed during the WHO meeting and a number of experts stressed the risks it poses. Although not included in the priority list, there was agreement that CHIKV continues to warrant further research and development.

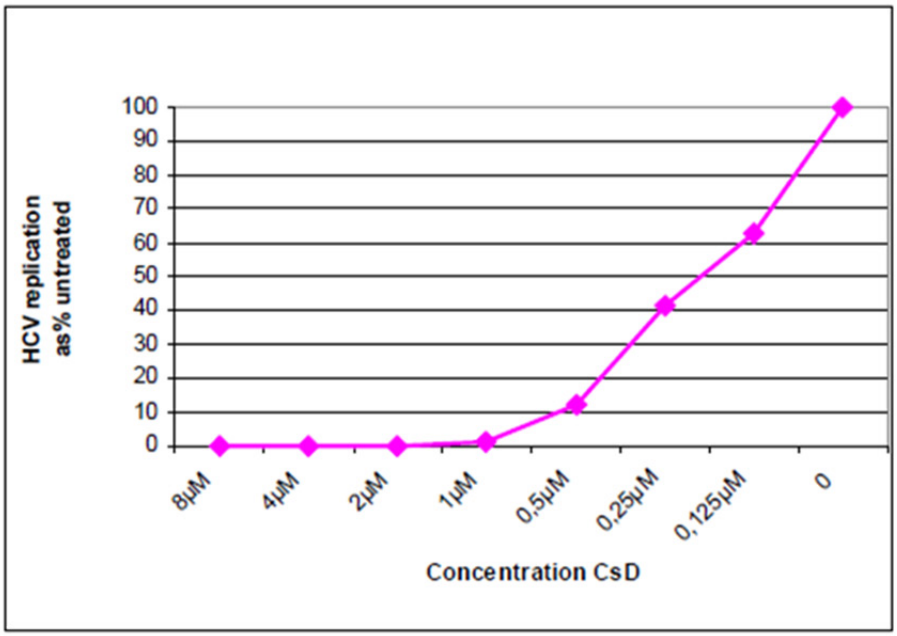
Abbreviations: CCHFV, Crimean-Congo haemorrhagic fever virus; CHIKV, Chikungunya virus; DENV, Dengue virus; hDPP4, Human Dipeptidyl Peptidase 4; IFNAR, interferon-α/β receptor; MERS, Middle East respiratory syndrome coronavirus; SARS, Severe acute respiratory syndrome virus; WT, wild type

Figure 1. *Example of Operating Procedure for antiviral drug testing within the ACTP*

Page 1 Operating Protocol Drug testing – Reference sheet	**DZIF-TTU Emerging Infection** **Broad-spectrum antivirals**	Datum / Date 8.01.2018 DZIF

- Drug Name	Cyclosporine D	
- Drug target	Cyclophilines	
- Drug stock solution	Order number:	sc-204702
	Supplier:	Santa Cruz Biotechnology, Inc.,2145 Delaware Avenue, Santa Cruz, California 95060
	Charge number:	Material Number: 63775-96-2
	Concentration stock solution:	10mM
	Solvent of stock solution:	Ethanol (100%)
	Storage of solvent	-20°C
	IC50/90 (incl. graph as attachment):	IC50 = 0,18µM IC90 = 0,71µM Calculation done with software GraphPad
- Cell line used for drug evaluation	Name of the cell line:	Lunet CD81 H EF cells
	Passage number:	p8
- Protocol used for drug test	Virus stock used (Titer):	Electoporation
	Cell line used:	Lunet CD81 H EF cells
	Passage number:	p12
	Medium:	DMEM complete
	Cell number/ plate format:	$1{,}5 \times 10^5$/ well (12 well plates)
	MOI of infection:	n.d.
	Protocol drug treatment (attachment)	attached

Attachment graph:



Attachment Protocol:

1. Cyclosporine D evaluated against HCV

Electroporation (EP):
- Lunet CD81 H EF cells (=Huh7 cells), 1x107 cells/ml
- electroporate 5µg Luc JFH1 NS2-5B (=HCV RNA) in 400µl cells; (=4x106 cells), resuspend cells in 41ml DMEM complete
- seed 1,5ml/well (12 well) in duplicate

Addition of Cyclosporine D (Cs):
- 4h post epo:
- remove DMEM, add 1,5ml DMEM + CsD serial diluted, in following concentrations: 0 (100% Ethanol; 1,2µl); 0,125; 0,25; 0,5; 1; 2; 4 and 8µM.

Analysis of HCV RNA replication capacity (Analysis):
- 48h post epo; analysis of HCV RNA replication (Luciferase assay as readout for HCV RNA replication):
- remove DMEM
- wash with 1xPBS
- add 350µl Luciferase Lysis Buffer + 1mM DTT/well (Assay to assess replication capacity)
- store at -20°C
- measure in duplicate (100µl/measurement)

2. Cell culture protocol

Cell culture media

DMEM complete: DMEM (Life Technologies #41965-039) supplemented with 10% FCS (heat-inactivated for 30 min. at 56°C), 2mM Glutamin (Life Technologies #25030-024), Penicillin (100 IU/ml) / Streptomycin (100µg/ml) (Life Technologies #15140-114) and 1x nonessentiell amino acids (Life Technologies #11140-035); 750µg/ml G418

G418 ("Geneticin", Life Technologies): Concentrations are given as weight per volume of the original substance. Specific activity of a typical batch is ca. 700 µg/mg as stated by the manufacturer. This value does not necessarily reflect the biological activity in our system. Therefore each new batch of G418 should be tested individually e.g. in an electroporation experiment using different selection conditions (0.2-1 mg/ml).

Freezing of cell cultures
- prepare cryotubes - label: name cell line, passage, amount (25 cm² or 75 cm²), date, split ratio, selection
  - prechill – 20°C
- detach good looking, confluent cells (0,05 % Trypsin/EDTA)
- resuspend in 10 ml complete DMEM
- centrifuge (700 rpm/ 5 min)
- remove supernatant

- resuspend the cell pellet in prechilled (4°C) cryosolution (volume depending on size of cryotubes, here: 1,8 ml/tube)
- mix gently (pipet) to homogenize the cell suspension
- aliquot cell suspension in cryotubes
- freeze o/n at -70°C
- store in liquid nitrogen

Note: it is very important that all material is ice-cold, that the work is done fast

Thawing of frozen cells

This protocol is used when frozen replicon cells are thawed.

- thaw the cells quickly at 37°C
- transfer cells to a 15 ml tube and suspend in 10 ml complete DMEM
- pellet cells carefully by 5 min. centrifugation (1000 rpm)
- discard supernatant
- resuspend cells in medium with the appropriate drug concentration and seed in a cell culture flask with the area given below