# Mechanisms of viral inactivation methods against hepatitis C virus

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**Running title:** Mode of action of HCV inactivation

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Conflict of interest: The authors do not have a conflict of interest.

Word count, abstract: 219

Word count, text: 3029

**List of Abbreviations**

HAV: hepatitis A virus

HCV: hepatitis C virus

HEV: hepatitis E virus

PK: proteinase K

PVP-I: povidon-iodid

qRT-PCR: quantitative real time-polymerase chain reaction

SD: standard deviation

$TCID_{50}$: tissue culture infectious dose 50

**Financial support:**

S. P. was supported by a stipend from the international research training group 1273 (IRTG 1273) provided by the DFG. E. S. was supported by the DFG (STE 1954/1-1) and intramural young investigator award of the Helmholtz Centre for Infection Research. T. P. was supported by a grant from the Helmholtz Association (SO-024).

**Abstract**

Virus inactivation by chemical disinfectants is an important instrument for infection control in medical settings, but the mechanisms involved are poorly understood. In this study, we systematically investigated the effects of several antiviral treatments on hepatitis C virus (HCV) particles as model for enveloped viruses. Studies were performed with authentic cell culture derived viruses and influence of chemical disinfectants, heat and UV treatment on HCV was analyzed by determination of infectious particles in a limiting dilution assay, quantitative RT-PCR, core ELISA and proteolytic protection assay. All different inactivation methods resulted in a loss of HCV infectivity by targeting different parts of the virus particle. Alcohols like ethanol and 2-propanol did not affect the viral RNA genome integrity, but disrupted the viral envelope membrane in a capsid protection assay. Heat and UV treatment of HCV particles resulted in direct damage of the viral genome as transfection of viral particle associated RNA into permissive cells did not initiate RNA replication. Additionally, heat incubation at 80°C disrupted the HCV envelope rendering the viral capsid susceptible to proteolytic digest. This study demonstrated the molecular processes of viral inactivation of an enveloped virus and should facilitate the development of effective disinfection strategies in infection control not only against HCV but also against other enveloped viruses.

Keywords: Hepatitis C Virus (HCV), enveloped viruses, disinfectants, capsid, inactivation

## Introduction

Virus inactivation procedures apply numerous treatment methods, for instance chemical inactivation, heat or UV irradiation. Although these methods have been widely used for a long time in industrial processes and public health systems, the understanding of the viral inactivation mechanisms remains relatively low. All viruses with the exception of iridoviruses can be assigned to either enveloped or non-enveloped viruses and are composed of a protein structure protecting the viral nucleic acid genome. Therefore, inactivation methods target either the lipid envelope membrane, the viral capsid and/or the viral genome. Hepatitis C virus is an enveloped, positive strand RNA virus belonging to the family of *Flaviviridae*. Its 9.6kb genome is composed of the 5´non-translated region (NTR), an open reading frame encoding a large polyprotein, and the 3´NTR (1). The polyprotein is cleaved into 10 individual proteins with the structural proteins building up the virus particle (Core, E1, E2) and the non-structural proteins required for RNA replication. HCV infection is considered a global health problem with an estimated 170 million people infected worldwide (2). Once a chronic infection is established there is a high risk for developing severe liver damage including hepatic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (3). In the last couple of years treatment options have been improved especially since the approval of direct-acting antivirals that could be used without interferon on an all oral combination therapy (4). However, there is still no protective vaccine available rendering health care workers at a constant risk to acquire HCV from occupational exposure. Additionally, nosocomial transmission of HCV still accounts for a large proportion of new HCV infections each year (5-9). Together with needle stick injuries or injections with contaminated syringes, especially among intravenous drug users,

which constitutes the main route of infection in developed countries (10) as well as other transmission routes involving vertical and sexual transmission (11-13), approximately three to four million people are newly infected each year (14).

Different studies have recently evaluated the environmental stability of HCV and its susceptibility to chemical biocides in quantitative suspension assays (15-19) or on dried surfaces (20, 21). However, virus inactivation mechanisms of these and other procedures and the question which parts of the virus particles are specifically disrupted have not been addressed so far. Therefore, with the help of a productive HCV cell culture system, we analyzed the effect of several inactivation methods on the HCV particle and show that different disinfectant procedures target different parts of the virus. A detailed understanding of the molecular processes involved in viral inactivation will assist the development of effective disinfection strategies against HCV.

## Materials and Methods

**Cell culture and reagents**. For HCV infection experiments a human hepatoma cell line, designated Huh7.5, was used which is permissive for HCV infection and replication (22). The cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10 % fetal calf serum (DMEM complete).

**Plasmids, *in vitro* transcription, electroporation, and production of cell culture-derived HCV.** The plasmid pFK-Jc1 has been described recently and encodes the intragenotypic 2a/2a chimeric virus Jc1 (23). Infectious HCV particles were produced as described elsewhere (24). Briefly, Jc1 plasmid DNA was linearized and transcribed into RNA, followed by the electroporation into Huh7.5 cells. Virus-containing culture fluids were harvested after 48, 72 and 96 h and concentrated using centricons (Centricon plus-70, Millipore, USA). For determination of viral infectivity cell-free supernatants were used to infect naive Huh7.5 target cells.

**Disinfectants and inactivation methods.** For viral disinfection the following disinfectants were used: 5% Triton X-100 (Roth, Karlsruhe, Germany), 100% ethanol (Roth, Karlsruhe, Germany), 2-propanol (Carl Roth GmbH, Karlsruhe, Germany), ©Betaisodona, active agent povidon-iodid, PVP-I (Mundipharma GmbH, Limburg an der Lahn, Germany). For heat inactivation Jc1 virus stock was first diluted 1:10 with

DMEM and then heated at 80°C for 5 min. For UV inactivation Jc1 virus stock was diluted 1:10 with DMEM and subsequently irradiated in a 6-well cell culture dish with an intensity of 0.6 J/cm$^2$ using a UV-crosslinker CX-2000 (UVP).

**Virucidal activity experiments, virus titration and controls.** To determine the effect of inactivation procedures on viral infectivity, virucidal suspension experiments were performed. Virus was incubated with chemical disinfectants at a ratio of 1:10, for 1 min at RT or treated as mentioned above. As control, virus was incubated with DMEM. After the incubation period, target cells were infected in a limiting dilution assay on Huh7.5 cells. The tissue culture infectious dose 50 ($TCID_{50}$) was determined 72 h post infection as described before (25). As interference control, the cell culture medium of Huh7.5 cell was therefore replaced by a non-toxic dilution of the test substance and incubated 1 hour at 37°C. As a corresponding negative control, the cell cultures are exposed to PBS in the same manner parallel to the disinfectant in the non-toxic concentration and are incubated for one hour under the same conditions. Following to the incubation the disinfectant dilution or PBS is removed from the cell cultures. Afterwards, the titers of a test virus suspension are determined on these cell cultures (26). To determine the cytotoxicity of the disinfectants, one part of PBS were mixed with nine parts of the disinfectant and inoculated into permissive cells. Cytotoxicity was determined by examining target cells by microscopy for any significant changes of the cell monolayer. The cytotoxicity was calculated in analogy to the determination of virus titer [TCID50/ml].

**Quantitative detection of HCV RNA and core protein.** To measure HCV specific RNA the viral RNA was isolated using the High-Pure Viral RNA Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. For the RT-PCR the LightCycler 480 RNA Master Hydrolysis Probes kit (Roche, Mannheim, Germany) was used with the JFH1-specific probe A-195 (TIB Molbiol, Berlin, Germany), and the primers S-147 and A-221 (MWG-Biotech) as described (Steinmann, Brohm 2008). Measurement was conducted at the LightCycler 480 (Roche, Mannheim, Germany). To quantify HCV core protein, samples were inactivated with 1 % (v/v) triton X-100 in PBS and core protein levels were measured using a core-specific ELISA (27).

**Proteolytic digestion (PK) and proteolytic protection assay.** Samples were treated with 50 µg/ml proteinase K (PK) (Roche, Mannheim, Germany) for 1 h on ice. To determine the amount of protease-resistant core protein after disinfectant treatment, 50 µl of the disinfectant/virus mixture were left untreated, 50 µl were treated with 50 µg/ml PK for 1 h on ice and another 50 µl were lysed with 2 % (v/v) triton X-100 prior to PK treatment. Protease digestion was stopped by addition of 5 mM PMSF (phenylmethylsulfonyl fluoride; AppliChem, Darmstadt, Germany), heating to 95 °C for 10 min and addition of 50 µl 2x protease inhibitor cocktail (1 pill in 5 ml TNE) (Roche, Mannheim, Germany). The amount of core protein was determined using a core-specific ELISA.

**Statistical analysis.**

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1A statistical analysis of all figures was performed using a one-tailed student′s t-test. *P* 2values were calculated, and differences are reported as significant if the *P* value were 3*< 0.05, **< 0.01 and ***< 0.001. Differences were considered not significant at *P* 4values of >0.05.

## Results

### Effect of viral inactivation procedures on HCV infectivity and RNA genome stability

In order to systematically analyze the effect of different viral inactivation methods, we used chemical disinfectants (triton, ethanol, 2-propanol, PVP-I) in a quantitative suspension assay as depicted in Figure 1 (Fig. 1) (15). In these assays, nine parts of disinfectants were mixed with one part of the HCV Jc1 virus (23) and the mixture was incubated at room temperature for 1 min. In case of heat and UV inactivation the virus was preincubated with nine parts DMEM before the respective inactivation. Following the chemical treatment or preincubation, viral infectivity was determined in a limiting dilution assay (Fig 1A) and viral particle associated RNA was determined by qRT-PCR (Fig. 1B). To investigate RNA genome stability, the virus associated RNA was purified and subsequently re-transfected into human liver cells highly permissive for HCV RNA replication. Successful RNA replication was measured by qRT-PCR (Fig. 1C) and release of infectious particles by inoculation of Huh7.5 cells with cell culture supernatants (Fig. 1D).

All the chemical disinfectants (triton, ethanol and 2-propanol) significantly reduced viral infectious titers at least two to three orders of magnitude to the level of detectable cytotoxicity induced by the disinfectants (Fig. 2A). To verify that the susceptibility of the target cells for the virus infection was not influenced negatively by the treatment with the disinfectant, an interference control experiment was performed. We observed no difference in susceptibility of the target cells due to the disinfectant treatment (data not shown). For heat and UV treatment no residual infectivity could be determined. Next, we purified the viral RNA from the differently treated samples and determined

the amount of HCV RNA copies by qRT-PCR (Fig. 2B). No difference between the non-treated control and the inactivation methods was observed. To determine whether the loss of infectivity was due solely to the inability of the virus particle to penetrate into cells via the normal route of entry, or whether the viral genome itself was no longer infectious, we transfected the virus particle associated RNA into highly permissive Huh7.5 cells. Successful initiation of viral RNA replication was assessed by qRT-PCR (Fig. 2C). Compared to the control treated virus sample no significant reduction was observed for the alcohol treated specimen, a significant reduction for the triton and PVP-I treated samples and no RNA replication was detected after treatment of the virus with UV radiation or heat. To further analyze whether infectious particles were released from the cells which still enable viral replication, the supernatants were harvested and used to inoculate naïve Huh7.5 cells in a limiting dilution assay. Productive infection of target cells similar to the control could be detected in the ethanol treated sample, while treatment with triton, 2-propanol and PVP-I resulted in a significant reduced virus production and heat or UV treatment completely abrogated virus production (Fig. 2D). In summary, these results indicate that some inactivation procedures exert a strong influence on viral RNA stability and integrity whereas others apparently inactivate HCV by targeting different parts of the virus particle.

**Effect of viral inactivation procedures on viral capsid and envelopment**

Besides the viral RNA, the viral capsid and envelope constitute possible targets for particle disruption by inactivation treatments. We analyzed whether the viral capsid was impaired due to the antiviral procedures. To this end, we measured the amount of core protein via core-specific ELISA after preincubation of virus with the respective

chemical or treatment. As seen in Figure 3A, the different inactivation methods had no effect on the total amount of viral capsid protein itself (Fig. 3A). To dissect the effect on the viral envelope, we performed a proteolytic protection assay to determine the amount of protease-resistant, enveloped core protein after treatment. In case of an intact envelope, externally added proteinase K (PK) is not able to cleave the viral capsid, because the protease has no access to the membrane enveloped core protein. In contrast, treatment-induced disruptions of the viral envelope permits access of the protease to the viral capsid and thus results in a digestion of core protein, which can be quantified via core specific ELISA (19). To control that the concentration of PK used was sufficient to cleave core protein we added a high dose of the detergent triton as positive control, which resolved all membranes. Only the UV treated virus showed still a protection against PK to comparable levels as the control treated virus indicating that the viral envelope was still mainly intact (Fig. 3B). The two different alcohols ethanol and propanol as well as heat treatment disrupted parts of the viral envelope, resulting in a PK protection of approximately 70 %, 40 % and 30 %, respectively. On the other hand, heat as well as triton completely destroyed viral envelopment whereas the PVP-I treated samples were not detectable in this assay setup (Fig. 3B). Taken together, UV light inactivation had no influence on the virus particle membrane while chemical disinfectants and heat treatment destroyed the viral envelope rendering HCV non-infectious.

## Discussion

The usage of viral inactivation methods is an essential part of infection control practices and plays an important role in the prevention of nosocomial infections. However, the exact antiviral mechanisms of these inactivation treatments are largely not well characterized (28). For measures allowing the interruption of infectious virus and sterilizing strategies knowledge about the specific mode of action should improve the application of inactivation procedure and disinfection strategies. In this study, we could show that different inactivation methods against HCV comprising treatment with triton, the alcohols ethanol and 2-propanol, PVP-I and heat, as well as UV irradiation resulted in a loss of infectivity for the HCV particle. Further analyses revealed that each disinfectant method targeted different parts of the viral particle (Fig 4). Heat and UV treatment resulted in an irreparable damage of the RNA and therefore to a loss of viral RNA replication. Heat treatment at 80°C, but not UV irradiation, further disrupted the viral envelope rendering the viral capsid susceptible to proteolytic digestion. Even though we did not see an influence of either treatment method on the viral capsid, we cannot exclude that the viral capsid itself might also be damaged since the core ELISA is based on the detection of only a small part of the capsid (27). It has been shown for other viruses that heat inactivation induces structural changes in viral proteins, which might cause the loss of infectivity (29, 30) and degrades the viral RNA (31, 32). Whether heat inactivation influences only the viral proteins or also the RNA might depend on the applied temperature as well as duration of heat administration. The same holds true for ultraviolet irradiation. Ultraviolet irradiation, typically at a wavelength of

254 nm (UVC) is known to target nucleic acids while leaving proteins largely preserved (29, 33). However, both viral genome as well as protein damage has been reported previously due to UV irradiation (30, 34, 35). Viral inactivation by alcohols is thought to be due to membrane damage and rapid protein denaturation (36) and indeed, HCV RNA integrity was not compromised after treatment of the virus with either ethanol or 2-propanol. However, the viral envelope was damaged and resulted in a reduced protection of the capsid from externally added proteinase K supporting the assumption that alcohols target the viral envelope. Both the actions of triton as well as PVP-I are thought to occur by targeting of the viral envelope. Triton is a non-ionic surfactant commonly used as a detergent in laboratories, which solubilizes proteins of the cell membrane (37) whereas PVP-I is a complex of iodine and a solubilising carrier, which acts as a reservoir of “free” active iodine (38). With both inactivation methods we observed a mild reduction in the ability of the RNA to replicate after transfection of viral associated genomes indicating that both treatments have an influence on the viral RNA. As expected, triton treatment resulted in complete destruction of the viral envelope rendering core susceptible to proteinase K digestion. However, the effect of PVP-I could not be completely solved as the disinfectant targeted the core protein even in the absence of proteinase K in the untreated control within this assay setup. It could be observed that longer incubation of HCV with PVP-I resulted in decreased amounts of core protein (data not shown) suggesting that this disinfectant has a direct effect on the viral capsid and therefore simultaneously on the viral envelope. The antimicrobial mechanism of PVP-I has been described as a direct delivery to the bacterial cell membrane, where it rapidly penetrates into the microorganism and targets key groups of proteins, nucleotides and fatty acids in the

cytoplasm and cytoplasmic membrane (38). The antiviral action against viruses has not been extensively studied but it is likely that iodine attacks the surface proteins of enveloped viruses, but it could also destabilize membrane fatty acids by reacting with unsaturated carbon bonds (39). Furthermore, lipid-enveloped viruses are in general more sensitive to chemical inactivation methods than non-lipid enveloped viruses (36) which would support our assumption that the viral envelope constitutes a target for PVP-I. Interestingly, recent evidence suggests that some non-enveloped viruses like hepatitis A virus (HAV) and hepatitis E virus (HEV) circulate in the blood of infected patient or animals enveloped in host-derived membranes but are shed as non-enveloped viruses. The two types of particle, enveloped and non-enveloped, appear to be equally infectious but are probably differently stable in the environment (40, 41).

In conclusion, different viral inactivation methods target specific parts of HCV particles as an example of an enveloped virus. While heat and UV treatment mainly damaged the viral genome stability, alcohol disinfectants caused a disruption of the virus particle membrane. Understanding virus inactivation on a basic mechanistic level will aid to predicting the susceptibility of non-culturable virus strains and should improve methods for combating viral transmission and inactivation.

1**Acknowledgments.**

2We are grateful to Takaji Wakita and Jens Bukh for JFH1 and HCV isolates,
3respectively and to Charles Rice for Huh7.5 cells and the E9E10 monoclonal antibody.
4Moreover, would like to thank all members of the Institute of Experimental Virology,
5Twincore, for helpful suggestions and discussions.

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**Figure legends**

**Figure 1: Experimental set up for studying the mode of action of HCV inactivation procedures.**

(A) Chemical disinfectants and virus were mixed at a ratio of ten to one and the mixture was incubated at room temperature for 1 min before infectivity was determined by tissue culture dose 50 assay ($TCID_{50}$). In case of heat treatment or UV irradiation the virus were mixed at a ratio of ten to one with DMEM and heated at 980°C for 5 min or UV irradiated before determination of $TCID_{50}$. (B) Virus particle associated RNA was extracted and measured by qRT-PCR. Purified RNA was used to transfect naïve Huh7.5 cells by electroporation. (C) After 72 h, Huh7.5 cells were lysed and HCV RNA was analyzed by qRT-PCR. (D) The supernatant of the cells was harvested and used to infect naïve Huh7.5 cells to determine viral titers.

**Figure 2: Influence of treatment procedures on HCV infectivity and RNA integrity**

(A) Chemical disinfectants, heat and UV treatment were tested in a quantitative suspension assay for their efficiency in inactivating HCV by determination of $TCID_{50}$. (B) HCV RNA of the respective supernatant was isolated and quantified by reverse-transcription polymerase chain reaction. (C) The isolated RNA was used for re-electroporation of Huh7.5 cells. After 72 h, RNA was extracted and quantified by reverse-transcription polymerase chain reaction. (D) Limiting dilution assay was used to determine the $TCID_{50}$ of the viral supernatants. Depicted is the mean + SD of three

independent experiments. Background level of the assay is shown in a dotted line. Statistical analysis was performed using a one-tailed student's t-test.

**Figure 3: Influence of inactivation methods on viral capsid and envelope**

(A) Chemical disinfectants and virus were mixed at a ratio of ten to one and the mixture was incubated at room temperature for 1 min before the amount of HCV core protein was determined via core-specific ELISA. In case of heat treatment or UV irradiation the virus were mixed at a ratio of ten to one with DMEM and heated at 980°C for 5 min or UV irradiated before core-specific ELISA. (B) Proteolytic digestion protection assay to determine protease resistant core protein. Therefore, one part was left untreated, one part was treated with 50 µg/ml proteinase K (PK) for 1 h at 4 °C, and another part was lysed in 2 % triton X-100 prior to PK treatment. The amount of protease-resistant core protein was quantified with a core-specific ELISA. Depicted is the mean + SD of at least three independent experiments. N.d. not detected. Statistical analysis was performed using a one-tailed student's t-test.

**Figure 4: Mode of action of inactivation treatments on the HCV particle**

Schematic depiction of the HCV particle, showing the glycoproteins E1 and E2 (blue), the viral envelope (yellow) and the capsid formed by the core protein (light blue), which protects the viral RNA (black). Each inactivation procedure affects the particle in a unique way, by either influencing the viral RNA and/or destroying the viral envelope (solid yellow line: intact envelope, thick dashed yellow line: envelope damaged, thin dashed line: envelope strongly damaged).