



# Double-stranded RNA Activated Caspase Oligomerizer (DRACO): Designing, Subcloning, and Antiviral Investigation

Mojtaba Sharti<sup>1</sup>, Hadi Esmaili Gouvarchin Ghaleh<sup>1</sup>, Bahman Jalali Kondori<sup>2,3</sup>, Ruhollah Dorostkar<sup>1\*</sup>

<sup>1</sup>Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran

<sup>3</sup>Marine Medicine Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

**Corresponding Author:** Ruhollah Dorostkar, Ph.D. in Virology, Assistant Professor, Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98- 9125467559, Email: r.dorost@yahoo.com

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## Abstract

**Introduction:** Antiviral therapy is an alternative for viral infection control when the virus is identified. As antiviral therapy has effectively used basic science to create very efficient treatments for severe viral infections, it is one of the most promising virology aspects. In the present study, a novel broad-spectrum antiviral method, dubbed double-stranded RNA (dsRNA) activated caspase oligomerizer (DRACO) have been developed, which induces apoptosis in cells with viral dsRNA selectively to rapidly kill infected cells with no damage to uninfected ones.

**Materials and Methods:** Following the design, development, expression, and purification of DRACO, the influenza virus-infected MDCK and uninfected MDCK cells were treated with 40, 60, and 80 mg/L concentration of DRACO to study its potential antiviral activity. Then, TCID<sub>50</sub> (50% tissue culture infectious dose) of the virus, together with the viability of cells, was measured.

**Results:** The findings of the present study showed that DRACO is nontoxic to uninfected MDCK cells and is effective for H1N1 influenza virus-infected MDCK cells dose-dependently. Also, the infected MDCK cells treated with DRACO have shown a significant reduction in TCID<sub>50</sub> compared with the control group.

**Conclusions:** The outcomes suggest that DRACO has a potential as a new anti-H1N1 therapeutic drug that its in-vivo antiviral efficacy requires to be examined through a clinical analysis of large quantities of animal models.

**Keywords:** Double-stranded RNA, Caspases, MDCK Cells, Cytotoxicity

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## Introduction

It is essential to improve human life, which is always confronted with the consequences of viral infections.<sup>1</sup> The viruses, which lead to the creation of diversified human diseases, have been developed along with human evolution.<sup>2</sup> More than 219 virus species have been identified to be able to infect humans. Many viruses can alter host metabolism and innate/immune responses.<sup>3</sup> Considering the human population, a vast number of viral pathogens are possibly used for treatments consistent with human vaccines and immunizations activities.<sup>4</sup> It is, therefore, necessary to produce a wide range of antiviral drugs to overcome human viruses.<sup>5</sup> Since available antiviral drugs and vaccination strategies are still inadequate, new antiviral tactics are required to control viruses, and novel nontoxic antiviral drugs must be developed to affect different types of virus in-vivo and in-vitro being potentially appropriate for either therapeutic or prophylactic administration.<sup>6-8</sup>

Recently, the influenza virus, as an important emerging pathogen, threatens human and animal populations. Influenza viruses are an important part of Orthomyxoviridae family, including three genera of influenza virus A, B, and C. Influenza viruses have primarily been introduced with high

virulence properties involving the risk of potential epidemic progression.<sup>9-11</sup> The influenza virus genome comprises eight negative-sense RNA molecules containing ten major proteins, such as hemagglutinin (HA) and neuraminidase (NA) glycoproteins as fundamental viral core antigens.<sup>12</sup> Influenza has been recognized as a key factor of human morbidity and mortality for a long time either caused by global pandemics or routine seasonal spread.<sup>13-16</sup> This virus, with a high mutation rate of the RNA genome besides a variety of its multiple genomic fragments, develops diversified antigens and novel subtypes, enabling the virus to attenuate the performance of vaccines and causing antiviral drug resistance.<sup>17-19</sup> Thus, a novel anti-influenza therapy would be required through creative strategies and abnormal targets.

Double-stranded RNA (dsRNA) activated caspase oligomerizer (DRACO) is intended for selective killing of virus-infected cells without damage to uninfected ones at the same time. The synthetic construction of DRACO consists of the dsRNA detection and apoptosis domains.<sup>20,21</sup> Generally, DRACO is known to be non-toxic, affecting a broad spectrum of viruses, including influenza virus, bunyaviruses, and flaviviruses.<sup>22</sup> Most virus-infected cells

have long dsRNA helices produced by the virus during genome replication and transcription. In contrast, there is no long dsRNA for uninfected cells. In this regard, DRACO can bind to viral dsRNA to activate the apoptosis pathway to kill virally infected cells.<sup>23,24</sup> The DRACO is united to a recognized protein transduction tag using the corresponding protein transduction domain 4 (PTD-4) at the N-terminus for cell delivery purposes. Meanwhile, all domains indicated the Homo sapiens sequence.<sup>25</sup>

In this study, a novel wide-range antiviral method, DRACO has been developed, which has toxic effects on cells with viral dsRNA selectively to kill quickly infected cells with no damage to uninfected ones.

## Materials and Methods

### Cell Culture

Influenza virus (A/Puerto Rico/8/34 (H1N1; PR8) and MDCK (Madin-Darby canine kidney) cell line were prepared from the Applied Virology Research Center, Baqiyatallah University of Medical Science of Iran. The culture medium of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) was applied in cultured MDCK cells, supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA) in a dampened incubator with 5% CO<sub>2</sub> at 37°C.<sup>26</sup>

### Construction, Expression, and Purification of DRACO

The protein kinase R (PKR) was used as DRACO's dsRNA detection domain with two dsRNA binding motifs in the N-terminal domain. The apoptosis induction domain was the caspase recruitment area of apoptotic protease activating factor 1 (Apaf-1) that binds to procaspase 9. DRACO was encoded by the DNA cassette to be cloned into the pET-32a vector for the expression. The induction of DRACO expression was conducted by adding IPTG to the Luria broth (LB) medium at a final concentration of 10 mM for 4 h. Afterward, the *E. coli* cells were centrifuged (9400 g, 4°C, 20 minutes) and suspended in PBS buffer by sonication. The purification of His-tagged target proteins was conducted under the protocol provided by the manufacturer using Ni-NTA agarose (Invitrogen, CA, USA). By using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA), the concentration of purified DRACO was found relative to the standard BSA. Imidazole was also removed through ultrafiltration by the use of a Macrosep® Advance Centrifugal Device (Pall, New York, USA).<sup>27</sup>

### TCID50 Assay

The virus titration was performed applying a standard TCID50 (50% tissue culture infectious doses) method. In brief, after culturing the MDCK cells in 24-well plates for 24 hours, the 200 µL of virus dilutions in DMEM with 0.5 µg/mL TPCK-trypsin was added to each well and incubated for two days. The cell monolayer was rinsed with PBS after removing the uninvolved viruses. Then cells incubated with various concentrations of DRACO (40, 60, and 80 mg/L) for 36 hours and the virus yield titration was conducted by the collected supernatants. Virus titers were identified as the TCID50.<sup>27</sup>

### Cell Viability Assay

The H1N1 influenza virus-infected MDCK and uninfected MDCK cells' viability was determined after DRACO treatment applying 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT; Sigma, USA) assay. The confluent cell monolayer was incubated with 100 µL/well and various concentrations of DRACO (40, 60, and 80 mg/L) or PBS (in triplicates) for further 2 days in 96-well microtiter plates. The supernatants were separated from the wells after the incubation period, and 50 µL of an MTT solution (1 mg/mL in PBS) was added to every well. The plates were again incubated for 4 hours at 37°C, and MTT crystals were dissolved by adding 100 µL of DMSO (Samchun, Korea). Using the ELISA reader (StataFax 2100, USA), the absorbance was read at 570 nm. The following formula was used for calculating the percentage of toxicity:

$$\text{Toxicity (\%)} = [(ODT/ODC) \times 100]$$

Where ODC and ODT stand for the control substance and the test's optical density, respectively.<sup>28</sup>

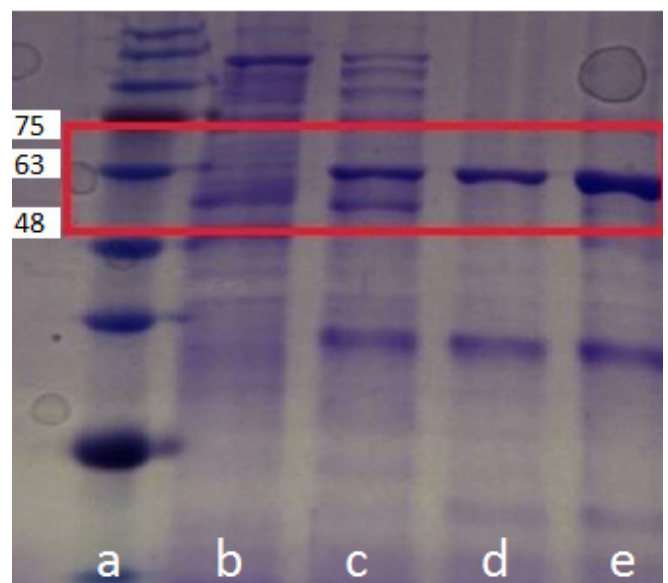
### Statistical Analysis

Experiments were carried out with three or more separate replicates. The SPSS 23.0 software was used to analyze data. Student one-way ANOVA and t-test were applied. Differences with *P* values below 0.05 were deemed to be significant.

## Results

### Construction, Expression, and Purification of DRACO

According to Figure 1, DRACO was produced with different transduction tags, apoptosis induction domains, and dsRNA detection domains. Then, the SDS-PAGE was used for confirming the DRACO expression and purification (Figure 1).



**Figure 1.** DRACO Protein Expression. (a): Protein ladder (b): negative control. (c to e): The 60 KDa band of the DRACO protein expression in different protein concentration.

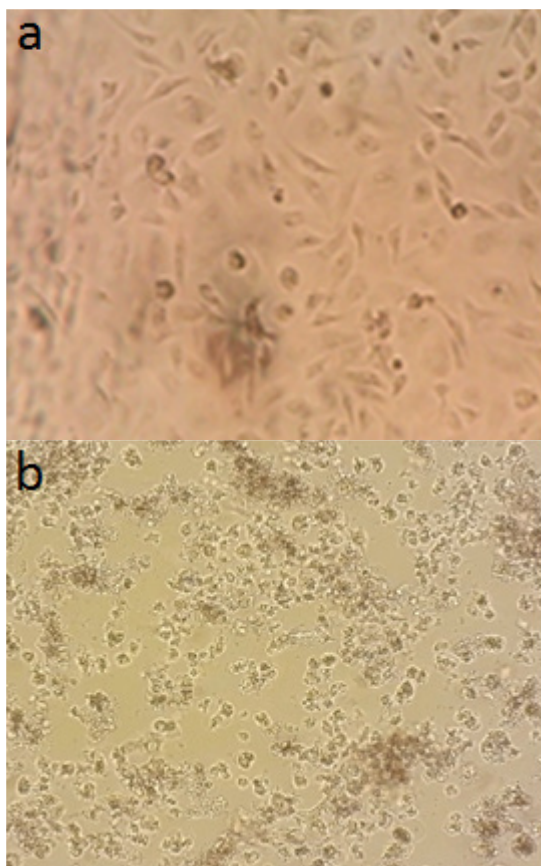
## Archive of SID Cell Viability and Cytopathic Effect Results

In Figure 2, the cytopathic effect (CPE) of the H1N1 influenza virus in MDCK cells have been presented. The cytotoxicity assay was necessary to determine the DRACO antiviral activity in the early stage of antiviral drug development. In the case of (infected and non-infected) MDCK cells, the cytotoxicity was investigated using the MTT assay. As it can be seen in Figure 3A, cells incubated in mediums with 40, 60, or 80 mg/L DRACO after treatment for 48 hours represent relative viability of around 100% compared to PBS control. The effect of DRACO on the H1N1 influenza virus infection was then evaluated through TCID<sub>50</sub> assays. The H1N1 influenza virus titer was dramatically dropped by DRACO relative to the PBS control (Figure 3B).

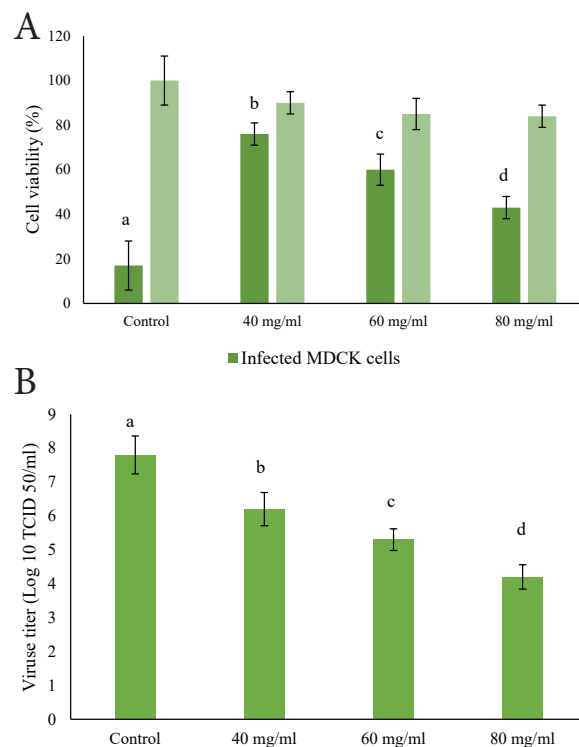
### Discussion

The influenza virus infection is still the main health issue with limited choices for treatment and control. Historically, the native and recombinant products, as well as their derivatives, have been regarded as valuable therapeutic agents.<sup>30</sup> Recent technological advancements have provided research with the evolution of antiviral, especially anti-influenza, drugs from native and recombinant products. The DRACO is one of the antiviral native and recombinant products.<sup>31</sup>

The synthetic construction of a DRACO consists of three sections, including an apoptosis induction domain, a dsRNA



**Figure 2.** Cytopathic Effect (CPE) of H1N1 Influenza Virus in MDCK Cells. (a): phase-contrast image of untreated MDCK cells. (b): H1N1 influenza virus treated cells. The values were normalized to those of control group.



**Figure 3.** (A) The cytotoxicity of DRACO in MDCK cells (infected and non-infected cells) was measured using the MTT assay. Cells were incubated with different concentrations of DRACO or PBS for 48 h, and then cell viability assay was performed. (B) Virus titers in virus infected cells (MOI=0.1) were determined after treatment with DRACO or PBS for 36 h. Different superscript letters on column of figure show statistically significant differences between the groups ( $P < 0.05$ ).

detection domain, as well as a transduction tag.<sup>32</sup> The DRACO can induce apoptosis selectively in cells involving viral dsRNA for killing virus-infected cells rapidly with no damage to uninfected ones. The DRACO is known to be non-toxic, affecting a wide range of viruses, such as bunyaviruses and flaviviruses.<sup>33</sup> Most virus-infected cells have long dsRNA helices produced by the single- or double-stranded RNA viruses during genome replication and transcription.<sup>34</sup> In contrast, there is no long dsRNA for uninfected cells. In this regard, DRACO can bind to viral dsRNA in order to activate the apoptosis pathway to kill virus-infected cells through the cleavage of a variety of cellular proteins.<sup>35</sup>

The DRACO inhibitory impact is also associated with two natural cellular procedures: one in dsRNA detection in the interferon pathway and the other in apoptosis induction in the apoptosis pathway.

In this study, DRACOs were generated with various dsRNA detection domains, apoptosis induction domains, as well as transduction tags (Figure 1). Construction, expression, and purification procedure of our study results revealed that synthesized DRACO comprised three sections of an apoptosis induction domain, a dsRNA detection domain, and a transduction tag. For this purpose, the MTT cytotoxicity assay method was used. This study evaluated the anti-influenza virus activity of DRACO *in vitro* on the MDCK cell line, as represented by the effectiveness against the H1N1

influenza virus. Regarding the cell viability tests, DRACO had a dose-dependent antiproliferative effect on infected MDCK cells compared with uninfected MDCK ones. Rider et al, revealed that DRACO rapidly entered into cells and induction the apoptosis in cells transfected with dsRNA.<sup>23,36</sup> In an uninfected cell, dsRNA was absent, cell viability was not reduced, and the apoptosis percentage was not significantly increased. While DRACO made no change in the survival of uninfected cells population, it reduced viral titers and CPE in virus-challenged cells. Thus, the reduced virus-infected cells directly led to a reduction in viral titers and CPE. Guo et al indicated that DRACO was capable of reducing viral titers, cell viability, IFA, and CPE in virally infected cells contributing to inhibit PRRSV infection. They also stated that DRACO would be a promising anti-PRRSV therapeutic drug.<sup>24</sup> Accordingly, DRACO has a significant dose-dependent antiviral impact on infected MDCK cells but not on uninfected MDCK ones.

### Conclusions

Overall, it can be concluded that DRACO has exhibited strong antiviral activity against the H1N1 Influenza virus-infected MDCK cells. The DRACO also has the potential to develop therapeutic and prophylactic strategies for the H1N1 Influenza virus infection. Nevertheless, the DRACO antiviral efficacy in-vivo must be examined through a clinical analysis of plenty of animals.

### Authors' Contributions

The authors contributed equally to this study.

### Conflicts of Interest Disclosures

No conflict of interest is declared by the authors.

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