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Short Communication

The "Janus-like" RNA-editing machinery in innate antiviral immunity

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ABSTRACT

Our innate immune systems are evolved to provide the first line of immune defense against microbial infections. A key effector component is the adenosine deaminase acting on the RNA-1 (ADAR-1)/ interferon (IFN) pathway of the innate cytoplasmic immunity that mounts rapid responses to many viral pathogens. As an RNA-editing enzyme, ADAR-1 targets viral RNA intermediates in the cytoplasmic compartment to interfere with the infection. However, ADAR-1 may also edit characteristic RNA structures of certain host genes, notably, the 5-hydroxytryptamine (serotonin) receptor 2C (5-HT_{2C}R). Dysfunction of 5-HT_{2C}R has been linked to the pathology of several human mental conditions, such as Schizophrenia, anxiety, bipolar disorder, major depression, and the mental illnesses of substance use disorders (SUD). Thus, the ADAR-1-mediated RNA editing may be either beneficial or harmful; these effects need to be tightly modulated to sustain innate antiviral immunity while restricting undesired off-target self-reactivity. In this communication, we discuss ideas and tools to identify the orphan drug candidates, including small molecules and biologics that may serve as effective modulators of the ADAR-1/IFN innate immunity and are thereby promising for use in treating or preventing SUD- and/or viral infection-associated mental illnesses.

KEYWORDS: 5-HT_{2C}R, ADAR-1, COVID-19, interferon, measles viruses, RNA editing, substance use disorders, SARS-CoV-2, Sudemycin D6, U87.

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ABBREVIATION

the 5-hydroxytryptamine (serotonin) receptor 2C, 5-HT_{2C}R; the 5' copy-back defective interfering RNAs, DI-RNAs; adenosine, A; adenosine deaminase acting on RNA-1, ADAR-1; double-stranded RNA, dsRNA; green fluorescent protein, GFP; guanosine, G; human immunodeficiency virus-1, HIV-1; inosine, I; interferon, IFN; interferon regulatory factor, IRF; measles viruses, MeV; Multiplicity of infection, MOI; nonstructural protein, NSP; nuclear export signal, NES; nuclear localization signal, NLS; open reading frame, ORF; red fluorescent protein, RFP; the Rev Response Element, RRE; splicing factor 3B subunit 1, SF3B1; Sudemycin D6, SD6; substance use disorders, SUD; TANK binding kinase 1, TBK1; the severe acute respiratory syndrome coronavirus-2, SARS-CoV-2.

INTRODUCTION

Many individuals with substance use disorders (SUD) develop mental illness, and about half of all patients with a mental illness also experience a SUD condition [1-3]. Although such high rates of comorbid SUD and mental illness have been recognized for years, the underlying mechanisms remain poorly understood. Recent studies on anti-viral immunity begin to uncover evidence about a pathophysiological link between dysfunction of the adenosine deaminase acting on the RNA-1 (ADAR-1) intracellular immunity and SUD-associated mental illness [4-7].

ADAR-1 plays multiple roles in a pathway of the innate cytoplasmic immunity that mounts rapid responses to viral infections [4, 8]. The ADAR gene family includes three members, ADAR-1, ADAR-2, and ADAR-3 [4, 8]. The ADAR-1 and ADAR-2 encode for enzymes with adenosine deaminase activity. ADAR-3 is expressed primarily in the brain but has not been shown to possess any RNA-editing activity. ADAR-1 is expressed to higher levels than the other ADARs. The protein encoded by this gene exists in two forms generated from alternative exon 1 structures that initiate from different promoters [9]. A smaller isoform (p110) is expressed constitutively while a larger isoform (p150) is upregulated in response to IFN. The p110 isoform is a truncated version of p150, which lacks one Z-DNA binding domain at the N-terminus that contains a nuclear export signal (NES) [9, 10]. Therefore, p110 is almost exclusively found in the nucleus while p150 is largely expressed in the cytoplasm [11]. Only the p150 isoform of ADAR-1 acts as an IFN-responsive anti-viral effector in the cytoplasmic compartment.

ADAR-1 p150 recognizes the double-stranded RNA (dsRNA) intermediates of invading viruses that replicate in the cytoplasm. As an RNA-editing enzyme, ADAR-1 catalyzes the conversion of adenosine (A) to inosine (I) in double-stranded RNA by hydrolytic deamination of purine C-6 (Figure 1) [12-14]. The A-to-I editing leads to nucleotide substitution editing, because "I" is decoded as guanosine (G) instead of as A by ribosomes during translation and by polymerases during RNA-dependent RNA replication. The A-to-I editing can alter RNA structure stability as I:U mismatches are less stable than A:U base pairs.

By altering the structure of the viral RNA genome and the encoded viral proteins, the A-to-I(G) editing may directly interfere with viral infection. For example, ADAR-1 may inhibit the human immunodeficiency virus-1 (HIV-1) replication by introducing A-to-I(G) mutations in the region encoding the Rev Response Element (RRE) binding domain of HIV-1 RNA. Since the binding of Rev to RRE is essential for the transport of primary transcripts from the nucleus to the cytoplasm, these mutations effectively block the transport of primary transcripts like *gag*, *pol*, and *env* from the nucleus to the cytoplasm [15, 16]. Additionally, genetic knockdown of ADAR-1 in human liver cells markedly enhances the replicon of the hepatitis C virus [17]. Recent studies revealed evidence that the ADAR-1-induced deamination of RNA also occurred in the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) responsible for the current outbreak of COVID-19 pneumonia [18, 19]. Such innate immune pressure may contribute to the generation and selection of mutant viruses and accelerate SARS-CoV-2 evolution in humans during the epidemic [19, 20].

However, ADAR-1 also recognizes and edits certain characteristic mRNA structures of cellular origins, notably, the 5-hydroxytryptamine (serotonin) receptor 2C (5-HT_{2C}R) mRNA [5]. The 5-HT_{2C}R is one of a family of receptors that coordinates the intracellular responses to serotonin in the mammalian nervous system [21-23]. This protein couples the

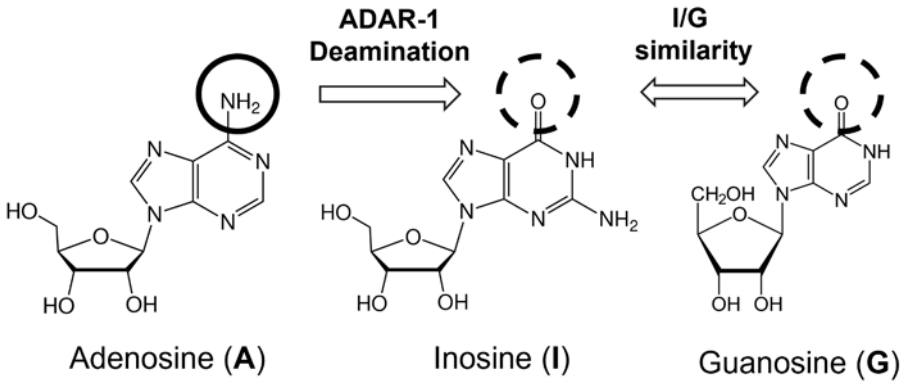

Figure 1. ADAR-1-mediated A to I(G) RNA editing.

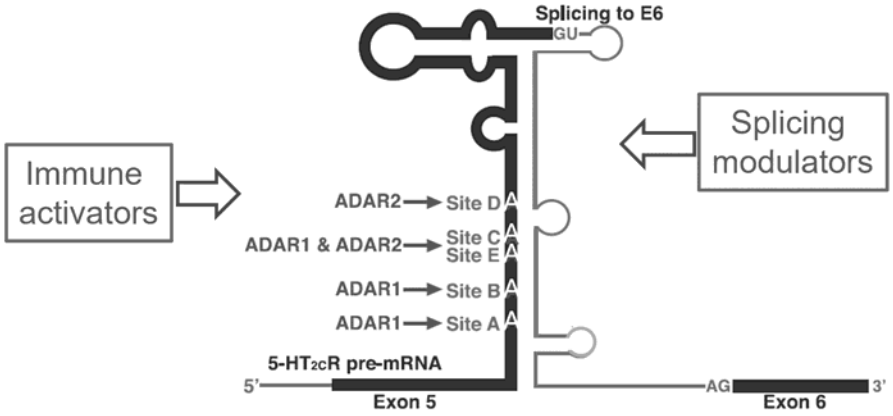

Figure 2. Schematic of ADAR-editing sites in the 5-HT_{2C}R pre-mRNA and potential modulating agents. Exons and introns of 5-HT_{2C}R pre-mRNA are shown by thick and thin lines, respectively. Five A residues subjected to A→I/(G) editing and the GU dinucleotides' splicing donor and AG splicing acceptor sites are indicated. Possible factors affecting the efficacy of 5-HT_{2C}R pre-mRNA editing are outlined here and discussed in the text. This illustration is a modified adaptation from Ref. [5].

binding of ligand to the surface receptor domain and mobilizes intracellular calcium *via* G-protein coupling and activation of protein kinase pathways. A cluster of ADAR-editing sites has been identified in the 5-HT_{2C}R (Figure 2) [5]. In particular, the ADAR-1 p150-mediated RNA editing may alter the 5-HT_{2C}R serotonergic plasticity involved in several pharmacological and behavioral processes and has the potential to influence the development of cocaine use disorder in humans. Increased expression of ADAR-1 and increased editing of 5-HT_{2C}R mRNAs have been associated with addictive substance abuse behavior in several model organisms [6, 24-26]. In fact, IFN-α treatment for chronic viral hepatitis or certain malignancies may cause severe depression in the treated patients [27-29]. Thus, the ADAR-1/IFN-arm of innate immunity may have the "Janus-like" effects to be either beneficial or harmful; these effects need to be tightly modulated to sustain innate antiviral immunity while restricting undesired off-target self-reactivity.

Mimicking concurrent viral infection and substance abuse in a cell culture model

ADAR-1 appears to be a key target for modulating the critical self-non-self-activities of the ADAR-1/IFN-pathway of cytoplasmic immunity. We sought to identify an *in vitro* model to monitor the complex interplay between viral infection, addictive substance abuse, and the ADAR-1 functionality, and then apply the model to screen for a new class of drug candidates that could effectively modulate ADAR-1 expression and its RNA-editing activities. Ideally, the model is built on a stable cell line that expresses functional 5-HT_{2C}R in response to addictive substances and is permissive to infection by a human virus. For this purpose, we tested a human glioblastoma cell line, U87 (U87MesG/ HTB14, ATCC, Rockville, MD, USA) by challenging it with a vaccine strain of measles viruses in the presence or absence of cocaine.

Specifically, we seeded cells at low confluency (5000 cells per well in a 96-well plate) to avoid cell-cell overlaying. In this way, we were able to follow the cell growth morphological changes in real-time, notably the dendritic-like cell differentiation and the neural network-like cell-cell connections, by scanning the plate using a cell imaging system without any staining. As shown in Figure 3, we treated the U87 cells in several groups: a) cells infected with an MeV vaccine strain, either vac2$(GFP)_H$ or vac2-$C^{KO}$$(mCherryNLS)_H$ [30]; b) cells co-challenged with a vaccine and a cocaine dose; c) cells treated with cocaine alone; and d) un-infected and drug- free controls.

Using the EVOS FL Auto Cell Imaging System (Life Technologies, Carlsbad, CA), we monitored

cell growth and differentiation by scanning the culture on the bright field channel. (Figures 3A and B) and examined the establishment of infection by scanning the cells on corresponding fluorescent channels, i.e., green fluorescent protein (GFP) for the vac2$(GFP)_H$-infected cultures (Figures 3C and D) and red fluorescent protein (RFP) for the vac2-$C^{KO}(mCherryNLS)_H$ cultures (Figures 3E and F).

The cell morphology shown in Figure 3A [vac2$(GFP)_H$ alone)] and Figure 3B [vac2$(GFP)_H$ plus cocaine (4mM)] indicates the co-treatment suppressed the typical dendritic-like cell-differentiation of U87 as early as 44 h after treatment. The reduced cell-differentiation is, however, associated with increased number of un-differentiated cells (Figure 3B). By contrast, the vac2$(GFP)_H$ alone group (Figure 3A) and control group (data not shown) maintained the cell differentiation phenotype. Notably, the intensity of the vac2-reporter expression in the co-treatment groups (Figure 3D-GFP; Figure 3F-RFP) was significantly lower than that detected in the vac-2 alone cultures (Figure 3C-GFP; Figure 3E-RFP).

Apparently, the U87 cells responded to the co-stimulation of MeV infection and cocaine with reduced dendritic-like differentiation, increased cell proliferation, and suppressed expression of the MeV-H-linked reporter signal. To our knowledge, such striking cellular responses, accompanying with suppression of viral gene expression, to concurrent stimulation of viral infection and a cocaine dose, were previously unrecognized.

Discussion

We examined the feasibility of using a relatively simple biological model, i.e., U87 cell line, to monitor the biological effects of concurrent viral infection and substance abuse. We demonstrate that U87 is permissive to infection by the two vaccine strains of MeV, vac2$(GFP)_H$ and vac2-$C^{KO}(mCherryNLS)_H$. Notably, co-treatment of U87 with MeV-vac2 and cocaine induced a unique pattern of cellular response, which is characterized by suppression of the dendritic-like cell differentiation and enhancement of cell proliferation. These observations highlight the potential pathogenic additive effects of substance abuse and viral infection and raise concern in a clinical situation when a SUD patient is challenged by a virus, which may render the subject at high risk of severe mental illness.

The COVID-19 pandemic poses new challenges to our healthcare system. As of July 27, 2022, there have been 570,005,017 confirmed cases of COVID-19, including 6,384,128 deaths, reported to WHO (https://covid19.who.int/). One of the post-COVID-19 pandemic effects is post-COVID depression, which affects up to 40% of people who have had SARS-CoV-2 infection [31-33]. An important question to be addressed is whether dysregulation of the ADAR-1/IFN-pathway of innate immunity is linked to the long-COVID mental illnesses.

SARS-CoV-2 infection may interrupt the IFN immunity *via* multiple mechanisms [34-37]. For example, nonstructural protein 6 (NSP6) of the virus binds to TANK binding kinase 1(TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation; NSP13 binds to TBK1 and blocks its phosphorylation activity; and open reading frame 6 (ORF6) binds to the importing karyopherin α 2 to inhibit IRF3 nuclear translocation [35]. The IRF3 nuclear translocation is further inhibited by ORF3b and NSP3, the truncated ORF3b of SARS-CoV-2, suppresses IFN induction more efficiently than that of SARS-CoV, which may contribute to the poor IFN response reported in COVID-19 patients [34, 37]. Nsp16 of SARS-CoV-2 can modify the 5′ cap with its 2'-O-methyl-transferase activities, allowing the virus to efficiently evade recognition by the innate immune system [36]. Moreover, the human cell-produced spike glycoprotein-positive exosomes may transport and release inhibitory microRNA of IRF9 to suppress the IFN innate immunity [38, 39].

New ideas and innovative strategies are needed to explore a new class of therapeutic agents that can effectively modulate the ADAR1/IFN-arm of innate immunity. Conceptually, the pre-existing cytoplasmic ADAR-1 p150 may play an important modulating role [4]. For example, this first-responder ADAR-1 activity may "neutralize" the viral dsRNA species by editing the dsRNA duplexes of invading viruses in the cytoplasmic compartment that would prevent further activation of a cascade of the cytoplasmic antiviral signaling

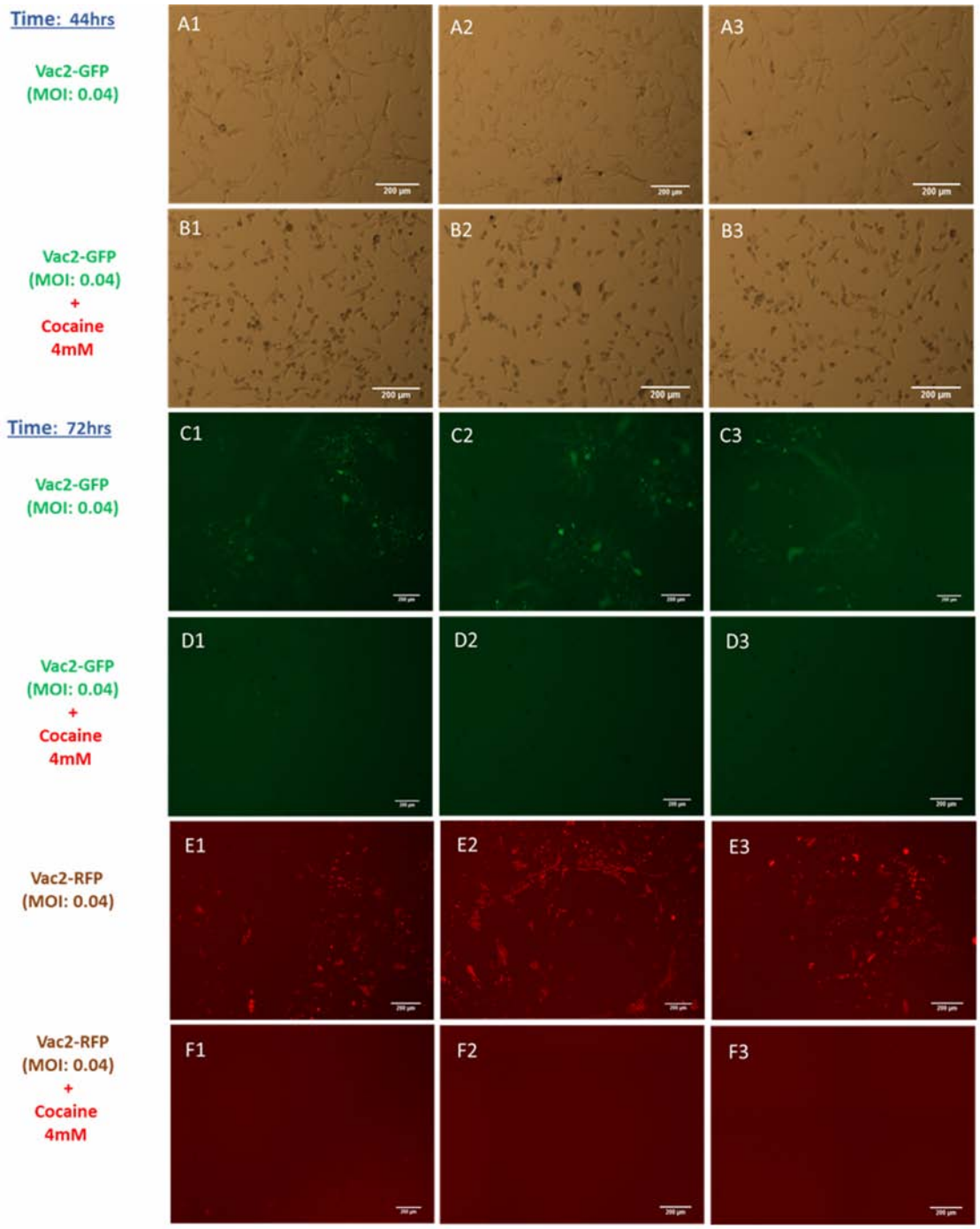

Figure 3. Co-treatment of U87 with MeV-vac2 viruses and cocaine induced striking alteration in cell-growth behavior. ***A and B:*** Cells were infected with MeV-vac2(GFP)$_H$ [Multiplicity of infection (MOI) 0.04)] in the absence (***A***) or presence (***B***) of cocaine (4 mM)(C1528, Sigma-Aldrich) and were scanned in the bright field phase 44 h-post infection; ***C and D:*** the same cell cultures as in ***A and B*** were scanned in the GFP channel 72 h-post infection; ***E and F:*** Cells were infected with MeV-vac2-C^{KO}(mCherryNLS)$_H$ in the absence (***E***) or presence (***F***) of cocaine (4 mM) and were scanned in the RFP channel 72 h-post infection. All cells were plated on a flat 96-well plate in triplicate per treatment group as specified and scanned in real-time using the EVOS FL Auto Cell Imaging System (Life Technologies, Carlsbad, CA). The scale bars are 200 μm in all panels.

pathways to down regulate IFN-I induction. By contrast, in the absence of viral dsRNA and cytoplasmic ADAR-1 p150, the cell-derived dsRNA-like structures (e.g., 5-HT_{2C}R mRNA) may be targeted by these antiviral machineries to cause pathogenic self-RNA editing. Mutations in ADAR-1 also confer autoimmunity in humans and in other animal models [40-45]; the Adar-$1^{-/-}$ mice are in fact associated with lethal autoimmunity [46, 47]. Thus, ADAR-1 is likely a druggable target for modulating the critical self-non-self-activities of the ADAR-1/IFN-pathway of innate immunity.

Potential ADAR-1-modulating agents may include small molecules and immune activating biologics (e.g., some established vaccine vectors) (Figure 2). RNA-splicing is required for producing functional ADAR-1 and 5-HT_{2C}R. Thus, RNA splicing modulators [48-53] are potential drug candidates. A promising example is, perhaps, the anti-cancer orphan drug Sudemycin D6 (SD6) that selectively induces cytotoxicity in tumor cells with minimal cytotoxic effect in normal cells [49, 54-57]. The mechanisms of action of SD6 have been extensively studied. SD6 has high-affinity to splicing factor 3B subunit 1 (SF3B1), a key component of the U2 snRNP complex required for mRNA splicing [58-60]. SF3B1 is also important in the chromatin modifications required for releasing nascent transcripts to the cytoplasmic compartment where mRNA translation takes place [59, 61-63]. By targeting SF3B1, SD6 may suppress ADAR-1 mRNA splicing to increase the expression and cytoplasmic production of the ADAR-1 p150 isoform to strengthen the innate cytoplasmic immunity. Alternatively, a splicing enhancing factor may reduce the expression of the p150 isoform to negatively modulate the ADAR-1/IFN-pathway.

Live-attenuated MeV has been administered as a vaccine to at least one billion children with outstanding safety and efficacy [64, 65]. The MeVs have been also explored as anti-cancer agents based on their oncolytic activity. Recent phase I and II vaccine clinical trials of recombinant MeV have confirmed safety and yielded promising efficacy indications as a class of novel oncolytic agents [66, 67]. Vaccine strains of MeV may also be explored as an effective modulator of the innate immunity. Interestingly, the vac2-C^{KO} strain shows a 10-fold increase in the abundance of the 5' copy-back defective interfering RNAs (DI-RNAs), although it is deficient in the host-response-modulating C protein of MeV (C^{KO}) [30]. Of note, the DI-RNAs carry characteristic sequences for ADAR-1 editing and are natural ligands of ADAR1 p150. Thus, vac2-C^{KO} may have potential to be used to redirect the ADAR-1-arm of the innate intracellular immunity to the "foreign" dsRNA and suppress the undesired "self" RNA-editing. Thus, vac2-C^{KO} and other MeV vaccine vectors are readily applicable for clinical investigation as potential immune activators of the innate immunity.

CONCLUSION

In summary, these potential ADAR-1 modulating agents warrant a focused investigation using multiple approaches. The U87-MeV model described here may be further developed to establish high throughput functional assays for screening and classifying the ADAR-1-targeting agents. Conceptually, both positive and negative modulators are valuable since they may be timely chosen based on a specific clinical situation to regulate the activities of the ADAR-1/IFN innate immunity to treat or prevent the SUD- and viral infection-associated mental illness.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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