Review Article

ANTI-NS1 PROTEIN OF INFLUENZA VIRUS: A POTENT ANTI-VIRAL STRATEGY

Samuel Pushparaj

The Lundberg-Kienlen Lung Biology and Toxicology Laboratory
Department of Physiological Sciences, Oklahoma State University
Stillwater - 74075, Oklahoma, USA

ABSTRACT

Influenza viruses are potent pathologic agents that cause lethal respiratory disease worldwide. The nonstructural protein (NS1) protein of the influenza virus plays a vital role in the virus replication by the evasion of host innate immune system. NS1 protein was found to be conserved functionally in both Influenza A and B viruses. Recent studies revealed that the NS 1 protein of influenza A and B viruses binds to TRIM 25 ubiquitin ligase that is needed for the post-translation modification of the RIG-I and thereby prevents the interferon signaling cascade. Protacs are the bifunctional molecules that bind to the target protein and an ubiquitin ligase, enabling ubiquitination and destruction of the target protein through the Ubiquitin-Proteasome system. In this minireview, based on earlier studies we propose novel molecules designed to target NS1 protein in order to elicit an interferon response and NEDD4 ligase to prevent the degradation of interferon (IFN)-induced transmembrane protein 3 (IFITM3).

Keywords: Antiviral, Influenza virus, Innate immunity, IFITM3, Interferon, NEDD4 ligase, NS1 Protein, Protac, RIG-1

INTRODUCTION

Influenza viruses are globally important and cause recognized pathogenic respiratory diseases of humans. It belongs to the family Orthomyxoviridae, which contains all enveloped viruses with segmented, negative sense RNA genome (Palese and Shaw, 2007). Influenza type A, B, C and D are the members of this family of which A, B are the common subtypes that infect humans often (Klenk *et al.,* 2004; Palese and Shaw, 2007; Wright and Webster, 2007). It causes acute, highly contagious respiratory illness that commonly affects the upper respiratory tract. Sometimes it causes primary viral pneumonia by affecting the lower respiratory tract (Wright and Webster, 2007). Severe infections are noticed in very young or elderly age group or in individuals suffering with chronic diseases. The unpredictability of the influenza virus is due to the phenomenon of antigenic shift and drift which causes reassortment and mutations in the viral surface proteins (Subbarao *et al.,* 2006). This paves the way for the zoonotic capability of the Influenza virus due to the evolution of antigenically novel viral strains that can efficiently replicate in humans.

*Corresponding author, email: samuel.pushparaj@okstate.edu

Generally, people affected by influenza virus recover from infection within a week or two without much medical treatment, but in people with high risks it can lead to pneumonia and ultimately death (Wright and Webster, 2007). Even though many vaccination programs were being followed, seasonal influenza infections results in 291,000 to 646,000 deaths every year worldwide (WHO, 2021). Influenza virus can also affect animals such as pigs, horses, and birds and thus it is of zoonotic importance. Hence, antiviral strategies against influenza virus must be include a one health approach for effective mitigation of the disease spread in human and animal population.

Influenza A virus

Influenza A viruses are pleomorphic organisms of 80-160nm diameter with a lipid envelope and has two surface glycoproteins namely, hemagglutinin and neuraminidase. About 16 hemagglutinins (HA) and 11 neuraminidases (NA) have been identified and their reassortment produces different subtypes of viruses. M2 is the viral integral membrane ion channel protein which is embedded within the lipid envelope bilayer. M1 is the viral matrix protein that lies beneath the envelope and acts as a supporting particle. The viral genome consists of eight single-stranded negative sense RNA segments (Table 1). Each viral strand is enclosed within the nucleocapsid protein and is associated with three proteins, Polymerase subunit basic PB1, PB2 and polymerase subunit acidic PA (the viral polymerase complex) that are responsible for the transcription and replication. The viral nucleoprotein complex is formed by the combination of nucleocapsid protein-encapsidated RNA and its heteromeric polymerase (Hale *et al.,* 2010).The only non-structural protein of the Influenza A virus which is produced in abundance in the early infection is the NS1 protein (Neumann and Kawaoka, 2011). The NS1 protein along with the viral nuclear export protein is encoded by the viral segment 8 and consists of 230-237 amino acids. The highly multifunctional nature of NS1 protein is mainly contributed by the presence of RNA binding domain (RBD) and the C-terminal effector domain (ED). The RBD binds to RNA in a non-specific manner and also needed for interactions with specific cellular proteins while ED interacts with various cellular proteins (Hale *et al.,* 2008; Krug and Aramini, 2009; Das *et al.,* 2010; García-Sastre, 2011). In this review, we have shown how the NS1 protein helps the virus in evading the host innate immunity and also the potential of NS1 protein as an antiviral target.

Antiviral innate immunity

Type I interferon response plays the most important role in limiting the influenza virus replication and enables the survival of the host (García-Sastre, 1998; Kugel, 2009; Szretter, 2009; Van Hoeven, 2009). During the process of virus replication, several types of pathogen-associated molecular patterns (PAMPs) are synthesized and the cytoplasmic viral RNA species that contain a triphosphate at the 5'end (PPP-5') (Pichlmair, 2006) is important as they helpthe host to detect the virus through pattern recognition receptors (PRRs) and initiate the antiviral response (Randall and Goodbourn, 2008). The PRRs are classified into several families which include Retinoic acid- inducible gene-I (RIG-I)-like helicases, Toll like receptors (TLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (Mogensen, 2009).

Table 1: Functions of Influenza A viral proteins

Segment	Viral protein	Function
1	PB2	Component of RNA transcriptase complex; Host cell capped mRNA recognition and binding
2	PB1	Component of RNA transcriptase complex; RNA-dependent RNA polymerase activity; capped mRNA endonuclease activity
3	PA	Component of RNA transcriptase complex; Required for replication; possible role in transcription
4	HA	Surface trimer glycoprotein; Cleaved intoHA1and HA2; Major antigenic determinant; Functions in virus binding to cell surface receptors and fusion
5	NP	Associated with RNA segments to form ribonucleoprotein
6	NA	Surface tetramer glycoprotein; neuraminidase activity; functions in virus release
7	M1	Major virion component; involved in RNP transport out of nucleus
	M2	Coded from spliced mRNA; ion channel activity; target of amantadine
8	NS1	Nonstructural protein; interferon antagonist; inhibits mRNA transport from nucleus

The RIG-I pathway

Retinoic acid inducible gene I like helicases are found in the cytoplasm of many cell types including the pulmonary epithelial cells which are the primary target of the influenza virus replication (Kawai and Akira, 2008). The RIG I like helicases contain DExD/box RNA helicases; RIG-I, laboratory of genetics and physiology-2 (LGP-2) and melanoma differentiation-associated gene-5 (MDA-5), which are involved in the recognition of viral cytoplasmic RNA during the infection (Randall and Goodbourn, 2008). RIG-I has two tandem caspase activation and recruitment domains (CARDS) at amino-terminal, a central ATP-dependent helicase domain and regulatory domain (RD) at carboxy-terminal and several other functional domains (Nakhaei *et al.,* 2009). The activation of RIG-I is due to the binding of RD to PAMPS, resulting in the conformational change and leading to RIG-I dimerization and exposure of the CARDS (Yoneyama, 2004). The second CARD domain of the RIG-I has to be ubiquitinized at lysine 172 which paves the way for the initiation of IFN response and this is done by tripartite motif (TRIM) 25 post-translationally. Upon ubiquitination, RIG-I translocates to mitochondria and binds with CARD of mitochondria antiviral signaling adaptor (MAVS) (Kawai, 2005). MAVS, in turn recruits two multiprotein signalosome complexes TRAF3 and its associated factors

such as TANK, TBK1, and IKKε which phosphorylates IRF-3 (IFN regulatory factor 3) and the second TRAF 6 and associated factors (RIP 1, NEMO, TAK1, IKKα and IKKβ,) which phosphorylates the inhibitor of κB (IκB), ultimately leading to NF-κB activation. Activated factors translocate into nucleus and promote IFN-β mRNA transcription. The induced IFN-β initiates a signaling cascade which in turn induces the ISGF 3 (Interferon stimulated gene factor – 3) transcription factor complex. This in turn induces the transcription of several other genes (Sadler and Williams, 2008) andresults in an effective anti-viral state through production of antiviral proteins such as PKR, OAS, MxA, viperin and ISG 15 (García, 2006; Lenschow, 2007; Silverman, 2007; Wang *et al.,* 2007; Haller *et al.,* 2009; Lai, 2009) within cells and helps in limiting the viral replication.

NS1- Effective inhibitor of RIG-I pathway

The nonstructural protein (NS1) of the influenza virus is found to be conserved functionally in both Influenza A and B viruses (García-Sastre, 1998; Talon, 2000; Dauber *et al.,* 2004; Donelan, 2004). The NS1 protein plays an effective role in prevention of the host innate immune response (Talon, 2000). Infection of mutant viruses without NS1 resulted in higher levels of IFN response in cell culture and animal model studies (Ferko, 2004; Falcón, 2005; Quinlivan, 2005; Solórzano *et al.,* 2005; Chambers, 2009; Steel, 2009). Recent studies revealed that the NS 1 protein of influenza A and B viruses binds to TRIM 25 (E3 ubiquitin ligase) that is needed for the post-translation modification of the RIG-I and thereby prevents the signaling cascade (Gack, 2007) (Fig. 1). TRIM 25 is a RING domain (‘really very interesting new gene’) containing E3 ubiquitin ligase with many conserved domains among the ten subfamilies of its family (Ozato *et al.,* 2008). It has a distinct carboxy SPRY domain (splA and ryanodine receptor) which binds to the first CARD domain of RIG-I and the RING domain causes ubiquitination of lysine 172 of the second CARD of RIG-I (Gack, 2007) Thus, the ubiquitination of the second CARD domain of RIG-I is the initial critical step in eliciting the IFN response. The NS1 protein of the influenza virus prevents the interaction between TRIM 25 and RIG-I by binding itself with the coiled coil domain of TRIM 25 (Gack, 2009) and inhibits the RIG-I pathway of innate immunity.Studies have shown that NS 1 protein also affects host cell gene expression (Nemeroff *et al.,* 1998) by interacting with cleavage and polyadenylationspecificity factor (CPSF 30). This interaction prevents CPSF 30 from its polyadenylation activity at the 3’ ends of pre-mRNA (Nemeroff *et al.,* 1998; Noah, 2003; Twu *et al.,* 2006; Kochs *et al.,* 2007).NS 1 also limits the effects of other IFN- inducible antiviral effectors such as PKR (Li *et al.,* 2006) and ISG15 (Yuan and Krug, 2001). The Influenza A virus NS1 protein can also activate PI3K signaling and suppress the host cell apoptosis (Ehrhardt, 2007; Shin, 2007; Zhirnov and Klenk, 2007) . Apart from innate immunity, NS1 protein also limits adaptive immune responses (Fernandez-Sesma, 2007) and was also found to reduce dendritic cell maturation (López *et al.,* 2003). Thus NS1 acts as an emerging antiviral target and there have been several ongoing studies to develop chemical compounds that inhibit its conserved interactions.

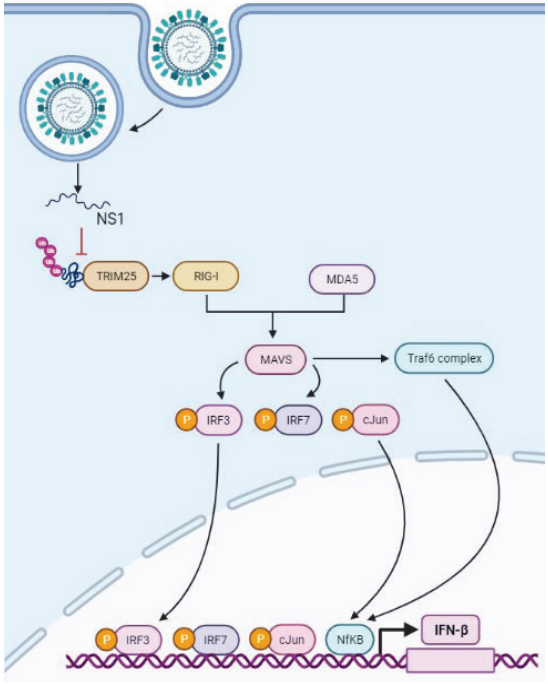


Fig. 1.Role of NS1 protein in inhibition of RIG-I mediated IFN pathway: TheNS1 protein of the influenza virus is shown to bind with TRIM 25 preventing the ubiquitination of RIG-I protein. Inactivation of RIG-I prevent the activation of steps involved in the downstream of the signaling cascade and thereby fail to elicit an interferon response

Small molecule inhibitors of NS1

The full length NS1 protein crystallization and structure determination (Bornholdt and Prasad, 2008) gave a concrete base for many structure-based drug design studies(Krug and Aramini, 2009). The crystal structure of NS1-dsRNA (Yin, 2007) and NSI-CPSF30 binding interactions reveal novel targets for development of specific inhibitors of NS1 protein. Compounds with antagonist activity against NS1 proteins have been identified through various approaches such as Biochemical assays (Cho, 2012; You, 2011), Cell-based export assays (Mata, 2011; Zhang, 2012), NS1-dependent assay in yeast (Basu, 2009; Walkiewicz *et al.,* 2011; Jablonski *et al.,* 2012). High-throughput screening using yeast based phenotypic assay lead to the discovery of a potent NS1 antagonist chemical series, represented by ML303 which specifically inhibit NS1 function (Patnaik, 2010) (Fig. 2).This antiviral small molecule showed an improved potency of IC90 of 155nM and overcame the weak activity limitations of the prior compounds without overt cytotoxicity to the host cells. Another novel approach in targeting a particular protein is through the concept of PROTACs which was first described by Zhou *et al.* (2000). PROTACs are bi-functional compounds, molecules or proteins that bind an E3 ubiquitin ligase on one of its end and another protein which is not the actual E3 substrate on other end. PROTACs have the ability to retarget the E3 ligase activity to new substrates (Skaar *et al.,* 2014). The other advantages of these PROTAC molecules include the fact that these are catalytic and can be used to recruit anydisease-causing protein that exists in a multi-subunit complex. These moleculesare highly specific and do not necessarily need transfection or transduction. PROTACs can be applied to cells; injected into animalsdirectly without the use of vectors. Since there are large numbers of E3 ligases the possibilities for different combinations of PROTACs to target specific disease-causing proteins to different ligases are unlimited (Zhou *et al.,* 2000). In this paper, we discuss the insights of applying the concept of PROTAC to target

the NS1 protein of the influenza virus and thereby inhibiting the NS1 interaction with TRIM 25 which in turn indirectly activates the RIG-I pathway. In the new proposed model of PROTACs we used ML303 probe at one end of the PROTAC to bind with the NS1 protein and a VHL ligandon the other end (Fig. 3) or a NEDD4 inhibitor (Fig. 4) can be attached to interact with VHL E3 ligase or NEDD4 E3 ligase respectively (Galdeano, 2014; Kathman, 2015). The VHL ligase isone of the well-studied E3 ligase that is applied to the concept of PROTAC molecules. The reason for choosing a NEED4 inhibitor was that the NEDD4 E3 ligase degrades the interferon (IFN)-induced transmembrane protein 3 (IFITM3), a cell-intrinsic factor that limits influenza virus infections (Chesarino *et al.,* 2015). Therefore, we expect the proposed PROTAC molecule with VHL ligand should elevate the IFN- β levels through the activation of RIG-I mediated pathway and the one with NEDD4 inhibitor additionally should increase the availability of IFITM3 protein.

Conclusion and future directions

Studies on NS1 protein in infected cells and animals have clearly shown that the virus replication, spread and pathogenesis are dependent on function of this protein. It is also shown that the viruses lacking NS1 have reduced replication and pathogenic capacity in animal models (García-Sastre, 1998; Talon, 2000; Falcón, 2005; Richt and García-Sastre, 2009; Zhou, 2010). These results indicate that specific inhibition of NS1 protein can affect the viral replication and pathogenesis. The importance of these novel drug studies is to overcome the hurdles due to the antigenic shift and drift of the influenza virus. Recent studies have shown that NEDD4 E3 ligase plays an important role in promoting the influenza viral infection by decreasing the levels of IFITM3 antiviral protein (Chesarino *et al.,* 2015). So, the proposed PROTAC molecule with NEDD4 inhibitor, if found to be successful in inhibiting both NS1 protein of the virus and also the NEDD4 E3 ligase host factor, could be further studied and used as an effective antiviral drug for the Influenza viral infection. As these PROTACs can target any disease-causingprotein for degradation, it is the prime time for these novel molecules to be transformed into potent drugs in the future.

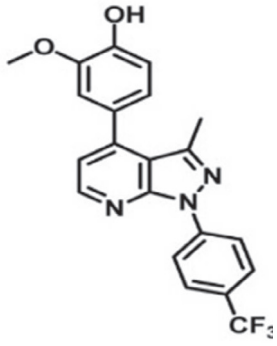
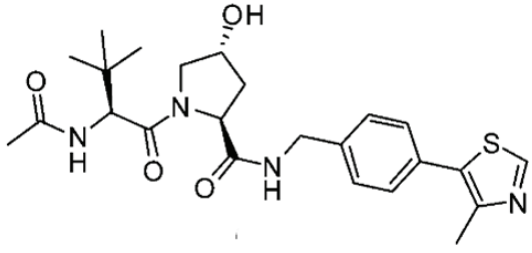
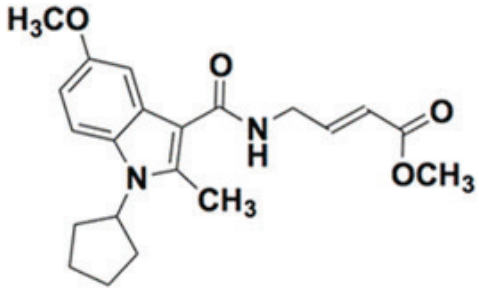


Fig. 2. Probe ML303. ML303 has an improved potency of IC90 of 155nM and was found to be less toxic to the cells compared to previously identified



molecules.

Fig. 3. VHL ligand:Can be employed on



one end of the PROTAC for binding to VHL ubiquitin ligase

Fig. 4. NEDD4 Inhibitor:Can be employed on one end of the PROTAC for binding to NEDD4 E3 ligase, so that it can prevent degradation of IFITM3 protein.

Abbreviations

NS1: Non-structural protein 1; RBD- RNA binding domain; ED-Effector domain; PRRs: pattern recognition receptors; RIG-I: Retinoic acid- inducible gene-I; NOD: nucleotide binging oligomerization domain; MDA-5: melanoma differentiation-associated gene-5; PAMPS: pathogen-associated molecular patterns; CARDS: caspase activation and recruitment domains; TRIM: tripartite motif; MAVS: mitochondria antiviral signaling adaptor/IPS-1/Cardiff/ VISA; TRAF: TNF receptor associated factor; TANK: TRAF-associated NF-κB activator; TBK1: TANK-binding kinase 1; IKK:IκB kinase; IRF: interferon regulatory factor; RIP 1: receptor interacting protein 1; NEMO: NF-κB essential modulator,TAK1: Transforming growth factor beta-activated kinase 1, NF-κB:Nuclear factor κB; ISGF: Interferon stimulated gene factor; PKR: Protein kinase R; OAS: Oligoadenylate synthase, MxA: Myxovirus resistance protein A; CPSF30: Cleavage and polyadenylation specificity factor 30; NEDD4:Neural precursor cell Expressed Developmentally Down-regulated protein 4

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

Samuel Pushparaj performed the literature review and wrotethe draft of the manuscript; reviewed the document; designed the figures, organized the tables, and supervised thewhole manuscript preparation.

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