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Manipulation of Human Immunodeficiency Virus Restriction Factors: RPRD2, SERINC3 and SERINC5

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Manipulation of Human Immunodeficiency Virus restriction factors: RPRD2, SERINC3 and SERINC5

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Abstract

Background: There is a need for a newer approach to tackle human immunodeficiency virus (HIV) due to limitations of the current antiretrovirals, innate cell restriction factors offer such opportunity. This study seeks to characterise three novel HIV restriction factors and explore their therapeutic potentials.

Methods: Jurkat cell line was treated with polyinosinic-polycytidylic acid (poly I:C), a toll-like receptor-3 agonist (TLR3), at different concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml) and (4 µg/ml, 8 µg/ml, 12 µg/ml) and untreated controls. Its effects on cellular proliferation were observed over many hours. A bioinformatic search of Regulation of nuclear pre-mRNA domain-containing 2 (RPRD2) and Serine incorporator 3 and 5 (SERINC3 and SERINC5) were conducted to predict for nuclear localisation signal (NLS) and potential ubiquitination and Sumoylation sites in the proteins using online NLS Mapper, UbPred and GPS-SUMO tools respectively. Small interference RNA (siRNA) transfection of Jurkat cell line was done to knockdown karyopherin alpha 2 (KPNA2) using Lipofectamine 3000. Western blot analysis was done to assess transfection efficiency.

Results: Jurkat cells treated with 5 µg/ml, 10 µg/ml and 20 µg/ml proliferated more than the control while those treated with 4 µg/ml, 8 µg/ml and 12 µg/ml proliferated less with statistical significance between the untreated and 4 µg/ml concentration at 72 hours ($p = 0.021$).RPRD2 was the only protein that has NLS (RDPFHSLKRPRPPFARGPPFFAPKRPFFP)at position 1430 with a score of 9.8. RPRD2 has a site predicted each for SUMO interaction and sumoylation consensus ($p = 0.022$), SERINC3 had six sites for SUMO interactions and 2 for sumoylation non-consensus, all with no statistical significance and SERINC5 has a significant SUMO interaction site at position 44-48 ($p = 0.049$). The predicted ubiquitination sites for RPRD2 were 44 (ten with high, 27 with medium and seven with low confidence respectively), SERINC3 had four sites (two with medium and low confidence each) and SERINC5 had three (one with high, medium and low confidence each). The KPNA2 knockdown was not successful.

Conclusion: HIV restriction factors present a potential therapeutic target; adequate characterisation of these proteins is important towards fashioning drugs in this regard.

Keywords: Toll-like receptor 3, Polyinosinic-polycytidylic acid (poly I:C), Nuclear localisation signal, Sumoylation, Ubiquitination, Karyopherin alpha 2 (KPNA2)

Introduction

The war against human immunodeficiency virus (HIV) is not yet over though some battles have been won with the help of combination antiretroviral therapy that has significantly reduced its morbidity and mortality [1–3]. However, nearly a million lives are still lost annually to HIV infection [1, 4]; this has necessitated the need to fashion new weapons to fight the disease [5, 6]. One of the areas of ongoing research is understanding how the immune cells can be able to control the viral infection [7, 8], prominent in this regard are proteins that have been reported to restrict HIV infection and are generally called HIV restriction factors [9–11]. Pathogen-associated molecular patterns (PAMPs) that are common to almost all organism activates cellular pattern recognition receptors (PRRs) of which toll-like receptors (TLRs) family is a group [12, 13], the activation of TLR3 has been reported to cause interferon-mediated expression of restriction factors [14]. TLR3 has been shown to recognise different forms of double-stranded RNA (dsRNA), a viral replication intermediate, giving way for the use of synthetic analogue of dsRNA to be employed in researches [15, 16]. A common example that has been used is poly I:C [17, 18]. For instance it leads to apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3G(APOBEC3G) and tetherin upregulation in macrophages [19, 20].However, the virus has accessory proteins that

counter the action of the restriction factors. The accessory proteins in HIV-1 are Vif, Vpr, Vpu, Nef (and Vpx in HIV-2) [21–23]. Many HIV restriction factors have been discovered, well-known example include (APOBEC3) family of protein especially APOBEC3G, tripartite-motif-containing 5a (TRIM5a), and tetherin/bone marrow stromal cell antigen 2 (BST2) [24–27]. The new ones include Zinc-finger antiviral protein (ZAP), Serine incorporator 3 and 5 (SERINC3/5) and RPRD2 (also called RNA-associated early-stage antiviral factor, REAF) [28–30]. The functions of restriction factors can be modified by post-translational modifications (PTM) such as the addition of ubiquitin and small ubiquitin-like modifiers (SUMO) proteins called ubiquitination and sumoylation respectively in addition to their nucleocytoplasmic transport [31–34]. Importin alpha such as karyopherin alpha 2 (KPNA2) has been documented to be involved in the latter [35]. Some restriction factors have been well characterised while interest is increasing about the novel one such as RPRD2, SERINC3 and SERINC5 [29, 30, 34, 36], as this could be an avenue to develop new therapy at tackling HIV[37–39].Therefore, this study seeks to undertake the following: (I) study the effect of poly I:C on cellular proliferation (II) prediction of NLS, ubiquitination and sumoylation sites, of RPRD2, SERINC3 and SERINC5 (III) Knockdown of KPNA2 protein.

Materials and methods

Cell Culture

Jurkat cell line (E6-1) was cultured in RPMI growth medium (Lonza) containing 10% foetal bovine serum (FBS, ThermoFisher Scientific), 1% penicillin (Sigma) and 1% streptomycin solution (Sigma) in a T 25 cm^3 flask. The cell culture was incubated at 37^0C and humidified air with 5% CO2. The cells passaging was done at 3 to 4 days intervals.

Poly I:C treatment

A sterile 24-well plate was plated in duplicate with 1×10^5 cells/well in RPMI containing 10% FBS and penicillin-streptomycin. Cells were then treated with high molecular weight (HMW) poly I:C (InvivoGen) at different concentrations (5 µg/ml, 10 µg/ml and 20 µg/ml). Untreated cells in duplicate wells served as a control. The plate was incubated at 37^0C and humidified air with 5% CO2. The total viable cells were counted at 72 hours, 120 hours, and 168 hours by the haemacytometer method using 0.4% trypan blue stain (Sigma). In another instance, the plating of a sterile 24-well plate was made in duplicate with 2×10^5cells/well in RPMI containing 10% FCS and penicillin-streptomycin. The cells were treated with HMW poly I:C at different concentrations (4 µg/ml, 8µg/ml and 12 µg/ml). There was a negative control also. The plate was incubated at 37^0C and 5% C02 humidified air. The total viable cells were counted at 24 hours, 48 hours, 72 hours and 96 hours by the haemacytometer method using 0.4% trypan blue stain (Sigma).

Bioinformatics resources

Three novel HIV restriction factors were selected: RPRD2, SERINC3 and SERINC5. The following searches were performed on each: prediction of NLS, potential ubiquitination and sumoylation sites. The FASTA format (text-based representation of peptide sequence) of each protein was accessed from https//www.uniport.org [40–43], figure 1.Classical NLS (cNLS) Mapper was used for the prediction of the NLS with the cut-off set at 5.0 and within the terminal 60-amino-acid region [44]. UbPred was used for the prediction of the potential ubiquitination sites [45], while group-based prediction system, GPS-SUMO was used for the prediction of the potential sumoylation sites [46]. High performance determined the choice of the tools selected [47, 48]. The threshold for the GPS-SUMO was set at medium for the sumoylation (sensitivity 67.93%, specificity 90.00%) and SUMO interaction (sensitivity 65.96%, specificity 90.03%, supplementary table 1.

siRNA transfection

Plating of a sterile 24-well plate was made with 2×10^5 cells/well. Lipofectamine 3000 reagent (ThermoFisher Scientific,), 0.75µl and 1.5µl were diluted in 25µl Opti-MEM medium each (Gibco) and mixed. 1 µg of KPNA2-specific Small interfering RNA (10 µM, ThermoFisher Scientific) 1µg was diluted in 50 µl of Opti-MEM medium (Gibco), this was divided equally and mixed with the diluted Lipofectamine 3000 reagents and the complex was incubated for 30 minutes at room temperature. The complex reagents were added to a well of cells each and untreated control was also set up. The plate was incubated at 37^oC with 5% CO2 humidified air for 5 days after which the cells were harvested. Western blot was used to confirm transfection efficiency.

Western blot

The harvested cells were lysed in RIPA buffer mixed with protease inhibitor cocktail (ThermoFisher Scientific) on ice-cold, LDS sample buffer (Novex) and sample reducing agent (Novex) was added to aid cell lysis. The cells were also frozen at -80^o C for 60 seconds and thawed to aid cell lysis further. The protein concentration was determined with the BCA protein assay kit (ThermoFisher) following the manufacturer's guide. Heat blocking was done at 85^o C for

a

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>sp|Q5VT52|RPRD2_HUMAN Regulation of nuclear pre-mRNA domain-
containing protein 2 OS=Homo sapiens OX=9606 GN=RPRD2 PE=1 SV=1
MAAGGGGGSSKASSSSASSAGALESSLDRKFQSVTNTMESIQGLSSWCIENKKHHSTIVY
HWMKWLRRSAYPHRLNLFYLANDVIQNCKRKNAIIFRESFADVLPEAAALVKDPSVSKSV
ERIFKIWEDRNVYPEEMIVALREALSTTFKTQKQLKENLNKQPNKQWKKSQTSTNPKAAL
KSKIVAEFRSQALIEELLLYKRSEDQIELKEKQLSTMRVDVCSTETLKCLKDKTGGKKFS
KEFEEASSKLEEFVNGLDKQVKNGPSLTEALENAGIFYEAQYKEVKVVANAYKTFANRVN
NLKKKLDQLKSTLPDPEESPVPSPSMDAPSPTGSESPFQGMGGEESQSPTMESEKSATPE
PVTDNRDVEDMELSDVEDDGSKIIVEDRKEKPAEKSAVSTSVPTKPTENISKASSCTPVP
VTMTATPPLPKPVNTSLSPSPALALPNLANVDLAKISSILSSLTSVMKNTGVSPASRPSP
GTPTSPSNLTSGLKTPAPATTTSHNPLANILSKVEITPESILSALSKTQTQSAPALQGLS
SLLQSVTGNPVPASEAASQSTSASPANTTVSTIKGRNLPSSAQPFIPKSFNYSPNSSTSE
VSSTSASKASIGQSPGLPSTTFKLPSNSLGFTATHNTSPAAPPTEVTICQSSEVSKPKLE
SESTSPSLEMKIHNFLKGNPGFSGLNLNIPILSSLGSSAPSESHPSDFQRGPTSTSIDNI
DGTPVRDERSGTPTQDEMMDKPTSSSVDTMSLLSKIISPGSSTPSSTRSPPPGRDESYPR
ELSNSVSTYRPFGLGSESPYKQPSDGMERPSSLMDSSQEKFYPDTSFQEDEDYRDFEYSG
PPPSAMMNLEKKPAKSILKSSKLSDTTEYQPILSSYSHRAQEFGVKSAFPPSVRALLDSS
ENCDRLSSSPGLFGAFSVRGNEPGSDRSPSPSKNDSFFTPDSNHNSLSQSTTGHLSLPQK
QYPDSPHPVPHRSLFSPQNTLAAPTGHPPTSGVEKVLASTISTTSTIEFKNMLKNASRKP
SDDKHFGQAPSKGTPSDGVSLSNLTQPSLTATDQQQQEEHYRIETRVSSSCLDLPDSTEE
KGAPIETLGYHSASNRRMSGEPIQTVESIRVPGKGNRGHGREASRVGWFDLSTSGSSFDN
GPSSASELASLGGGGSGGLTGFKTAPYKERAPQFQESVGSFRSNSFNSTFEHHLPPSPLE
HGTPFQREPVGPSSAPPVPPKDHGGIFSRDAPTHLPSVDLSNPFTKEAALAHAAPPPPPG
EHSGIPFPTPPPPPPPGEHSSSGGSGVPFSTPPPPPPPVDHSGVVPFPAPPLAEHGVAGA
VAVFPKDHSSLLQGTLAEHFGVLPGPRDHGGPTQRDLNGPGLSRVRESLTLPSHSLEHLG
PPHGGGGGGGSNSSSGPPLGPSHRDTISRSGIILRSPRPDFRPREPFLSRDPFHSLKRPR
PPFARGPPFFAPKRPFFPPRY
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b

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>sp|Q13530|SERC3_HUMAN Serine incorporator 3 OS=Homo sapiens
OX=9606 GN=SERINC3 PE=2 SV=2
MGAVLGVFSLASWVPCLCSGASCLLCSCCPNSKNSTVTRLIYAFILLLSTVVSYIMQRKE
METYLKKIPGFCEGGFKIHEADINADKDCDVLVGYKAVYRISFAMAIFFFVFSLLMFKVK
TSKDLRAAVHNGFWFFKIAALIGIMVGSFYIPGGYFSSVWFVVGMIGAALFILIQLVLLV
DFAHSWNESWVNRMEEGNPRLWYAALLSFTSAFYILSIICVGLLYTYYTKPDGCTENKFF
ISINLILCVVASIISIHPKIQEHQPRSGLLQSSLITLYTMYLTWSAMSNEPDRSCNPNLM
SFITRITAPTLAPGNSTAVVPTPTPPSKSGSLLDSDNFIGLFVFVLCLLYSSIRTSTNSQ
VDKLTLSGSDSVILGDTTTSGASDEEDGQPRRAVDNEKEGVQYSYSLFHLMLCLASLYIM
MTLTSWYSPDAKFQSMTSKWPAVWVKISSSWVCLLLYVWTLVAPLVLTSRDFS
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c

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>sp|Q86VE9|SERC5_HUMAN Serine incorporator 5 OS=Homo sapiens
OX=9606 GN=SERINC5 PE=2 SV=1
MSAQCCAGQLACCCGSAGCSLCCDCCPRIRQSLSTRFMYALYFILVVVLCCIMMSTTVAH
KMKEHIPFFEDMCKGIKAGDTCEKLVGYSAVYRVCFGMACFFFIFCLLTLKINNSKSCRA
HIHNGFWFFKLLLLGAMCSGAFFIPDQDTFLNAWRYVGAVGGFLFIGIQLLLLVEFAHKW
NKNWTAGTASNKLWYASLALVTLIMYSIATGGLVLMAVFYTQKDSCMENKILLGVNGGLC
LLISLVAISPWVQNRQPHSGLLQSGVISCYVTYLTFSALSSKPAEVVLDEHGKNVTICVP
DFGQDLYRDENLVTILGTSLLIGCILYSCLTSTTRSSSDALQGRYAAPELEIARCCFCFS
PGGEDTEEQQPGKEGPRVIYDEKKGTVYIYSYFHFVFFLASLYVMMTVTNWFNHVRSAFH
LLP
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Figure 1 FASTA format of amino acids for HIV restriction factors. (a)RPRD2 sequence with sequence identifier at the beginning (b) SERINC3 sequence with sequence identifier at the beginning (c) SERINC5 sequence with sequence identifier at the beginning. In the FASTA format, amino acids are represented with single-lettered code and start with a single-line description. However, they are presented in two lines here in other to make the sequences fit into the available space.

10 minutes to denature the proteins. The extracted protein (25µg) was separated by sodium dodecyl sulfate, SDS (Novex) polyacrylamide gel electrophoresis, PAGEs (precast, ThermoFisher Scientific) at 200 Volts for 1 hour 30 minutes. The separated proteins were transferred to a nitrocellulose membrane using the iBlot2 Dry Blotting System at 25 Volts for 6 minutes. The membrane was blocked with 5% milk in TBS (50 mM Tris-HCl, pH 7.6, 150mM NaCl) for 30 minutes at room temperature. Then the membrane was incubated with rabbit anti-KPNA2 primary antibody (Abcam), 1:1000 dilution in 5% milk in TBS-T (50 mM Tris-Cl, pH 7.6, 150mM NaCl, containing 0.1% Tween-20) overnight at 4^0C. The membrane was then washed in TBS three times before it was incubated in goat anti-rabbit Ig-linked horseradish peroxidase (HRP) secondary antibody (Abcam), 1: 5,000 dilution in 5% milk in TBS-T for 1 hour. Anti-LAC antibody (Abcam), 1: 2,500 dilution served as a control. The membrane was washed finally three times and West femto chemiluminescent substrate (ThermoFisher Scientific) was added and incubated for 5 minutes in the dark. Chemiluminescence imaging (Gensys) for band detection was done.

Statistical analysis

Data for the poly I:C treatment were expressed as the mean ± standard deviation of duplicate wells. A comparison was made between the mean of the treated versus control and statistical significance was assessed by the Student`s t-test using SPSS (version 25, IBM). Statistical significance was set at $p < 0.05$

Results

Poly I:C treatment

Jurkat cells were treated with poly I:C at different concentrations versus untreated control. In figure 2a, the proliferation of the treated cells was more than that of the untreated control from 72 hours through 168 hours. However, this was not statistically significant. In figure 2b, the proliferation of the untreated Jurkat cells was generally more than that of those treated with poly I:C up till 72 hours (except with 12 µg/ml concentration at 72 hours), before it fell below that of others at 96 hours. There was a statistically significant difference in the growth of the untreated cells compared with that of the cells treated cells with 4 µg/ml at 72 hours ($p = 0.021$) with proliferation being more in the untreated cells. This gives a contrasting finding on the effect of poly I:C on the cellular proliferation between the two sets of poly I:C concentrations.

Nuclear localisation signal prediction

The NLS signal was seen in RPRD2 at position 1430 with an NLS score of 9.8. There was no NLS seen for SERINC3 and SERINC5 at the cut-off of 5.0 and within the terminal 60-amino-acids region used in this analysis. However, when the cut-off was set at the lowest value of 2.0 and within the entire region, positions for NLS were detected in SERINC3 and SERINC5 although the values were low, supplementary tables 5 and 6.

Sumoylation prediction

Many SUMO substrates contain tetrapeptide represented by ΨK×D/E (Ψ is hydrophobic residue, K is lysine, that is conjugated to the SUMO, × is any amino acid and D/E represent acidic residue. This conserved tetrapeptide is termed the SUMO consensus motif (SCM). Enzymatic covalent binding of SUMO to proteins involves Ubc9 enzyme. SUMO interacting motif (SIM) involves the non-covalent interaction of SUMO with proteins [47, 49, 50]. GPS-SUMO showed that when the threshold was set at medium, there was a position of statistical significant sumoylation consensus predicted for RPRD at 658 ($p = 0.022$) and one SUMO interaction site at position 383-387 and has p-value that approached statistical significance (0.056), figure 3a. For SERINC3, there were six positions predicted for SUMO interaction and two sumoylation non-consensus sites. There was none that was statistically significant. The closet to significance was SUMO interaction at position 177-181 ($p =0.07$) and position 246-250 ($p =0.072$) figure 3b. SERINC5 has a statistically significant SUMO interaction at position 44-48 ($p=0.049$) figure 3c.

Ubiquitination sites prediction

Ubiquitination occurs at lysine residue denoted by (K)[43, 51].The predicted potential ubiquitination sites for RPRD2 was 44. There were ten residues with high confidence, 27 with medium confidence and seven with low confidence, figure 4a. For SERINC3 there were 2 predicted sites each with medium and low confidence (figure 4b) while SERINC5 had one site each predicted for ubiquitination with high, medium and low confidence, figure 4c. The prediction confidence is based on the score range shown in figure 4d. The sensitivity and specificity of the corresponding range of scores are also stated.

Western blot

The western blot showed that KPNA2 was present in both the siRNA treated cells and the control (band 58 kDa). Detection of Lamin A/C(LAC) served as a control (between bands 65- 70 kDa, supplementary figure 6. Therefore, the siRNA transfection to knockdown KPNA2 was not successful.

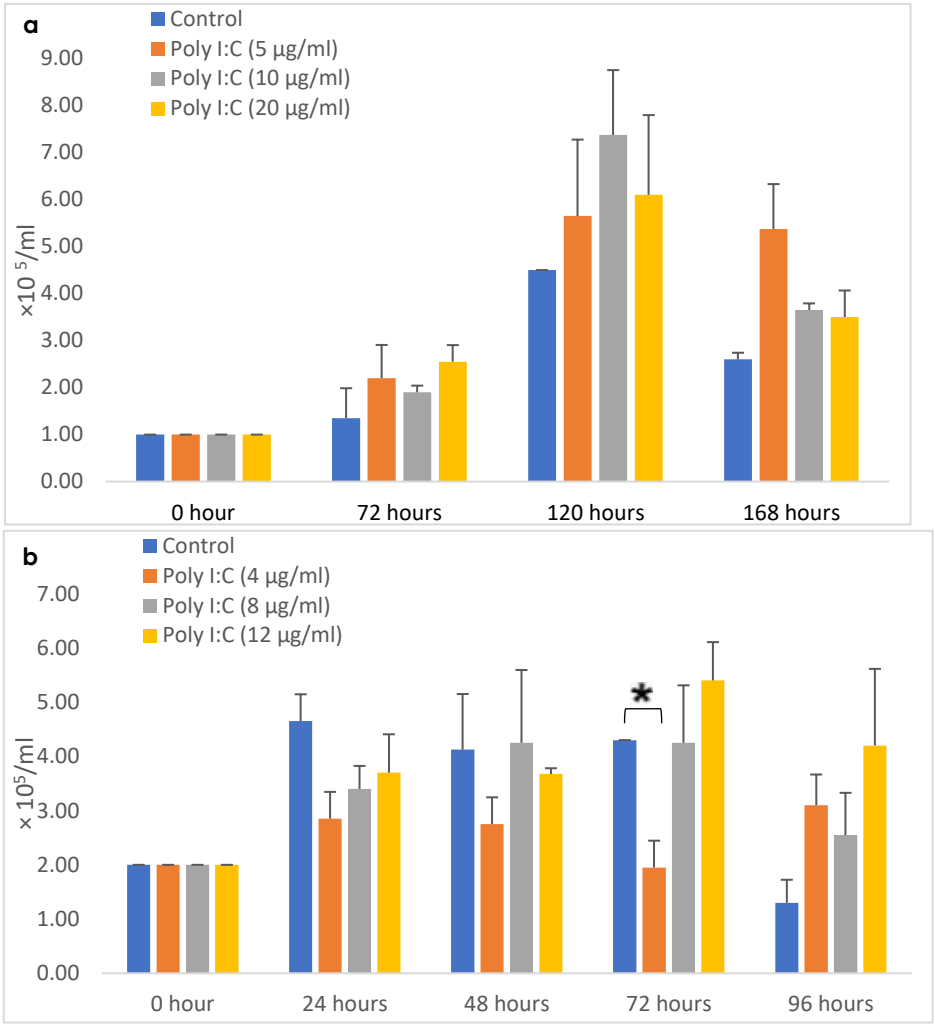

Figure 2 The effects of poly I:C on Jurkat cell proliferation. (a) Jurkat cells treated with different concentrations of poly I:C (5 µg/ml, 10 µg/ml and 20 µg/ml) versus untreated control. The cells were incubated at 37^{0}C and total viable cells were counted at 72 hours, 120 hours, 168 hours. (b) Jurkat cells treated with different concentrations of poly I:C (4 µg/ml, 8 µg/ml and 12 µg/ml) versus untreated control. The cells were incubated at 37^{0}C and total viable cells were counted at 24 hours, 48 hours, 72 hours and 96 hours. The results were expressed as mean ± SD of duplicate wells. * P = 0.021

Table 1 Predicted bipartite NLS of RPRD2

Position	Sequence	Score
1430	RDPFHSLKRPRPPFARGPPFAPKRPFFP	9.8

The predicted NLS of RPRD2 showing the position, sequence and score with the cut-off set at 5.0 and within the terminal 60-amino-acids region of the protein.

a

Position	Peptide	Score	Cut-off	P-value	Type
383-387	VEDDGSKIIVEDRKEKPAE	41.065	29.92	0.056	SUMO Interaction
658	SSEVSKPKLESESTS	11.496	2.13	0.022	Sumoylation consensus

b

Position	Peptide	Score	Cut-off	P-value	Type
177-181	LFILIQLVLLVDFAHSWNE	36.631	29.92	0.07	SUMO Interaction
218-222	SAFYILSIICVGLLYTYYT	32.354	29.92	0.104	SUMO Interaction
230	LLYTYYTKPDGCTEN	3.831	3.32	0.378	Sumoylation Non-consensus
246-250	FFISINLILCVVASIISIH	35.457	29.92	0.072	SUMO Interaction
253-257	ILCCVAIISIHPKIQEHQ	32.888	29.92	0.148	SUMO Interaction
274-278	SGLLQSSLITLYTMYLTWS	34.683	29.92	0.143	SUMO interaction
328	PTPTPPSKSGSLLDS	3.867	3.32	0.73	Sumoylation Non-consensus
465-469	VWTLTAPLVLTSRDFS	31.385	29.92	0.137	SUMO Interaction

c

Position	Peptide	Score	Cut-off	P-value	Type
44-48	FMYALYFILVVVLCCIMMS	38.411	29.92	0.049	SUMO Interaction

Figure 3 Prediction results of sumoylation sites and SUMO interaction at a medium threshold. (a) RPRD2 with a SUMO interaction and sumoylation consensus each. (b) SERINC3 with six SUMO interaction sites and two sumoylation non-consensus. (c) SERINC5 with a SUMO interaction site

The modified position, modified peptide, predicted scores, prediction cut-off, and regulation type are presented. $P < 0.05$ is significant

Discussion

Toll-like receptors recognise and respond to PAMPs leading to intracellular signal pathways activation, this process is important in the immune response to pathogens [12, 13]. There are 11 known TLRs, TLR-3 is involved in the key process of the immune response to viruses by the recognition of dsRNA (a common viral replication intermediate molecule) [12, 15]. The development of synthetic dsRNA analogue affords the opportunity to study the effect of TLR-3 activation in cellular processes. poly I:C is a dsRNA analogue that has been employed in this regard [17]. In this study HMW poly I:C was used in preference to low molecular weight counterpart (LMW) as the former has been reported to have higher activation efficiency than the latter [52]. Jurkat cells were treated with different concentrations of HMW poly I:C and its effects on the proliferation of the cells were observed. In figure 2a, the proliferation of cells that were treated with HMW poly I:C was more than that of the control up to 168 hours, although this difference was not statistically significant. In figure 2b however, the effect of HMW poly I:C on Jurkat cell proliferation was different even though the concentrations used in both instances were similar. Overall, there was less proliferation of the treated cells up to 72 hours before a reduction in the growth of the control below that of others at 96 hours. A significant difference was noted at 72 hours between the control and 4 µg/ml concentration of poly I:C with more proliferation in the untreated cells. The effects of poly I:C on cell proliferation in the two instances in figure 2 are contrasting. The effect seen in figure 2b is similar to some extent with what has been reported in other studies that documented that poly I:C is anti-proliferative [53–56]. This was reported in different cell lines and human macrophages. There was associated inhibition of HIV replication following the treatment of cells with poly I:C [14, 18, 19, 52]. One of the mechanisms underlying these has been documented to be related to the anti-viral response via the activation of intracellular signals mediated by interferons leading to the expression of restriction factors such as APOBEC3G and tetherin [14, 18, 19, 52, 57].

a

Residue	Score	Ub	Residue	Score	Ub
11	0.66	Yes	527	0.81	Yes
30	0.78	Yes	574	0.69	Yes
150	0.67	Yes	588	0.76	Yes
201	0.63	Yes	608	0.79	Yes
210	0.78	Yes	623	0.77	Yes
212	0.77	Yes	656	0.81	Yes
249	0.68	Yes	658	0.85	Yes
259	0.62	Yes	671	0.87	Yes
262	0.66	Yes	677	0.79	Yes
310	0.91	Yes	741	0.88	Yes
355	0.90	Yes	755	0.74	Yes
382	0.89	Yes	801	0.79	Yes
389	0.82	Yes	820	0.84	Yes
391	0.65	Yes	862	0.78	Yes
395	0.82	Yes	933	0.91	Yes
405	0.76	Yes	960	0.70	Yes
412	0.74	Yes	995	0.80	Yes
431	0.82	Yes	1032	0.88	Yes
455	0.75	Yes	1081	0.93	Yes
468	0.76	Yes	1168	0.80	Yes
494	0.81	Yes	>1221	0.77	Yes
513	0.76	Yes	1246	0.85	Yes

b

Residue	Score	Ub
87	62	Yes
328	66	Yes
363	78	Yes
398	71	Yes

c

Residue	Score	Ub
282	0.73	Yes
293	0.63	Yes
393	0.86	Yes

d

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 ≤ s ≤ 0.69	0.464	0.903
Medium confidence	0.69 ≤ s ≤0.84	0.346	0.950
High confidence	0.84 ≤ s ≤1.00	0.197	0.989

Figure 4 Ubiquitination of RPRD2, SERINC3 and SERINC5. (a) RPRD2 has 44 ubiquitination sites, seven sites with low confidence, 27 sites with medium confidence and ten sites with high confidence. (b) SERINC3 ubiquitination sites, two sites with low confidence and two sites with medium confidence. (c) SERINC5 ubiquitination, one site each with low, medium and high confidence. (d) Label showing the range of scores with classification into low (green), medium(blue) and high (red) confidence of ubiquitination. The sensitivity and specificity of each are shown. Ub- ubiquitinated

Therefore, TLR3 agonists could be developed as a therapy for HIV by activating this receptor. In this study, the detection of HIV restriction was not assessed, therefore, further research to detect and quantify RPRD2, SERINC3 and SERINC5 can be carried out to know if they are part of restriction factors that TLR3 activation can

upregulate. A possible advantage of this mechanism of therapy may be non-emergence of resistant strains that are associated with the current anti-retroviral therapy as TLR3 agonists will enhance the immune cells to curtail or inhibit the virus. This may offer a chance for a cure as a study has demonstrated the reversal of HIV latency in microglial cells following TLR3 receptor stimulation [17]. A recent clinical trial assessed poly I:C and poly-L-lysine (Poly ICLC) complex among 15 HIV positive adults that are on antiretroviral therapy. In addition to the therapy being safe, it elicited immune responses that suggested the possible use of TLR3 as adjuvant among HIV patients [58]. More trials to understudy this is expected.

The localisation of cellular proteins has influences on their functions and many transport systems are involved in nucleocytoplasmic trafficking [59, 60]. There are features of proteins that influence this, this includes the possession of NLS that is required for transportation of a protein into the nucleus. Importin a is important for this process and an example in humans is KPNA2 (also called importin alpha 1) [59, 61]. In addition, post-translational modification (PTM) of proteins is important in cellular pathways [51, 62]. A common example is ubiquitination and recently the addition of SUMO has been identified as another PTM that is important in the regulation of proteins [31, 33]. The traditional experiment to identify NLS and types of PTM is usually cumbersome and the development of computational methods to predict this has been useful [63]. In this study, NLS Mapper was used for predicting the NLS for the proteins with the cut-off set at 5.0. The range is from 2.0 to 10.0, the higher the score the higher the NLS activities. For example score of 8 to 9 and 1 to 2 means exclusive localisation to the nucleus and cytoplasm respectively [64]. At this cut-off, only RPRD2 showed NLS with a score of 9.8, table 1. This result implies an importin a shuttling of RPRD2 into the nucleus. SERINC3 and SERINC5 had NLS when the cut off was set at 2.0 but this was generally low giving credence to their cytoplasmic location, supplementary tables 5 and 6. It has been evidenced that HIV accessory protein capitalise on the intranuclear transport of restriction factors to initiate their degradation. Nuclear localisation of SAMHD1 has been shown to be required for Vpx-mediated ubiquitination and subsequent degradation. SAMHD1 was not degraded if localised to the cytoplasm [34, 35]. It was reported that intranuclear RPRD2 decreases in macrophages bearing Vpr accessory protein but increases if the virus lack Vpr. A decrease in RPRD2 was associated with higher susceptibility to HIV infection [65]. The cytoplasmic variants of the restriction factors were resistant to degradation mediated by the accessory proteins [34, 65]. Therefore, drugs that inhibit the nuclear transport of these restriction factors could be useful at ensuring their cytoplasmic presence to restrict HIV infection.

In the computational prediction of sumoylation and ubiquitination study, RPRD2 has a site for SUMO interaction and another for sumoylation consensus, figure 3a. The latter was statistically significant. The ubiquitination process is used for protein degradation among other functions and this mechanism of cellular protein disposal could be hijacked by HIV to degrade restriction factors [31]. RPRD2 has 44 residues of predicted lysine (K) ubiquitination with ten at high confidence, figure 2a. This implies a high chance of its being tagged with ubiquitin through a series of steps by the HIV accessory protein and causing its degradation [65]. A similar instance is a report that Vif induces the ubiquitination and subsequent degradation of APOBEC3G [31, 66, 67]. SERINC3 and SERINC55 had few predicted ubiquitination sites (SERINC5 has one with high confidence) as compared to RPRD2, figure 2. The ubiquitination of SERINC5 was reported not to be mediated by Nef but in the presence of Nef, ubiquitinated SERINC5 colocalise with lysosome-associated membrane protein 1 (LAMP) for lysosomal degradation [68]. Protein sumoylation has been reported to cause modification that reduces the addition of ubiquitin and changes in conformation. Sumoylation is the covalent binding of SUMO to lysine reside while non-covalent interaction of proteins with SUMO or its chains is via SUMO-interacting motifs (SIM) One of the effects of this is downregulation of ubiquitination system [49, 69, 70]. SERINC3 has six predicted SUMO interaction and two sumoylation non-consensus but none attain statistical significance, figure 3b, while the lone SUMO interaction seen in SERINC5 was statistically significant, figure 3c. The presence of significant SUMO interaction site in SERINC5 may be responsible for its higher potency than SERINC3. SERINC5 was shown to reduce HIV infection up to 90% while SERINC3 had 20% reduction in infection rate [32, 71, 72]. There were significant sumoylation consensus and almost statistically significant SUMO interaction in RPRD2, figure 3a. This may protect RPRD2 from Vpr-mediated ubiquitination and degradation. The knowledge of ubiquitination and sumoylation of SERINC3 and SERINC5 can be harnessed for therapeutic intervention of HIV. For stance, small molecules that inhibit Vif regulation of APOBECG3 and increasing its antiviral activities are in development [73, 74]. Study on the development of similar molecules that can potentiate the antiviral activities of RPRD2, SERINC3 and SERINC5 based on the ubiquitination and sumoylation interactions could be developed as a novel HIV therapy. This may be combined with current antiretrovirals for synergy. It has been documented that SERINC5 increased the sensitivity to HIV neutralising antibodies and maraviroc [75].

In the study for the KPNA2 knockdown, the siRNA transfection procedure was not successful making KPNA2 bands to be present in the siRNA transfected

sample and the control. However, this was not surprising due to the low transfection efficiency of Jurkat cell line with Lipofectamine 3000 (<30%) [76]. Optimisation of the transfection process through modifications and trials may be undertaken towards increasing transfection efficiency. Replacing Jurkat cells in the experiment with other cell lines with higher transfection efficiency such as K-562 (human myelogenous leukemia, 30-50%) or MDA-MB-231 (human breast cancer, 51-79%) may be undertaken [76, 77]. However, this may not be ideal or economical. Optimisation of other reagents in the procedure can be done. The volume of Lipofectamine 3000 reagent used in this study was 0.75 µl and 1.5 µl as stated in the manufacturer`s protocol. This can be scaled up to for example 2.5 µl or 5.0 µl in succession [78]. The concentration of siRNA is another factor that may determine the success of siRNA transfection. The range of concentrations recommended ranges from 5-100 nM [79]. The concentration that was used in the study was 10 nm, another working concentration could be tried, a dose-response curve can be used to work out the optimal concentration for siRNA, but this will require large amount of siRNA. Therefore, titration of the siRNA amount with different concentrations can be tried [79, 80]. Another mechanism that can be used to improve transfection efficiency for siRNA is to tag it with fluorescent labels. This could help to identify cells that receive the siRNA and may be useful in correlating the transfection with the protein knockdown [80, 81]. The use of positive and negative controls has also been shown to help in the optimisation of transfection and in distinguishing non-specific effects of the siRNA respectively, for example siRNA GAPDH is commonly used for positive control [82, 83]. Transfection process could cause cellular toxicity and death, therefore assessing for cellular viability can be undertaken with the use of stain such as trypan blue to check for dead cells [80, 84]. It is expected that stepwise application of these optimisation alterations will lead to a successful KPNA2 knockdown and subsequent experiment such as localisation of RPRD2 to confirm its use since RPRD2 possesses NLS with high score

Limitations in this study include time constraint which precludes for example adequate optimisation for the KPNA2 knockdown. The laboratory for this study had a high number of other researchers sharing facilities at the same time (for example, the incubator was frequently open and close), this may affect the optimal condition for the cultured cells and might have affected the results of the poly I:C treatment.

Conclusion

The need for the development of a new anti-HIV therapy is very crucial in other to address the present limitations of the available antiretrovirals. There has been a significant success with the current antiretrovirals but the loss of about a million lives to HIV every year is not acceptable. The knowledge of how the immune cells resist HIV infections via cellular restriction factors offers a potential therapeutic target. Researches on the characteristics of the immune restriction factors and the mechanism of how they interact with viral accessory proteins hold the key to the possibilities of developing drugs that utilises these proteins to inhibit HIV infections. While some restriction factors have been characterised, newer ones are being discovered and characterising them is an ongoing project. RPRD2, SERINC3 and SERINC5 are among the novel restriction factors. The activation of TLR3 has been showed to upregulate some restriction factors via interferon-mediated cellular signalling. It is yet to be determined if RPRD2, SERINC3 and SERINC5 are part of the restrictions factors that are upregulated via this route. Studies to determine this is recommended. Report of a clinical trial that assessed the safety of a TLR3 agonist was satisfactory and it suggested the possible use of the drug as adjuvant to antiretrovirals. Similarly, the understanding of post-translation modifications and how it alters the functions of HIV restriction factors can be harnessed as therapeutic targets. The development of small molecules that work through this mechanism such as preventing HIV accessory proteins degradation of restriction factors is being evaluated. It is anticipated that this may soon progress to the stage of clinical evaluation. The restriction factors may be able to address the problem of resistance associated with the antiretroviral drugs as their effects are exerted by increasing the capacity of the immune cells to ward off infection. This may also be one of the keys that will make HIV cure possible. More researches on characterising the novel HIV restriction factors are required and this may lead to a lethal addition to human armamentarium for the last battle that may end the war against HIV.

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