Optimization of Inhibitory Peptides Targeting Phosphoprotein of Rabies Virus

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Abstract
Rabies is a serious zoonosis caused by rabies virus (RABV) of the genus Lyssavirus, and immunotherapy is now the only approved, effective method for post-exposure prophylaxis against rabies in humans, whereas an effective antiviral therapy is still unavaiable if the central nervous system is invaded. Phosphoprotein (P) is known to play pivotal roles in the life cycle of RABV, and has been regarded as a prime target for inhibitors of viral replication. This study aimed to carry out intracellular administration of a kind of P-binding peptide for RABV inhibition. A group of reported P-binding peptides were focused on for activity improvement by quantitative structure–activity relationship (QSAR) method, and then were mediated by cell penetrating peptide (CPP) for intracellular activity evaluation. The QSAR models had good performance in reliability and predictability ($R^2 \geq 0.852$, $Q^2 \geq 0.601$, $Q^2_{ext} \geq 0.595$), and the peptide screened by partial least squares (PLS) QSAR model ($R^2 = 0.994$, $Q^2 = 0.937$, $Q^2_{ext} = 0.981$) exhibited even higher antiviral activity when it was delivered into the cells by CPP. Above all, this study provided an effective way for development of peptide drug against RABV.

Keywords Cell penetrating peptides · Descriptor · Post-exposure prophylaxis · QSAR · Vaccination

Introduction
Rabies is a fatal zoonosis caused by rabies virus (RABV) of the genus Lyssavirus, and has long been a serious public health threat in most areas of the world, especially in the developing countries (Gnanadurai et al. 2015; Yousaf et al. 2012; Zhu and Guo 2016), every year it kills up to 59,000 people worldwide (Lama et al. 2019; Zhu and Guo 2016).

Vaccination combined with administration of RABV neutralizing antibodies, is the only approved, effective method for post-exposure prophylaxis (PEP) against rabies in humans (Zhu and Guo 2016). However, owing to the cost, accessibility and complexity of treatment, PEP usually can’t be given promptly and correctly, rabies still has a high death rate (Jackson 2016; Kaur et al. 2015; Lama et al. 2019; Zhu and Guo 2016).

Antiviral therapy is thought to be an important component of combination therapy for the management of human rabies, especially for patients who have missed the deadline for valid vaccination or have developed clinical symptoms (Appolinario and Jackson 2015). Till now, no effective therapy can prevent the virus from invading the central nervous system (CNS) once it successfully replicate intracellularly (Banyard et al. 2017; Gnanadurai et al. 2015; Zhu and Guo 2016). Thanks to the progress in RABV molecular biology (Albertini et al. 2011; Lingappa et al. 2013), many targets have been identified for antiviral therapy development (Brunner et al. 2015; Castel et al. 2009; Gümpper et al. 2018; Lama et al. 2019; Meshram et al. 2013; Real et al. 2004; Singh et al. 2014).

The RABV phosphoprotein (P) is a multifunctional protein, besides the role in viral transcription and replication, it can interact with many host proteins to hijack the signaling pathways in favor of viral replication (Fouquet et al. 2015; Masatani et al. 2016; Okada et al. 2016; Wiltzer et al. 2014), or result in mitochondrial dysfunction in neurons causing acute degenerative changes (Kammouni et al. 2015, 2017). Therefore, it is regarded as a promising target for inhibitors development (Albertini et al. 2011; Castel et al. 2009; Kaku et al. 2011).
Real et al. (2004) once screened out a group of antiviral peptides targeting RABV P from combinatorial peptide libraries, which can be used as new leads for pharmacologically active RABV inhibitors design.

In order to analyze the prospect of these peptides in rabies control, in this work we mainly focused on their optimization as well as intracellular application.

Materials and Methods

Data Source

The data of 29 RABV inhibitory peptides targeting RABV P were retrieved from the literature, a reverse genetic viral ribonucleoprotein (RNP) complex reconstitution assay was used to test the peptide activity, which was negatively correlated with the luciferase intensity (Real et al. 2004). In this work the inhibitory activity was indicated by the logarithm of reciprocal of the luciferase intensity (Table 1).

Table 1 Sequences and activity of RABV P-binding peptides

| Name  | Sequences                      | Luciferase intensity | Activity |
|-------|--------------------------------|----------------------|----------|
| C1    | CKFCYGSAQCPTFLFIVRLLRFVVW      | 0.04                 | 1.40     |
| C2    | CTMCYQQNCFTRLIVGGMVFLV      | 0.02                 | 1.70     |
| C3    | CYSCPCERRCHIARGLILRSVLF      | 0.04                 | 1.40     |
| C4    | CQRGWTGVGVGSFLVRILRFVV       | 0.04                 | 1.40     |
| C5    | CTQCCAPSTCLNYRFVGLLRFVV1     | 0.06                 | 1.26     |
| C6    | CDSERCWYVWLILLRVLRLVSL       | 0.03                 | 1.52     |
| C7    | CKSCTDRCTLRLRRLRVGLPCMGCG    | 0.05                 | 1.30     |
| C8    | CRCCELSKCPTLMRVRLGLVL      | 0.01                 | 2.0      |
| C9    | CLCCDVRTCRRLLLGLVMVLSVRC     | 0.03                 | 1.52     |
| C10   | CGECGGGHIVGFCMVVRFLV       | 0.03                 | 1.60     |
| C26   | CVTCKSTVLCDKMFQHPCRGPSCIS    | 0.24                 | 0.62     |
| C27   | CGRLQRAACCYCRKLRLFLVIF      | 0.02                 | 1.70     |
| P11   | PPPIIPDPQRRPWWRFISLMVIRH     | 0.46                 | 0.34     |
| P12   | PPRLLDSPEVMVLHGFRGLVRWLIH   | 0.07                 | 1.19     |
| P13   | PPASSPMPNPPLRRIILLRLFVH     | 0.10                 | 1.0      |
| P14   | PPLPYGPNNEGPHLRVLLRLCIRLH   | 0.05                 | 1.30     |
| P15   | PPRPTIPHLVSLHLLRLRVRVH      | 0.04                 | 1.46     |
| P16   | PPDVHTPHALWRLHLRVLCLVRMWI    | 0.04                 | 1.40     |
| P17   | PPTSLPLLTPNLRPPPIIIWVLRLWVFH | 0.04                 | 1.44     |
| P18   | PLVYGRDTPTTRMPHLLRCCLRLVHV   | 0.08                 | 1.10     |
| P19   | PPDTQTTYPSAECPPPNPLSLILLGLWLH| 0.45                 | 0.34     |
| P20   | PPRAGHRPNSTVVLHVLIRCLLRVFVH | 0.04                 | 1.40     |
| P21   | PPDMSLPPVGHLVRLFLLRLSVH     | 0.05                 | 1.30     |
| P22   | PPAGAPPFRTHTPPRMVVLIRVWCH   | 0.06                 | 1.26     |
| P23   | PPAGAPPQDSVCHELHLVRLRLVIRH  | 0.02                 | 1.70     |
| P24   | PSHSFRPESLERHLRLRRVLLLMMRVH | 0.07                 | 1.16     |
| P25   |PPCYERMPPRLIRPPLSLLILLRRLLCH| 0.12                 | 0.92     |
| P28   | PPLFEDTPMVNSIPPLRVRFLLRLVVFH| 0.05                 | 1.30     |
| P29   | PPRGTETPQRCRRHLVEMCLVLRVFH  | 0.03                 | 1.60     |

The luciferase intensity was a relative value, it was indicated as 100% in the control experiment with the empty peptide expression vector (Real et al. 2004), so the experimental activity of the peptides was indicated with the logarithm of reciprocal of the luciferase intensity.

Descriptor Calculation

Two web servers were applied to compute the features and number of descriptor values of the peptides. The updated PROFEAT web server could provide 9 groups of structural and physiochemical descriptors with more than 2000 values, including amino acid composition (G1), dippeptide composition (G2), autocorrelation descriptors (G3), composition-transition-distribution (G4), quasi-sequence order descriptors (G5), pseudo-amino acid composition (G6, PAAC), amphiphilic pseudo-amino acid composition (G7, APAAC), atomic-level topological descriptors (G8), and total amino acid properties (G9, TAAPs) (Zhang et al. 2017), while 12 groups of descriptors could be got from iFeature, including
six groups of common features (G1–G6) and six groups of features different from PROFEAT (Chen et al. 2018).

**Variable Selection and Modeling**

Descriptors from each server were used to construct models respectively. Genetic algorithm (GA) was mainly used for variable selection, by default, the variance cut-off value was set to 0.0001 and correlation coefficient cut-off value was set to 0.99, the other parameters were as follows: total number of iterations (100), cross-over probability (1), mutation probability (0.5%). Data pretreatment was performed to remove constant and inter-correlated descriptors prior to GA execution.

MLR (multivariable linear regression) and partial least squares (PLS) were selected to construct models. A good QSAR model should be validated both internally and externally, and for such purpose, the dataset was divided into training and test sets by Kennard stone method, Euclidean distance method, and Activity-based method, respectively, PLS QSAR models were built using the training sets, and then validated (externally) by the test sets, the validity and stability of the models were assessed by determination coefficient ($R^2$), correlation coefficients of leave-one-out cross-validation ($Q^2$) and external validation ($Q^2_{ext}$).

**Optimization of the Inhibitory Peptide P16**

P16 was one of the most active peptides according to Real et al. (2004), which was selected for further improvement. A series of mutated peptides were first derived by single amino acid residue substitution, and the potential activity of these peptides was predicted based on the optimal QSAR model; after three rounds of mutation and selection, those with the highest predicted activity would be the candidates for experimental assay.

**Docking Analysis of the Peptides and the Phosphoprotein**

Molecular docking was performed using HDock server to analyze the interaction between the peptide and the target phosphoprotein (Yan et al. 2017), the amino acid sequence of which was acquired from GenBank (https://www.ncbi.nlm.nih.gov/protein). The calculated structures were ranked in terms of the lowest energy, and the top-ranked ones were selected.

**Activity Testing of the Candidate Peptide**

One of the candidates and P16 as a control were chemically synthesized. In order to deliver them into the cells, the cell penetrating peptide (CPP) ‘RRRRRRRRRR’ was linked to the N terminus of each synthesized peptide. The cultured BSR cells were infected with 0.01 MOI of RABV strain CVS, and 1 h later, the peptide was added to each culture with final concentrations from 0 to 50 μg/ml, after incubation at 37 °C for 72 h, fluorescence focus units (FFU) assay was performed to detect viral titers.

**Results**

**QSAR Modeling of the Inhibitory Peptides**

450 structural and physicochemical variables from feature groups G3, G4, G5 and G9 were obtained by PROFEAT server, while 64 variables from groups G1-G4 and G6 were got by iFeature server. Since redundant variables could lower the robustness and predictive capability of a model, especially when the number of variables was large, 15 and 8 variables were selected by GA for modeling respectively. The results showed that all the models exhibited good performance in stability and predictability ($R^2 > 0.852$, $Q^2 > 0.601$, $Q^2_{ext} > 0.595$) (Table 2). The PLS model with the best performance ($R^2 = 0.994$, $Q^2 = 0.937$, $Q^2_{ext} = 0.981$) was applied for peptide optimization (Fig. 1).

**Interaction Between the Peptides and the Phosphoprotein**

1596 sequences were derived from P16 by amino acid substitution, about 20 derivatives were predicted to have the highest activity based on the QSAR model (Table 3). Molecular docking was performed to analyze the interaction between the peptides and the phosphoprotein. The peptide P16b6 with the highest predicted activity was selected to compare with P16. The docking results showed that both peptides could bind to the phosphoprotein, but the docking energy scores indicated that P16b6 could bind more tightly than P16 (Fig. 2).

**Inhibitory Activity Testing of the Candidate Peptide**

The antiviral activity of P16b6 was then tested for validation. The results showed that both P16 and the derivative P16b6 could inhibit the replication of RABV with dosage effect, and the activity of P16b6 was even higher as predicted (Fig. 3). Therefore it could be concluded that the CPP was efficient in intracellular delivery of antiviral peptides.

**Discussion**

The complete management of human rabies needs future efforts on antiviral therapy development (Appolinario and Jackson 2015). A variety of new antiviral agents and
approaches are under development and evaluation, including favipiravir (T-705) and RNA interference. T-705 is a broad-spectrum RNA polymerase inhibitor, which has been shown to have antiviral activity against RABV, but recent studies showed its effect was limited (Banyard et al. 2017); siRNA-based silencing of target genes has been considered as one of the most promising approaches to fight against this virus, yet how to deliver the drug to the central nervous system safely and efficiently still remains in suspense (Zhu and Guo 2016).

Peptide drugs have been of great interest due to the unique advantages, such as low molecular weight, specificity, and low toxicity (Castel et al. 2009; Kaku et al. 2011). The RABV P-binding peptides screened by Real et al. (2004) provided another choice to fight against RABV, and higher activities will be the primary consideration for their further development.

QSAR is a useful tool for peptide optimization, which can not only reduce the load of experiments, but also explore the action mechanisms (Jenssen 2011). It could be seen from Table 2 that both MLR and PLS models were in good performance, indicating both methods were capable of reflecting the relationship between the peptide features and their activity well, and were suitable for peptide optimization. The peptide P16b6 had proved that by elevated activity. As for the servers to compute the peptide descriptors, both could extract the main features related to the biological activity, but due to fewer descriptors got by iFeature, the models seemed somewhat different.

It was believed that the antiviral peptides functioned by binding with the phosphoprotein to destabilize both the interaction and functionality of the lyssavirus N–P complex (Real et al. 2004), so the inhibitory activity might be determined by the binding force, which was influenced by many properties of the amino acids, such as hydrophobicity, polarity, and charge. Docking analysis showed the peptides could bind to the big pocket of the phosphoprotein, which included several helixes, besides 2 Lys residues on the second helix, there were many hydrophobic amino acids on helix 2 to 6, so substituting Ser17, Cys21 and Ile27 of P16 for Ile17, Glu21 and Trp27 could improve both the hydrophobic and the electrostatic interaction between the peptide and the phosphoprotein, which should account for the activity improvement of P16b6, whereas the secondary structure of the peptide was still in a random coil form mingled with a short helix, which didn’t seem to be related to the activity of the peptide (Fig. 4).

Due to the poor permeability, how to use the peptides intracellularly is another issue to be considered. Arginine-rich segments have been proved to be able to mediate the transmembrane process of a peptide (Bolhassani et al. 2017; Castel et al. 2009; Kristensen and Nielsen 2016; Tashima 2017). In this work, the CPP ‘RRRRRRRRR’ was effective in transferring the inhibitory peptides into cells.

Figure 3 showed that at low concentrations the antiviral effect of the two peptides had no obvious difference, while
at high concentrations P16b6 was more active than P16, and better antiviral effect depended on higher dosages of peptides, which indicated the inhibitory activity was related not only to the binding force, but also to the amount of peptide in the cell. High concentration might lead to side-effects, such as hemolysis (data not shown), to further improve the activity of the peptide and at the same time to decrease the side-effects should be considered in the future study. Another way to utilize these peptides is to express them in the host cells, provided that a safe and efficient delivery system is available. Alternatively, the optimized peptides can be used as drug leads for nonpeptide design (Real et al. 2004).

### Table 3 Derivatives of P16 and their predicted activity

| Derivatives | Sequences | Predicted activity |
|-------------|-----------|--------------------|
| P16a1       | PPDVHFPPHALWRLHLIRLVCLVRMWFH | 2.75724 |
| P16a2       | PPDVHWPPHALWRLHLIRLVCLVRMWFH | 2.74093 |
| P16a3       | PPDVHTPPHALWRLHLIRLVCLVRMWFH | 2.70629 |
| P16a4       | PPDVHTPPHALWQLIRLVCLVRMWFH  | 2.70447 |
| P16a5       | PPDVHTPPHALWRLIRLVDELVRMWFH  | 2.67841 |
| P16a6       | PPDVHTPPHALWRLHLIRLVELVRMWFH | 2.76685 |
| P16a7       | PPDVHTPPHALWRLHLIRLVILVRMWFH | 2.72462 |
| P16a8       | PPDVHTPPHALWRLIRLVKLVLMWFH  | 2.70661 |
| P16a9       | PPDVHTPPHALWRLHLIRVQLVRMWFH  | 2.74579 |
| P16a10      | PPDVHTPPHALWRLHLIRLVELRMWFH  | 2.68142 |
| P16b1       | PPDVHFPPHALWRLHLIRLVCLVRMWWH | 2.75724 |
| P16b2       | PPDVHWPPHALWRLHLIRLVCLVRMWWH | 2.74093 |
| P16b3       | PPDVHTPPHALWRLHLIRLVKLVRMWWH | 2.70629 |
| P16b4       | PPDVHTPPHALWRLHLIRLVQVLVRMWWH | 2.70447 |
| P16b5       | PPDVHTPPHALWRLHLIRLVDELVRMWWH | 2.67841 |
| P16b6       | PPDVHTPPHALWRLHLIRLVELVRMWWH | 2.76685 |
| P16b7       | PPDVHTPPHALWRLHLIRLVILVRMWWH | 2.72462 |
| P16b8       | PPDVHTPPHALWRLHLIRLVKLVLMWWH | 2.70661 |
| P16b9       | PPDVHTPPHALWRLHLIRVQLVRMWWH  | 2.74579 |
| P16b10      | PPDVHTPPHALWRLHLIRLVCLVRMWWH | 2.68142 |

The activity was predicted with the PLS QSAR model ($R^2 = 0.994, Q^2 = 0.937, Q^2_{ext} = 0.981$)

**Fig. 2** Docking analysis of the peptides and the phosphoprotein. a Docking result of P16 and the phosphoprotein, the docking energy score was -205.75; b Docking result of P16b6 and the phosphoprotein, the docking energy score was -224.56; Docking analysis was performed by HDOCK server, the yellow part indicated the peptide, the brown part indicated the phosphoprotein (Color figure online)

**Fig. 3** Antiviral activity comparison of P16 and the derivative P16b6. The cultured BSR cells were infected with RABV strain CVS, the peptides were delivered into the cells by cell penetrating peptide (CPP), and their antiviral activity was compared by testing the viral titers. The plot was produced by Microsoft Excel 2007
Conclusions

Stable and predictable QSAR models were built, which were proved to be useful for anti-RABV peptides design, and these peptides mediated by CPP could inhibit RABV intracellularly. Above all, this study provided an effective way for development of peptide drug against RABV.

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Author Contributions

LYZ designed the study and wrote the manuscript, CLY built the models, LJ checked and polished the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval No human or animal subjects were involved in the experiments, so an ethical approval was not required.

Informed Consent Informed consent was not required in this study.

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Fig. 4 Binding of P16b6 with the phosphoprotein. The labeled amino acid residues on the peptide were replaced ones, those on the second helix of phosphoprotein were lysine residues. Docking analysis was performed by HDOCK server, and the result was visualized via Swiss-PdbViewer
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