https://doi.org/10.17952/37EPS.2024.P1042

Preclinical Development of Myrcludex B Derivatives: Physicochemical Properties

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INTRODUCTION

When developing novel drugs, preclinical characterization is required to narrow down the number of lead candidates. For this purpose, several physicochemical properties have to be evaluated and compared.

About 5% of the 296 million people chronically infected with Hepatitis B (HBV) are estimated to be coinfected with the satellite virus Hepatitis D (HDV). HDV is a defective virus that is dependent on the envelope proteins of HBV to infect liver cells[1] (Fig. 1). Eventually, chronic HDV coinfection will cause more severe courses of disease, leading to hepatocellular carcinoma or liver cirrhosis.

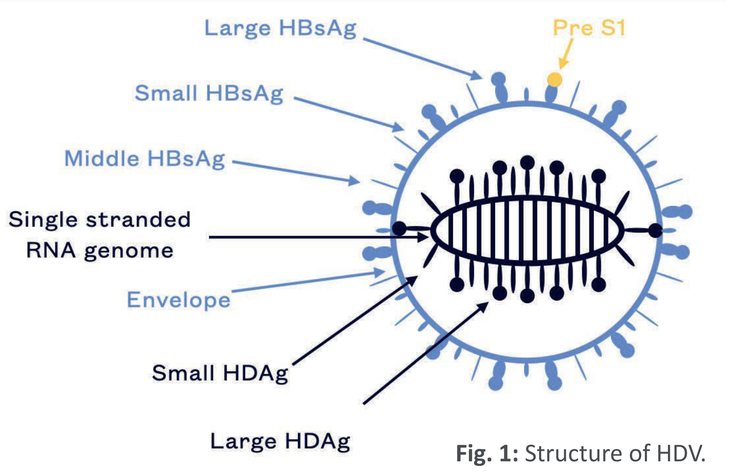

Fig. 1: Structure of HDV.

Until recently, the therapy was restricted to nucleosides and cytokines that induce antiviral response. In 2020, the first in class entry inhibitor Myrcludex B, sold under the name HepcludexTM, was approved by the FDA and the EMA. It is derived from the myristoylated pre S1 protein of the large HBsAg and consists of the first 47 amino acids. In order to further improve the treatment, derivatives of the original drug were designed and tested for their physicochemical properties as compared to the original drug.

RESULTS

LogD Determination

LogD determination revealed a wide range of logD values at varying pH values (Fig. 3). For some pH values, no logD values could be determined because the compounds did not dissolve at sufficient concentrations. In general, logD values decreased with increasing pH values. Extending over 1 log step, Myrcludex B showed the widest range of logD values.

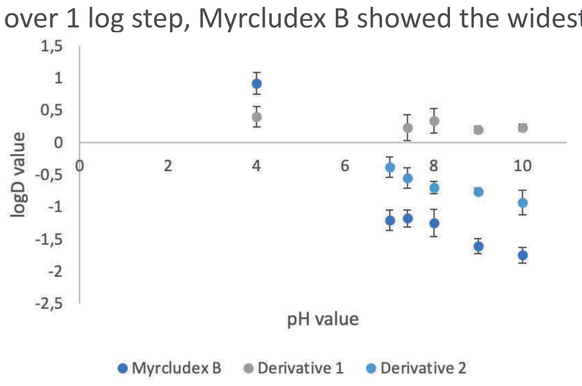

Fig. 3: Results of logD determination.

At pH 7.4, Myrcludex B was found to be the most hydrophilic compound with a logD value of -1.18 ± 0.13, Derivative 1 the most hydrophobic with logD7.4 0.23 ± 0.20. Derivative 2 was in between those two but closer to Myrcludex B with -0.55 ± 0.16. Because all compounds indicate to be in general more hydrophilic in behavior at pH 7.4, it is suggested that they will probably show poor permeation across biological membranes when administered orally.

Isoelectric Focusing

For the determination of the pIs, a calibration curve was generated from marker components with known pIs. This resulted in a linear equation used to calculate the pIs of Myrcludex B and the two derivatives. Additionally, the buffer used to dissolve the samples was applied in order to exclude interference (Fig. 4). Because Myrcludex B resulted in several pI values, three pIs were calculated representing the highest and lowest measurable pIs and middle pI. The calculated pIs distribute between 5.99 ± 0.09 and 4.35 ± 0.03 with a calculated mean of 5.17 ± 0.04. The pIs were calculated to be 5.95 ± 0.16 for Derivative 1 and 5.27 ± 0.07 for Derivative 2. Concluding, all three examined compounds are slightly acidic but the pIs of Myrcludex B and Derivative 2 are more alike to each other than the pI of Derivative 1.

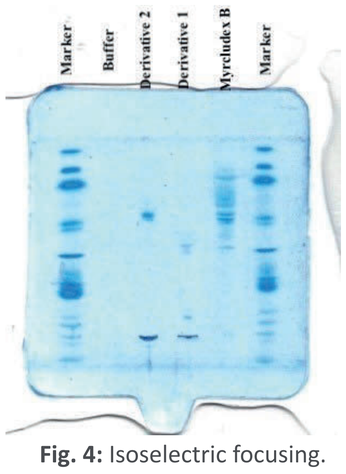

Fig. 4: Isoselectric focusing.

Serum Stability

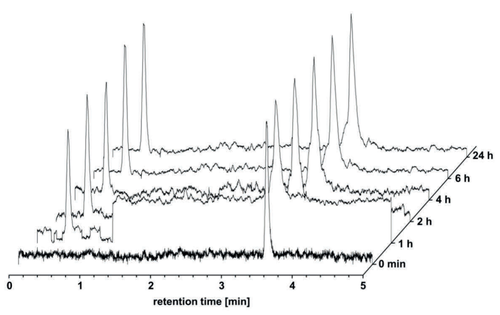

Fig. 5: Myrcludex B serum stability results.

Serum stability was determined using 125I-labeled compounds. All three compounds show high stability for 24 h without emerging side peaks (Fig. 5). The measurements for Myrcludex B are shown representatively. The first peak represents free 125I, which the samples were not purified from before the measurements, and the second peak represents Myrcludex B itself.

Protein Binding

Determination of protein binding of Myrcludex B or its derivatives proved to be quite difficult. In methods that include membrane separations, Myrcludex B notoriously clogs the membranes, prohibiting the compound from passing and distributing according to its albumin affinity. Precipitation of the serum proteins with acetonitrile delivered unstable results when measuring the remaining proportions of compound in the supernatant via HPLC. The magnetic beads and ultracentrifugation provided the most recent approaches, with the latter being the most promising one. However, these methods are still in their validation steps and so far, no conclusive experiments with either Myrcludex B or the derivatives could be done. The correct ratios of serum and compound have still to be determined and especially Myrcludex B showed to precipitate, when centrifuged with albumin, in several pre-trails.

METHODS

LogD Determination

Buffers with different pH values were produced and saturated with octanol. Myrcludex B and the two derivatives chosen were dissolved in the different buffers at a concentration of 0.5 mg/ml. Afterwards, the water-saturated octanol was added in a 1:1 ratio. The experiments were performed according to the OECD prescription[2] with some improvements:

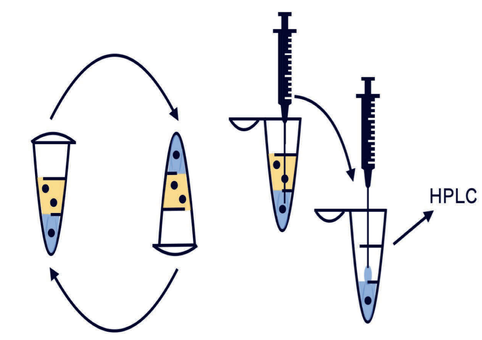

Fig. 2: LogD determination workflow.

1. No different solvent ratios were used
2. The microcentrifuge tubes were inverted for 45 ± 15 min, briefly centrifuged and allowed to stand for about 30 min (Fig. 2).
3. The octanol phase was discarded, and a small amount of the buffer phase taken using a syringe in order to avoid contamination of the sample with octanol.
4. The buffer phase was measured via HPLC because the signal-to-noise ratio was better than the one of the octanol phase.

Isoelectric Focusing

A separation and control unit was used to determine the isoelectric points (pI) of Myrcludex B and its two derivatives, together with pH gradient gels that separate samples according to their pI between 3 and 9.

The samples were dissolved in phosphate buffer pH 7.4 with a concentration of 0.5 mg/ml and 100 µL of the dissolved samples were loaded onto the gel together with a 1:5 diluted marker that consists of different substances with a known pI.

The experimental set up was cooled to 15 °C and after the electrical parameters reached the desired magnitude, the applicator, that was placed closest to the cathode, was automatically applied to the gel. One run lasted about 1.5 h and was completed, when 500 Vh were reached. Afterwards, the gels were stained with a Coomassie brilliant blue solution.

Serum Stability

Myrcludex B and the two derivatives were dissolved in phosphate buffer at pH 8.5 and labeled with 125I. Subsequently, they were incubated with human AB serum and measurements were made at incubation times of 1, 2, 4, 6 and 24 h. For this purpose, the serum proteins in the samples were precipitated with acetonitrile and the supernatant was measured by HPLC.

Protein Binding

To determine protein binding, several different approaches were pursued:

1. The first method tried was ultrafiltration. Filters sizes of 30 kDa and 100 kDa were used. As a pre-trail, 1 mg/ml Myrcludex B was centrifuged without serum, but this method had to be discontinued because Myrcludex B clogged the membranes.
2. The second approach contained dialysis. For this purpose, a chamber was manufactured that consisted of two halves between which a membrane could be placed. Into one half, the serum was pipetted, while the other half contained buffer with Myrcludex B or one of its derivatives. After a certain incubation time, a sample was supposed to be taken from the chamber containing the buffer and measured by HPLC and afterwards, the integrated area under the curve was intended to be compared to a stock solution measurement.
3. For the third approach, it was tried to precipitate the serum proteins with acetonitrile and measure the amount of Myrcludex B or the derivatives in the supernatant, compared to a stock solution.
4. The fourth approach included the use of magnetic beads which were coated with anti-HSA antibodies. The beads were first treated with 1% casein solution in order to block unspecific binding sites for Myrcludex B or its derivatives. Then, a saturating concentration of HSA was added and incubated, before Myrcludex B was added gradually. After each addition, a sample was taken and measured by HPLC. When the integrated areas under the curve were added to a graph, the slope should be greater after the saturation point was reached. Using this saturation point, the protein binding should have been calculated.
5. The last approach employed ultracentrifugation. HSA was diluted with phosphate buffer pH 7.4 and Myrcludex B and its derivatives were dissolved at 1 mg/ml. Centrifugation lasted 17 to 18 hours and a sample was taken from the top 1 ml after centrifugation, measured by HPLC and compared to the measurement of the stock solution.

SUMMARY

Several physicochemical properties of the first in class entry inhibitor Myrcludex B and two derivatives were determined. LogD determination showed mostly a decreasing behavior with increasing pH value. At pH 7.4, Myrcludex B and Derivative 2 were hydrophilic, while Derivative 1 was hydrophobic. Calculating the pIs revealed them to be slightly acidic, with Myrcludex B and Derivative 2 being very close to each other. Serum stability results proved all three compounds to be stable for at least 24 h. The results of protein binding are still subject of investigation because of the high complexity of the required workflow. Further investigations must be done, and improvements must be done to achieve oral availability of Myrcludex B and its derivatives.

References

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[2] OECD (1995). Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method. OECD Publishing *Section 1.*

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