Unfractionated heparin inhibits live wild-type SARS-CoV-2 cell infectivity at therapeutically relevant concentrations.

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Abstract

Background and Purpose: Currently there are no licensed vaccines and limited antivirals for the treatment of COVID-19. Heparin (delivered systemically) is currently being used to treat anticoagulant anomalies in COVID-19 patients. In addition, in the UK, nebulised unfractionated heparin (UFH) is currently being trialled in COVID-19 patients as a potential treatment. A systematic comparison of the potential antiviral effect of various heparin preparations on live wild-type SARS-CoV-2, in vitro, is thus urgently needed. Experimental Approach: A range of heparin preparations both UFH (n=4) and low molecular weight heparins (LMWH) (n=3) of porcine or bovine origin were screened for antiviral activity against live SARS-CoV-2 (Victoria/01/2020) using a plaque reduction neutralisation assay and Vero E6 cells. ND50 values for each heparin were calculated using a mid-point probit analysis. Key Results: UFH had potent antiviral effects, with ND50 values of 12.5 and 23 μg/ml for two porcine mucosal UFH tested. Bovine mucosal UFH had similar antiviral effects although it was ˜50% less active (ND50, 50-75 μg/ml). In contrast, LMWHs such as Clexane and Fragmin were markedly less active by ˜100-fold (ND50 values of 2.6-6.8 mg/ml). Conclusions and Implications: This comparison of a panel of clinically relevant heparins, including the UFH preparation under trial in the UK, demonstrated that distinct products exhibit different degrees of antiviral activity against live SARS-CoV-2. Porcine mucosal UFH has the strongest antiviral activity followed by bovine mucosal UFH, whereas LMWHs had the lowest amount of antiviral activity (by 100-fold). Overall the data strongly support further clinical investigation of UFH as a potential treatment for patients with COVID-19.

1 INTRODUCTION

Currently there are no licensed vaccines and limited antivirals for the treatment of COVID-19. Repurposing existing clinical drugs with proven safety profiles provides a rapid approach to address this gap in treatment. Throughout the COVID-19 pandemic anticoagulant drugs such as unfractionated heparin (UFH) and low molecular weight heparin (LMWH) delivered systemically, have been widely used across the world as part of standard treatment for patients in intensive care units (ICU). Use of these drugs has been shown to be effective for dealing with coagulopathies seen during the late stage of the disease (Kollias, Kyriakoulis et al., 2020; Tang, Li et al., 2020). Furthermore, heparin exhibits a wide range of anti-inflammatory properties (Mulloy, Hogwood et al., 2016), thus providing an additional rationale of its clinical use to treat the hyperinflammatory response observed in patients with COVID-19.

There is a recent and growing body of evidence suggesting that UFH has antiviral properties against SARS-CoV-2, the causative agent of COVID-19. UFH has been shown to bind to the receptor binding domain (RBD) of the SARS-CoV2 spike protein, which induces a conformational change (Mycroft-West, Su et al., 2020a) and also inhibits binding of spike protein to cells (Mycroft-West, Su et al., 2020b; Partridge, Green et al., 2020). Additional work with a pseudotyped assay indicated that UFH has an IC_{50} of 0.6 μg/ml (Tandon, Sharp et al., 2020). Recent studies, performed with live SARS-CoV-2 and Vero E6 cells, were limited to one heparin brand (Celsus, USA) and showed SARS-CoV-2 virus was inhibited by 44 - 80% with 6.25 μg - 200 μg/ml of UFH*in vitro* (Mycroft-West, Su et al., 2020b). Taking these interesting results together, there is an urgent need for a systematic assessment of the antiviral activity of a variety of clinically relevant heparins, *in vitro* , with live wild-type SARS-CoV-2, to establish the relative antiviral potencies of different heparins.

Independent from the systemic use of heparin in COVID-19 patients, nebulised heparin has also been proposed as a unique and potentially effective treatment for different stages of COVID-19 disease (van Haren, Page et al., 2020). In the UK a clinical trial evaluating the effectiveness of nebulised UFH in hospitalised COVID-19 patients is currently underway (https://accord-trial.org). The strategy underpinning this treatment stems from the positive observations made, based on anticoagulant and anti-inflammatory activity, when running trials of nebulised heparin in patients with acute lung injury and related conditions (van Haren, Page et al., 2020). The delivery of an aerosolised antiviral agent directly to the lungs where SARS-COV-2 virus is known to be present (both upper and lower respiratory tract) (Schaefer, Padera et al., 2020) may help to treat the alveolar coagulopathy that is often a feature of COVID-19, reduce the hyperinflammatory response and prevent patients from developing acute respiratory distress syndrome (ARDS) and pulmonary fibrosis (PF), the major complications of coronavirus infection.

Here we report for the first time a systematic assessment of the antiviral activity of a range of heparins (UFH and LMWH) *in vitro*against live wild-type SARS-CoV-2 thereby shedding light on the antiviral potency of a range of different heparin preparations, including the nebulised UFH already undergoing clinical trials in COVID-19 patients.

2 METHODS

2.1 Heparin preparations

Clinical LMWH: Innohep (tinzaparin sodium, LEO Pharma), Clexane (enoxaparin sodium, Sanofi), Fragmin (dalteparin sodium, Pfizer) and a porcine mucosal UFH (heparin sodium, Wockhardt, UK [currently being investigated by nebulisation in a UK human clinical trial]) were investigated. Further preparations investigated were, a porcine mucosal UFH preparation (Celsus, USA) and both a bovine lung heparin (Calbiochem) and a bovine mucosal heparin (15/110, NIBSC, UK).

Specific activity for the Wockhardt and NIBSC bovine sample were measured as described in the United States Pharmacopeia general monograph for assay of heparin (U.S.P.). The molecular weights for the UFH and LMWH samples were measured as previously described (Mulloy & Hogwood, 2015). The specific activity of the clinical LMWHs was taken from the clinical product information (Clexane, 100 IU/mg; Innohep, 100 IU/mg; Fragmin 130 IU/mg). The molecular weight and specific activity for Celsus porcine mucosal heparin and bovine lung heparin were as provided by the suppliers.

2.2 Plaque reduction neutralisation assay

SARS-CoV-2 Victoria/01/2020 (Caly, Druce et al., 2020) was generously provided by The Doherty Institute, Melbourne, Australia at P1 and passaged twice in Vero/hSLAM cells [ECACC 04091501]. Whole genome sequencing was performed, on the working stock at Passage 3, using both Nanopore and Illumina as described previously (Lewandowski, Xu et al., 2019). Virus titre was determined by plaque assay on Vero E6 cells [ECACC 85020206]. Cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC) PHE, Porton Down, UK. Cell cultures were maintained at 37°C in minimal essential media (MEM) (Life Technologies, California, USA) supplemented with 10% foetal bovine serum (FCS) (Sigma, Dorset, UK) and 25 mM HEPES (Life Technologies).

All heparin compounds were diluted, 5-fold in MEM (Life Technologies) containing 1% (v/v) FCS (Life Technologies), 1x antibiotic/antimycotic (Life Technologies) and 25 mM HEPES buffer (Sigma) (PRNT media) by diluting down in a 96 well plate. Dilutions were made fresh on day of assay. SARS-CoV-2 was diluted to a concentration of 933 pfu/mL (70 pfu/75µl) in PRNT media and mixed 50:50 with heparin dilutions, in a 96-well V-bottomed plate. The plate was incubated at 37°C in a humidified box for 1 hour to allow the virus to be exposed to heparin. The neutralised virus was transferred onto the wells of a washed 24-well plate that had been seeded with Vero E6 cells the previous day at 1.5 x 10^5 cells/well. The virus/heparin mixture was left to adsorb for an hour at 37°C, then plaque assay overlay media was applied (MEM containing 1.5% carboxymethylcellulose (Sigma), 4% (v/v) FCS and 25mM HEPES buffer). After incubation at 37ºC in a humidified box, for 5 days, plates were fixed overnight with 20% (v/v) formalin/PBS, washed with tap water and then stained with methyl crystal violet solution (0.2% v/v) (Sigma) and plaques were counted. Heparin dilutions were performed in either triplicate or quadruplicate. Heparin dilutions with cells only were also run in duplicate, to determine if there was any cell cytotoxicity.

An internal positive control for the PRNT assay was run in duplicate using a sample of heat-inactivated human MERS convalescent serum known to neutralise SARS-CoV-2 (National Institute for Biological Standards and Control [NIBSC], UK).

2.3 Statistical analysis

A mid-point probit analysis (written in R programming language for statistical computing and graphics) was used to determine the amount of heparin (µg/ml) required to reduce SARS-CoV-2 viral plaques by 50% (ND_{50}) compared with the virus only control (n=10). Analysis was conducted in R (Project, 2019) and the script was based on a source script from Johnson *et al* 2013 (Johnson, Dahlgren et al., 2013).

3 RESULTS

The characteristics of the different UFH and LMWHs are detailed in Table 1. A concentration dependent relationship between the amount of heparin (µg/ml) and the antiviral activity against SARS-CoV-2 was observed (Figures 1a-f).

3.1 Unfractionated heparin

The porcine UFHs had the greatest antiviral effect against SARS-CoV-2, with Wockhardt UFH (Clinical Trial batch) having a slightly greater effect with a mean ND_{50} value of 12.5 µg/ml when compared with Celsus UFH (mean ND_{50} 23 µg/ml). The antiviral activity of bovine UFH (mucosa) was approximately three-fold lower (ND_{50}, 75 µg/ml) than porcine UFH, whereas UFH from bovine lung was approximately 50% lower (ND_{50}, 51 µg/ml).

3.2 Low molecular weight heparins

LMWHs varied in activity but were typically ˜100-fold lower in antiviral activity compared with UFHs (Table 1). Of the LMWHs, Innohep was the most potent with a ND_{50} value of 2,600 µg/ml. Clexane was the least effective with an inhibitory activity ND_{50} 6,800 µg/ml.

3.3 Effect of Molecular Weight

The antiviral activity shown by the different heparins appeared to correlate broadly with their molecular weight. Overall UFHs with the highest molecular weights ranging between 12,500 – 16,300 Da were more potent inhibitors of SARS-CoV-2 than low molecular weight heparins ranging between 4230 - 6,630 Da. Of the LMWHs the brand with highest molecular weight Innohep (6,630 Da) had the highest antiviral effect whereas Clexane (lowest molecular weight, 4,230 Da) had the weakest antiviral effects.

4 DISCUSSION

Some studies have already reported the association between treatment with systemically administered heparin and lower mortality in patients admitted to hospital with COVID-19 (Ayerbe, Risco et al., 2020; Tang, Bai et al., 2020), assumed to be a consequence of the known anticoagulant effect. Coagulopathies have

caused major problems in late stage COVID-19 disease (Kollias, Kyriakoulis et al., 2020) and the use of heparin in the treatment of COVID-19 has become part of the standard care for patients in ICU. However, the dose and type of heparin (UFH or LMWH) delivered either by the sub-cutaneous or intravenous route varies across the world. Regarding standard systemic usage, the therapeutic range for UFH is typically 0.3 - 0.7 IU/ml (˜2 - 4 μg/ml) (Hirsh, Anand et al., 2001), with peak dosing concentrations reaching ˜10 - 20 μg/ml. It is reassuring to see that the ND_{50} of 12.5 μg/ml falls within the peak dosing range suggesting that at least partial antiviral effects could be expected in this setting.

In the UK, in addition to systemic heparin use, a novel approach of delivering UFH via nebulisation directly to lungs of COVID-19 patients is undergoing evaluation in a clinical trial (by the ACCORD clinical trials platform (https://accord-trial.org/), seeking to gain benefit from the additional known anti-inflammatory activity of heparin. Nebulisation allows for the targeting of lung tissue directly and therefore impact upon the local hyperinflammatory response and alveolar coagulation resulting from SARS-CoV-2 viral load in the lung. During the UK trial UFH (Workhardt) will be administered at 25,000 IU (125 mg) every 6 h to patients. The efficiency of nebulising UFH through a high efficiency mesh nebuliser is estimated to be about 20 %, thus the delivered dose to the lung is ˜ 25mg. Assuming the normal human airway surface fluids are in the range 10 - 60 ml (Frohlich, Mercuri et al., 2016), the peak amount of UFH delivered to the lung should be ˜400 - 2500 μg/ml, though these values could be lower if diluted by increased fluid volumes as a result of pulmonary oedema. Even allowing for this, these values greatly exceed the ND_{50} of 12.5 μg/ml reported here for the same batch of Workhardt UFH as used in the current UK clinical trial. Thus, nebulisation of UFH should provide strong antiviral effects, in vivo. Importantly, inhaled UFH does not cross the bronchial mucosa; intravenous or subcutaneous routes are required for systemic delivery of heparin as an anticoagulant.

Previous work has demonstrated that LMWHs also bind to the SARS-CoV-2 (Mycroft-West, Su et al., 2020b). However, in the present study we observed that LMWHs were markedly less potent in live SARS-CoV-2 virus assays (ND_{50} values of 2.6 - 6.8 mg/ml) than UFH. Using the results reported here LMWH would appear unlikely to reach sufficient concentration to achieve significant antiviral activity for either systemic or nebulisation delivery. The typical therapeutic range for LMWH for anticoagulant therapy is ˜5 - 8 μg/ml (0.6 - 1 IU/ml). Consistent with our data, the relationship (fold difference, 295-fold) between UFH and enoxaparin seen here was similar to that observed by Tandon et al, 2020 using a pseudotyped lentivirus inhibition assay where 180-fold difference was seen (Tandon, Sharp et al., 2020). In addition, LMWHs were also observed to be less potent than UFH for inhibition of cell binding by spike protein (Partridge, Green et al., 2020). However, an important caveat is that the potency of UFH and LMWHs remain to be determined in a suitable range of human cells relevant to those affected in individuals infected with SARS-Cov-2, perhaps especially from respiratory tract tissues.

The results of the present study also suggest a dependency on molecular weight for different UFH and LMWH preparations. A positive correlation between molecular weight and antiviral activity was noted for the various porcine LMWHs (4,200 - 6,600 Da) and UFH preparations tested which supports the hypothesis that UFH is more active due to its higher molecular weight (12,500 - 16,300 Da). Consistent with these live virus data, molecular weight dependency for binding of heparin and heparan sulfate saccharides to spike protein has been observed (Liu, Chopra et al., 2020; Mycroft-West, Su et al., 2020a).

In the present study bovine mucosal UFH (ND_{50}, 75 μg/ml) had a slightly lower antiviral potency (˜three-fold) compared to porcine UFH. The potent antiviral activity seen for both porcine and bovine heparins, suggests that this property is not species dependent. Bovine UFH may provide an additional source of heparin to use during the coronavirus pandemic. Currently therapeutic UFH available in Europe and US is of porcine mucosal origin; however, owing to supply issues there is now interest, specifically in the US, employing bovine mucosal UFH as an additional source to improve the robustness of supply chains (Hogwood, Mulloy et al., 2017; Keire, Mulloy et al., 2015).

The antiviral ND_{50} data for the different UFH and LMWHs display no obvious correlation with anticoagulant activities (IU/mg), indicating that different structure-activity relationships exist for antiviral activity. Importantly, this suggests that further investigation of non-anticoagulant heparins (Cassinelli, Torri et al.,

2020; Lindahl & Li, 2020) and heparin mimetics (Guimond, Mycroft-West et al., 2020; Lindahl & Li, 2020) is warranted. Mimetics have significant potential to target similar antiviral mechanisms and could be delivered systemically at higher doses to improve efficacy without potential side effects such as bleeding. Moreover, mimetics would also provide a fully synthetic route to bypass limitations of heparin supply.

Here we provide evidence for the first time that various types of commercially available and clinically used UFH preparations exhibit potent antiviral efficacy against live wild-type SARS-CoV-2 *in vitro.* This activity was seen across different brands of UFH and was also observed with both porcine and bovine heparins. These data indicate that current clinical use of systemic UFH in the treatment of COVID-19 patients in an ICU setting may provide useful antiviral benefits. Moreover, we predict that the delivery of UFH to the lung (via nebulisation) should provide a strong direct antiviral therapy in addition to other documented beneficial effects of heparins.

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