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In vitro study of BromAc on SARS-CoV-2 spike and envelope protein shows synergy and disintegration at modest concentrations

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Abstract

Objectives

SARS-CoV-2 infection is the cause of a worldwide pandemic, currently with limited therapeutic options. It is characterised by being highly contagious and nasal mucosa appears to be the primary site with subsequent spread to the lungs and elsewhere. BromAc (Bromelain & Acetylcysteine) has been described to disrupt glycoproteins by the synchronous breakage of glycosidic linkages and disulphide bonds. The spike protein of SARS-CoV-2 is an attractive target as it is essential for binding to the ACE2 receptor in host cells and is formed of glycoprotein and disulphide bridges for stabilisation. Hence, we sought to determine whether BromAc has activity on the spike and envelope protein specific to SARS-CoV-2 virus.

Design

Gel electrophoresis analysis was carried out on recombinant spike and envelope proteins that were treated with a range of concentrations of single agents and BromAc. For UV analysis of disulfide bonds reduction, both spike and envelope protein were treated with Acetylcysteine with the determination of loss of disulfide bonds.

Results

Recombinant spike and envelope SARS-CoV-2 protein were fragmented by BromAc whilst single agents had minimal effect. Spike and envelope proteins disulphide bonds were reduced by Acetylcysteine.

Conclusion

BromAc disintegrates the spike and envelope protein from SARS-CoV-2 and may render it non-infective. In vitro tests on live virus have been encouraging and clinical testing through nasal administration in patients with early SARS-CoV-2 infection is imminent.

Keywords: SARS-CoV-2, COVID-19, Bromelain, Acetylcysteine, BromAc

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Introduction

SARS-CoV-2 is a highly infectious virus, progressing to the clinical syndrome known as COVID-19 in many. As at 7th September 2020, over 27 million people worldwide had been infected with an overall mortality of 3.26% (1). Whilst older age and comorbidities are clear risk factors, a significant number of younger adults also succumb to the disease. There are currently few therapeutic agents that have been found to provide modest benefit in early and late stage disease (2). There are many vaccine candidates, however if an effective vaccine is developed, the length of protection may be limited (3, 4). Effective treatments are still needed.

Structurally, SARS- COV-2 contains spike protein (S), nucleocapsid protein (N) that contains the RNA, membrane protein (M) and envelope protein (E). The S protein is a complex glycoprotein made of three main sub-units with different roles accomplished through dynamic conformational modifications, based on disulphide bonds (5). It allows binding to the human angiotensin-converting enzyme (ACE2) receptors (and others) which triggers proteolysis by transmembrane protease serine 2 (TMPRSS2), furin, and perhaps other proteases, leading to capsule disintegration (6, 7) with infection of human cells, where it replicates.

Bromelain is an enzyme extracted mainly from the stem of the pineapple plant (*ananas comosus*) and contains a number of enzymes that gives it the ability to hydrolyse glycosidic bonds in complex carbohydrates (8). As a therapeutic molecule, it is used for debriding burns (NexoBrid) and as a mucolytic in combination with Acetylcysteine for the treatment of mucinous tumours, such as pseudomyxoma peritonei (in clinical trial) (9, 10) and has synergistic effects with certain anticancer drugs (11). It also has anti-inflammatory, immunomodulating and antithrombotic properties (8). As a mucolytic, Bromelain hydrolyses the O-linked glycosidic bonds whilst the disulfide bonds are reduced with Acetylcysteine (12). Although, the glycoproteins in SARS-CoV-19 spike protein and envelope protein are primarily N-linked (13), bromelain can also hydrolyse these linkages since it contains a number of enzymes such as phophatase, glycosidase, cellulase etc (8).

Previous studies have indicated that bromelain removes the spike protein and haemagglutinin of Semliki Forest virus, Sindbis virus, mouse gastrointestinal coronavirus, hemagglutinating encephalomyelitis virus and H1N1 influenza viruses, rendering these non-infective (14, 15). Acetylcysteine has already been used as an anti-viral. In a murine model of lethal influenza infection, an increased survival was seen in the acetylcysteine combination with oseltamivir

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group (100% vs 60%) (16). Acetylcysteine has also been reported to inhibit replication of influenza A and B, and MUC5, IL-6 and TNF which are part of the cytokine storm (17). Acetylcysteine is already being nebulized into the lungs for cystic fibrosis and chronic obstructive pulmonary disease (COPD). The three cystine disulfide bridges that are targeted by acetylcysteine for cleavage, lead to disruption of S protein and reduction in infectivity (18).

Since the integrity of the four different proteins of SARS-CoV-2 (S, N, M & E) is vital for viral functions. The presence of cysteine residues in the glycoprotein of both S and E indicates that disulfide bonds are present suggesting that these bonds play a vital role in the geometrical arrangement of these structures and hence their biochemical or physiological role (19). Hence, reduction of these disulfide bridges in cysteines of the spike protein will affect its binding to ACE2 (20). Similarly, the ACE2 receptors of the host cells also contain disulfide bridges that when disrupted will not synchronise with S protein with internalisation of the virus and in fact the joint disruption of cysteine residues in the spike and ACE2 receptors would produce a total null effect (21).

It is important to note that the removal of the viral spike protein is an entirely different means of treatment in comparison to many of the existing antiviral drugs. It is not targeted at the viral replication machinery but rather the prevention of viral entry into the host cells. More notably, recent studies have shown that the nose contains the highest percentage of ACE2 and TMPRSS2 in the human body. The ratio for ACE2 was over 5x in the nose than the distal respiratory tract (22). The pattern of lung pathology also supported the aspiration from the nose as being the likely mode of COVID-19 infection. Hence, therapeutic methods that targets the nose may serve as a very useful tool in controlling the viral entry.

Therefore, in the current study we set out to determine whether BromAc can disrupt the integrity of SARS-CoV-2 spike and envelope proteins and subsequently aim at testing their effect on viral infection in in vitro systems. We used gel-electrophoresis to determine whether BromAc hydrolyses the spike and envelope protein and subsequently we used UV spectral determination to show that the disulfide bridges in the spike and envelope protein are reduced by Acetylcysteine. Initial studies showed complete hydrolysis of both spike and envelope protein whilst UV spectral analysis showed in addition, the reduction of disulfide bonds in these proteins suggesting that the spike and envelope protein will be denatured or deconfigured and hence their biological role will be affected.

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Methods

Materials

Bromelain API was manufactured by Mucpharm Pty Ltd (Australia) as a sterile powder. Acetylcysteine was purchased from Link Pharma (Australia). The SARS-COV-2 spike protein was obtained from SinoBiological (Cat#40589-V08B1). The envelope protein was obtained from MyBioSource (Cat#MBS8309649). SDS-PAGE precast gel from Bio-Rad (Cat#456-1095). All other reagents were from Sigma Aldrich.

Recombinant spike and envelope gel electrophoresis

The spike or envelope protein was reconstituted in sterile distilled water according to manufacturer's instructions and aliquots were frozen at -20^{0}C. Spike or envelope protein 2.5µg were placed in micro-centrifuge tubes and 50µg or 100µg/ml Bromelain plus 20mg/ml Acetylcysteine in Milli-Q water. The total reaction volume was 15µL per tube. Control contained no drug. All tubes were incubated at 37^{0}C for 30min, after which 5µl of sample buffer was added into each tube. Each well of the SDS-PAGE gel was loaded with 20µL of each processed sample described above. Protein electrophoresis was performed at 100w for 1hr. The gels were stained using Coomassie blue.

UV Spectral detection of disulfide bonds in spike and envelope proteins

SARS-COV-2 spike protein at a concentration of 3.0 µg/ml in PBS (pH7.0) containing 1mM EDTA was prepared. Two sets of 5 tubes were prepared. To one set of tubes 0, 10, 20, 40 & 50 µl of Acetylcysteine (0.5 M) was added and agitated at 37^{0}C/30min followed by equivalent addition of DTT (Dithiotretiol) (0.5 M) and agitated for further 30min at 37^{0}C. To the next set of tubes containing spike protein, only DTT (0.5M) was added as before without any Acetylcysteine, agitated at 37^{0}C for 30min. The absorbance was then read at 310 nm. UV spectral detection of disulfide bonds in envelope protein has been done in a similar manner.

Results

Disintegration of SARS-COV-2 spike and envelope proteins

Gel electrophoresis indicated that with the addition of 20 mg/ml Acetylcysteine to the spike protein, the band showed a reduction in band thickness and intensity (Figure 1A), although

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still present, indicating that the protein has been altered but not removed (Lane 2). However, with Bromelain (50 µg/ml), the band becomes very faint (Lane 3). The combination of Bromelain (50 µg/ml) with 20 mg/ml Acetylcysteine (Lane 4) shows a very faint thin band. With the addition of 100 µg/ml Bromelain, the band is very thin and faint but still present (Lane 5). In combination, Bromelain (100 µg/ml) with 20 mg/ml Acetylcysteine (Lane 6), no visible original band but faint bands at lower molecular weight are seen, indicating fragmentation of the original protein. BromAc has affected the integrity of the spike protein by disintegration in a concentration-dependent manner. The results, however, show clear evidence of joint action between the components of BromAc in the same way that we have seen in cancer [13, 14].

Treatment with Acetylcysteine on the envelope protein (Figure 1B) did not disintegrate the protein, however it extended sideways (Lane 1). Treatment with 50 µg/ml of Bromelain alone resulted in complete disintegration as shown by a very faint and almost absent band (Lane 3). A combination of Bromelain 50 µg/ml + Acetylcysteine 20 mg/ml also had a similar effect (Lane 4). Increasing the concentration of Bromelain to 100 µg/ml alone or in combination with Acetylcysteine 20 mg/ml also resulted in disintegration of the protein. The gel electrophoresis indicates that BromAc is effective in disintegrating the envelope protein of SARS-CoV-2.

UV Spectral detection to show the disintegration of disulfide bonds in spike proteins

The differential assay between Acetylcysteine and DTT for the reduction of disulfide bonds found in the spike protein indicates that in the presence of Acetylcysteine, the reduction of disulfide bonds by DTT falls by 42% indicating that Acetylcysteine reduces 42% of the disulfide bonds before the addition of DTT (Figure 2A). The remaining is reduced by DTT to produce the chromogen that is detected at 310 nm. Hence, this assay indicates that the disulfide bonds are the targets for Acetylcysteine that is found in BromAc® formulation for treating (disinfecting) the SARS-COV-2 virus.

UV Spectral detection to show the disintegration of disulfide in envelope proteins

The differential assay between Acetylcysteine and DTT for the reduction of disulfide bonds found in the envelope protein indicates that in the presence of Acetylcysteine, the reduction of disulfide bonds by DTT falls by 40% indicating that Acetylcysteine reduces 40% of the disulfide bonds before the addition of DTT. The remaining is reduced by DTT to produce the

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chromogen that is detected at 310 nm. Hence, this assay indicates that the disulfide bonds in the envelope protein are the targets for Acetylcysteine that is found in BromAc formulation for treating (disinfecting) the SARS-COV-2 virus (Figure 2 B).

Discussion

SARS-Cov-2 is a highly infectious virus that is currently causing a worldwide pandemic. Prospective, several randomised clinical trials are undergoing of repurposed drugs including remdesivir, lopinavir, umifenovir, arbidol, nitazoxanide favipiravir, baricitinib, ritonavir, hydroxychloroquine, oseltamivir, baloxavir, tocilizumab, azithromycin, losartan, and ribavirin to treat the SARS-CoV-2 infection [2]. Unfortunately, regardless of these efforts, there remains no real effective treatment for the SARS-Cov-2 infection. Hence, there is an urgent need for the development of new or other repurposed drugs and perhaps new therapeutic strategies that aim to nullify SARS-CoV-2 infectivity.

The spike protein is the cornerstone of virion binding to host cells and hence, it is an ideal therapeutic target. The electrophoresis analysis showed very effective action of BromAc in disintegrating the spike protein. A study of spike's amino acid sequence identified some predetermined sites where BromAc could preferably act, the S2' sites rich in disulphide bonds and with abundant N-glycosylation (23). This suggests that the viral inactivation observed after BromAc application is linked to a spike protein alteration. The main difference in amino acid sequence between SARS-CoV-2 and previous beta coronaviruses is the inclusion of a furin cleavage site between S1 and S2 domains. Three disulphide bonds were identified in the SARS-CoV spike receptor binding domain (24). Considering the high homology between the amino acid sequences of the two beta coronaviruses, notably regarding cysteine position (23), we presume these stabilising covalent linkages are still present, and of importance in SARS-Cov-2 spike function. The receptor binding domain on the spike is notably the basis of the ACE2 receptor binding.

We developed a formulation consisting of Bromelain and Acetylcysteine (BromAc) that has shown anticancer and mucolytic properties and is currently undergoing clinical evaluation for the treatment of peritoneal malignancies (9, 10). As Bromelain has the capacity to hydrolyse glycosidic linkages in protein molecules and Acetylcysteine has reductive potential that effectively disrupts disulfide bonds, these two agents have tremendous effect on rearranging

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the molecular geometry of proteins. Both drugs have already been shown to affect viral proteins (14-17).

SARS-Cov-2 infects the respiratory tract cells by fusion of the spike protein with the ACE2 receptor, it is conceivable that targeting of the spike protein will essentially disrupt its fusion and ultimately its infective potential. Our first study on the spike protein using gel-electrophoresis showed that the proteins were hydrolysed into fragments. Subsequent study using UV spectroscopy to investigate the reductive action of Acetylcysteine indicated that it reduces the disulfide bonds found within cysteine residues in the spike protein. The results indicated that BromAc can affect the molecular geometry of the spike protein that contains essential domains S1 and S2, which are vital for fusion after binding to the ACE2 receptors. Further investigation on the envelope protein indicated a similar result, that BromAc disintegrated the protein. The stability of spike protein depends on the integrity of the envelope protein and hence the alteration of the envelope protein may affect the spike protein and its biological significance.

Recent literature has suggested a potential for early nasal-directed treatment for managing SARS-CoV-2. Hou et al. suggested that the first site of infection is nasopharyngeal mucosa with a secondary movement to infect lung by aspiration (25). The authors showed a gradient receptor expression with levels decreasing from the upper respiratory tract to the alveolar tissue. The pattern of infectivity of the respiratory tract cells followed ACE2 receptor expression. The nasal region exhibited higher proportion of ciliated, ACE2 positive and MUC negative cells together with a higher infectivity by SARS-CoV-2 when compared to lower tract epithelium (25).

We do have very encouraging live virus results with VERO and BGM indicating inhibition of infectivity of virus at similar concentration which will be reported shortly in a separate publication. Preliminary animal and clinical safety data for airway administration of BromAc is encouraging. However, further investigation into both the prevention and therapeutic potential of BromAc using live virus in systems with Vero and Calu-3 are required before assessing the efficacy of the agent.

In particular, Calu-3 experiments are important because the entry of virus into these cells is TMPRSS2 dependent, as in airway epithelium. In contrast, Vero cell entry is Cathepsin L dependent. In addition, studies on cells that have already been infected with SARS-CoV-2,

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rather than pre-treatment replicating a prophylactic treatment, are being conducted. Target cells membranous protease activity is a key parameter of spike modification after binding allowing the virus entry, currently with a serine-like protease. A further understanding of the mechanism of action, namely the effect on ACE2 receptor and other molecules helping virion entry into host cells (TMPRSS2, ADAM17, cathepsin L, CD147, and GRP78) is also required. Whilst studies to determine the safety of BromAc as a nasal delivery agent are incomplete, the lowest active concentration investigated was 50µg/ml in these in vitro studies, which is more than 10 times lower than that used in our cancer treatment with safety and efficacy (10). Initial studies with Vero cells are promising, showing effective control of virus entry into with BromAc, however, further evaluations are currently being conducted. Hence, there seems to be some strong indication that BromAc can be developed into an effective therapeutic agent for the treatment of COVID-19.

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Disclosures & conflicts of interest

Professor David Morris is the co-inventor and assignee of the Licence for this study and director of the spin-off sponsor company, Mucpharm Pty Ltd. Dr Javed Akhter, Dr Krishna Pillai and Dr Ahmed Mekkawy are employees of Mucpharm Pty Ltd. Miss Sarah Valle is partly employed by Mucpharm for its cancer development and is supported by an Australian Government Research Training Program Scholarship.

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Figure Legends

Figure 1. A) *The recombinant SARS-CoV-2 spike protein (red arrow) S1+S2 subunits treated with Bromelain or Acetylcysteine alone or BromAc® combinations.* ***B)*** *The recombinant SARS-CoV-2 envelope protein (red arrow) treated with Bromelain or Acetylcysteine alone or BromAc® combinations.*

Figure 2 A). *The percentage reduction of disulfide bonds by Acetylcysteine: 162/384 (100) was 42%. The differential assay between Acetylcysteine and DTT for the reduction of disulfide bonds found in the spike protein indicates that in the presence of Acetylcysteine (Ac), the reduction of disulfide bonds by DTT falls by 42% indicating that Acetylcysteine reduces 42% of the disulfide bonds before the addition of DTT. The remaining is reduced by DTT to produce the chromogen that is detected at 310nm. This assay indicates that the disulfide bonds are targets for BromAc®.* ***B)*** *In the envelope protein the reduction of disulfide bonds by DTT fall by 40% indicating that Acetylcysteine in BromAc reduces 40% of the bonds in the cysteine residues.*

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Fig. 1

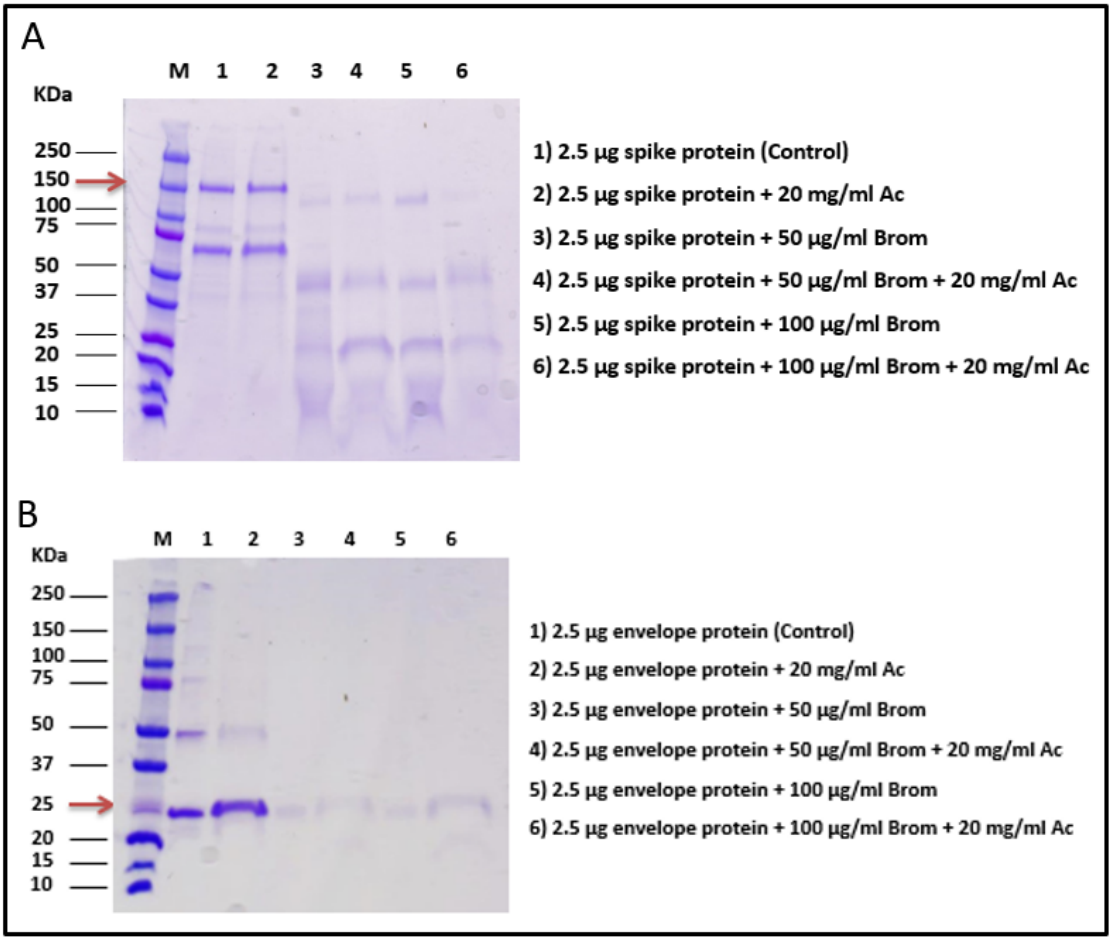

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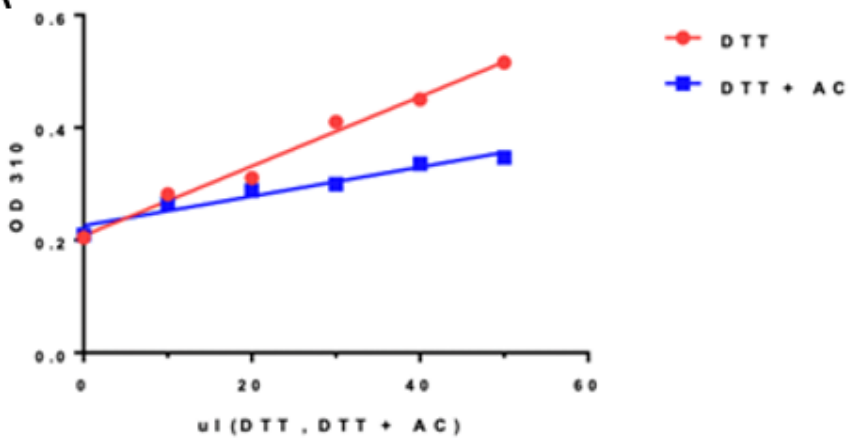
Fig. 2

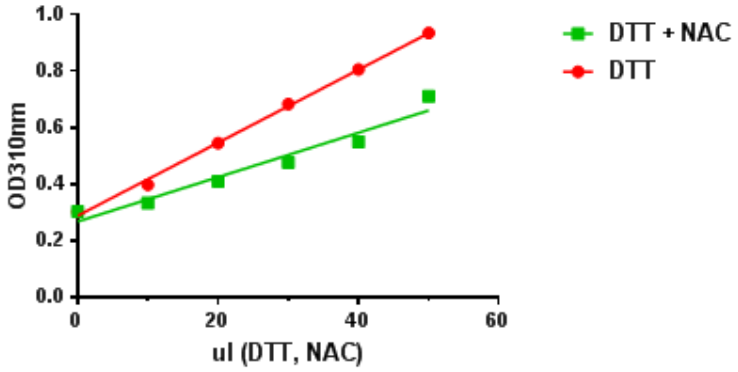
A



	DTT	DTT + AC
Best-fit values ± SE		
Slope	0.006171 ± 0.0003581	0.002599 ± 0.000309
Y-intercept	0.2082 ± 0.01084	0.2261 ± 0.009354
X-intercept	-33.74	-87.02
1/slope	162	384.8

B

Reduction of disulfide bonds in envelope protein by DTT and NAC



	DTT	DTT + NAC
Best-fit values ± SE		
Slope	0.01293 ± 0.0003048	0.007866 ± 0.0009175
Y-intercept	0.2885 ± 0.009228	0.2679 ± 0.02778
X-intercept	-22.31	-34.05
1/slope	77.34	127.1

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