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Virucidal action of sore throat lozenges against respiratory viruses parainfluenza type 3 and cytomegalovirus

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ABSTRACT

Most respiratory tract infections are self-limiting and caused by viruses, and do not warrant antibiotic treatment. Despite this, patients with respiratory tract infections often receive antibiotics, fuelling the rise of antibiotic resistance. Therefore, there is a need to encourage patients to try alternative non-antibiotic therapies, which ideally treat the symptoms and the cause. Lozenges containing amylmetacresol and 2,4-dichlorobenzyl alcohol (AMC/DCBA lozenges) as well as lozenges containing hexylresorcinol have been shown to provide effective symptomatic relief for sore throat. In this study, we investigated whether these lozenges also have virucidal effects *in vitro* against two viruses associated with respiratory tract infections, parainfluenza virus type 3 and cytomegalovirus. Both viruses were incubated with AMC/DCBA lozenge, placebo lozenge or the active ingredients (AMC/DCBA) as free substances, and parainfluenza virus type 3 was incubated with hexylresorcinol lozenge, placebo lozenge or hexylresorcinol as a free substance. Virucidal effects were observed with the active lozenges and the active ingredients as free substances against both parainfluenza virus type 3 and cytomegalovirus. Mean reductions in viral titre were significantly greater compared with placebo lozenge and peak effects were observed for the shortest incubation time, 1 min. These findings suggest that AMC/DCBA lozenge and hexylresorcinol lozenge have the potential to have local antiviral effects in patients with sore throat due to viral respiratory tract infections. Use of such over-the-counter treatments for self-limiting respiratory tract infections may satisfy patients' desire for an anti-infective medication and reduce the demand for antibiotics.

1. Introduction

Respiratory tract infections (RTIs) are the most common illnesses to affect humans (Denny, 1995). Typical symptoms include sore throat, rhinitis, cough and fever (Dasaraju and Liu, 1996; Eccles, 2007). Most RTIs are caused by a viral infection (Denny, 1995), for example, the symptom of sore throat is reported to be caused by viruses in 85–95% of cases in adults, 95% in children under 5 years and 70% in children aged 5–15 years (Worrall, 2011). Viruses associated with RTIs include orthomyxoviruses (influenza), paramyxoviruses (parainfluenza viruses [PIV], respiratory syncytial virus [RSV]), coronaviruses, picornaviruses, adenoviruses and herpes viruses (cytomegalovirus [CMV], Epstein–Barr virus [EBV]) (Collier and Oxford, 2000).

Although RTIs are generally self-limiting and have a predominantly viral cause, patients visiting healthcare professionals often expect antibiotics (or are perceived to expect antibiotics) and receive inappropriate antibiotic treatment (van der Velden et al., 2013). In Europe, one-quarter of those who took antibiotics in the last year did so to provide symptom relief, and there remains much consumer confusion about the effects of antibiotics with 49% of Europeans falsely believing that antibiotics kill viruses and 41% believing they were effective against colds and flu (European Commission, 2013). Physicians respond to sore throat patients' demands (actual and perceived) by prescribing an antibiotic in approximately 60% of cases (Barnett and Linder, 2013; Gulliford et al., 2014) but only about 5–15% of sore throats in adults are caused by a bacterial infection (Shulman et al., 2012).

Abbreviations: AMC, amylmetacresol; ASTM, American Society for Testing and Materials; ATCC, American Type Culture Collection; CMV, cytomegalovirus; DCBA, 2,4-dichlorobenzyl alcohol; EBV, Epstein–Barr virus; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MEM, minimum essential medium; PIV, parainfluenza virus; PIV3, parainfluenza virus type 3; RPMI, Roswell Park Memorial Institute; RSV, respiratory syncytial virus; RTI, respiratory tract infection; SARS-CoV, severe acute respiratory syndrome coronavirus; $TCID_{50}$, tissue culture infectious dose 50%.

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This unnecessary use of antibiotics drives the development of antibiotic resistance (Goossens et al., 2005), which is an increasingly serious threat to global public health (WHO, 2014). Therefore, there is a need to encourage patients with RTIs to try alternative treatments, while antibiotics should be reserved for patients with a serious illness or those at increased risk of complications (Essack and Pignatari, 2013).

An ideal treatment would provide the symptomatic relief that patients seek as well as treat the cause. Locally delivered formats such as lozenges and sprays are useful as they enable active ingredients to reach the site of infection directly; the localized delivery means that side effects are lower compared with systemically acting treatments (Farrer, 2011). Lozenges containing the antiseptics and local anaesthetics amylmetacresol (AMC) and 2,4-dichlorobenzyl alcohol (DCBA) or hexylresorcinol have been developed to treat the symptoms of sore throat (Buchholz et al., 2009; Foadi et al., 2014; McNally et al., 2010, 2012; Wade et al., 2011). These lozenges have demonstrated statistically significant reductions in sore throat symptoms in placebo-controlled clinical trials (McNally et al., 2010, 2012; Wade et al., 2011). AMC/DCBA lozenges have demonstrated antibacterial effects *in vivo* (Richards et al., 1989) and *in vitro* (Richards and Xing, 1993). AMC/DCBA lozenges have also been shown to have some virucidal effects *in vitro* on three enveloped viruses – RSV, influenza A virus and severe acute respiratory syndrome coronavirus (SARS-CoV) (Oxford et al., 2005).

This study investigated the *in vitro* virucidal activity of AMC/DCBA alone and in a lozenge on two other enveloped viruses that can cause sore throat and respiratory illness – PIV type 3 (PIV3) and CMV (Bisno, 2001). It also assessed the *in vitro* virucidal activity of hexylresorcinol alone and in a lozenge on PIV3. To our knowledge, this is the first study to examine the virucidal effects of throat lozenges on these viruses.

2. Material and methods

The American Society for Testing and Materials (ASTM) international standard method E1052-11 for "Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension" was followed.

The challenge viruses, PIV3 (strain C243 ATCC VR-93) and CMV (strain ATCC-2011-8 ACTT VR-1788) were obtained from the American Type Culture Collection (ATCC). PIV3 was propagated in Vero cells (ATCC CCL-81) and CMV in MRC-5 cells (ATCC CCL-171). The test substances were honey and lemon AMC/DCBA lozenge, cherry menthol hexylresorcinol lozenge, placebo lozenge, AMC/DCBA as free active substances and hexylresorcinol as a free active substance (Table 1). A positive control (sodium hypochlorite) and negative control (dilution medium) were also tested.

A 2.7 mL aliquot of each test substance (Table 1) was transferred into a 50 mL conical tube. Then 0.3 mL of the virus stock (titre of 10^6–10^8 $TCID_{50}$/mL for PIV3 and $10^{5.5}$–$10^{7.5}$ $TCID_{50}$/mL for CMV) was added and mixed immediately (by vortexing) at ambient room temperature (21 °C). Different contact times (1, 5 or 10 min) were used for each test (Table 1). Upon completion of the contact time, the reaction mixture was immediately mixed (by vortexing) with an equal volume of neutralizer (RPMI medium + 10% newborn calf serum + 1% HEPES + 1% $NaHCO_3$ for the AMC/DCBA and PIV3 experiments, MEM for the hexylresorcinol and PIV3 experiments and MEM + 10% fetal bovine serum + 1% HEPES + 1% $NaHCO_3$ for the AMC/DCBA and CMV experiments). The quenched sample was serially diluted 10-fold with dilution medium. Serial dilutions were inoculated onto host cells in a 24-well plate (1 mL inoculum per well, $n = 4$ per dilution). The inoculated plates were incubated at 36 ± 2 °C in 5 ± 1% CO_2 for 7 days for the AMC/DCBA and PIV3 experiments, 5–9 days for the hexylresorcinol and PIV3 experiments and 14 days for the AMC/DCBA and CMV experiments. After the incubation period, the titre of infectious virus was determined by $TCID_{50}$ assay using the Spearman-Karber formula. The results from the negative control was used as the input viral load and compared with the test substances to evaluate the viral reduction by each test substance. Each experiment (for each test substance, each control and each contact time) was run in triplicate. The mean reductions in viral titre were compared between the test substances and the placebo lozenge using the two-sided Student *t*-test.

Additional control tests were also conducted to assess the effectiveness of the neutralizer and cytotoxicity of the test substances. A 2.7 mL aliquot of the test substance (each lozenge and each combination of free active substances) was mixed with 0.3 mL of dilution medium, incubated at ambient room temperature (21 °C) for each contact time, and then an equal volume of neutralizer was added. Following serial dilution of the reaction mixture in dilution medium, 100 µL of a low titred virus stock (containing no more than approximately 5000 units of virus) was added to 4.5 mL of each dilution and incubated at ambient room temperature (21 °C) for at least 10 min. These were then inoculated onto the host cells which were assessed for the presence of infectious virus at the end of the incubation period. To evaluate cytotoxicity, the

Table 1
Test substances and controls.

Test substance	Contact time
AMC/DCBA lozenge Strepsils® Honey and Lemon lozenge, containing 0.6 mg AMC and 1.2 mg DCBA, dissolved in 4.5 mL of artificial saliva* Lozenge ingredients: AMC, DCBA, peppermint oil, terpeneless lemon oil, honey, tartaric acid, liquid glucose, liquid sugar, quinoline yellow	1, 5 and 10 min
Placebo lozenge for AMC/DCBA experiments Placebo lozenge, dissolved in 4.5 mL artificial saliva* Lozenge ingredients: peppermint oil, terpeneless lemon oil, honey, tartaric acid, liquid glucose, liquid sugar, quinoline yellow	1 and 10 min
AMC/DCBA as free active substances 0.6 mg AMC and 1.2 mg DCBA dissolved in 4.5 mL artificial saliva*	1 and 10 min
Hexylresorcinol lozenge Strepsils Extra Cherry lozenge, containing 2.4 mg hexylresorcinol and 4 mg menthol, dissolved in 4.5 mL of artificial saliva* Lozenge ingredients: hexylresorcinol, carmoisine edicol (dye), cherry flavour, menthol, liquid glucose, liquid sugar	1, 5 and 10 min
Placebo lozenge for hexylresorcinol experiments Placebo lozenge, dissolved in 4.5 mL artificial saliva* Lozenge ingredients: carmoisine edicol (dye), cherry flavour, menthol, liquid glucose, liquid sugar	1 and 10 min
Hexylresorcinol as a free active substance 2.4 mg hexylresorcinol as a free active substance, dissolved in 4.5 mL artificial saliva*	1 and 10 min
Positive control Sodium hypochlorite, 1000 ppm (1500 ppm for the hexylresorcinol experiments)	1 min
Negative control Dilution medium (MEM + 3 µg/mL trypsin for the PIV3 experiments and MEM + 5% fetal bovine serum for the CMV experiments)	10 min

AMC, amylmetacresol; CMV, cytomegalovirus; DCBA, 2,4-dichlorobenzyl alcohol; MEM, minimum essential medium; PIV3, parainfluenza virus type 3.

* 4.2 g sodium bicarbonate, 0.5 g sodium chloride, and 0.2 g potassium carbonate was added to ~900 mL of sterile deionized water. 3 g of bovine serum albumin was added to the solution and the pH adjusted to 6.5 ± 0.5. Sterile deionized water was added to reach a total volume of 1000 mL.