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Medical Hypotheses 144 (2020) 110027

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Why the lower reported prevalence of asthma in patients diagnosed with COVID-19 validates repurposing EDTA solutions to prevent and manage treat COVID-19 disease

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A B S T R A C T

There currently is no specific antiviral drug or a vaccine for SARS-CoV-2/COVID-19 infections; now exceeding 10,300,000 infections worldwide. In the absence of animal models to test drugs, we need to find molecular explanations for any unforeseen peculiarities in clinical data, especially the recent reports describing an unexpected asthma paradox. Asthma is considered a high medical risk factor for susceptibility to SARS-CoV-2/COVID-19 infection, yet asthma is not on the list of top 10 chronic health problems suffered by people who died from SARS-CoV-2/COVID-19. Resolving this paradox requires looking beyond the binary model of a viral receptor-binding domain (RBD) attaching to the ACE-2 receptor. A NCBI pBlast analysis revealed that the SARS-CoV-2 surface spike protein contains key two calcium-dependent fusion domains that are almost identical to those that were recently discovered SARS-CoV-1. These viral calcium-dependent binding domains can facilitate membrane fusion only after cleavage by the host surface protease TMPRSS2. Importantly, TMPRSS2 also requires calcium for its SRCR (scavenger receptor cysteine-rich) domain and its LDLRA (LDL receptor class A) domain. Thus, the presence of EDTA excipients in nebulized β_2-agonist medicines can disrupt SARS-CoV-2/COVID-19 infection and can explain the asthma paradox. This model validates repurposing EDTA in nebulizer solutions from a passive excipient to an active drug for treating COVID-19 infections. Repurposed EDTA delivery to respiratory tissues at an initial target dose of 2.4 mg per aerosol treatment is readily achievable with standard nebulizer and mechanical ventilator equipment. EDTA warrants further investigation as a potential treatment for SARS-CoV-2/COVID-19 in consideration of the new calcium requirements for virus infection and the regular presence of EDTA excipients in common asthma medications such as Metaproterenol. Finally, the natural history of Coronavirus diseases and further analysis of the fusion loop homologies between the Betacorona SARS-CoV-2 virus and the less pathogenic Alphacorona HC0V-229E virus suggest how to engineer a hybrid virus suitable for an attenuated alpha-beta SARS-CoV-2/COVID-19 vaccine. Thus, replacing SARS-CoV-2 fusion loops (amino acids 816–855) with the less pathogenic HCoV-229E fusion loop (amino acids 923–982) may provide antigenicity of COVID-19, but limit the pathogenicity to the level of HCoV-229E.

Introduction

Basic taxonomy and coronavirus virology

While the Coronavirus family of single-stranded enveloped RNA viruses is divided into four genera: α-CoVs, β-CoVs, γ-CoVs, and δ-CoVs, only alpha and beta can infect mammals [1]. After binding to their respective receptors, the Coronavirus viruses enter cells through endocytosis with the viral spike proteins driving the fusion of viral and endosomal membranes to enable insertion of the viral genome into the cytoplasm [2]. The less pathogenic Alphacorona virus 229E (HCoV-229E) was isolated from students suffering from the common cold in 1966 [3,4]. HCoV-229E is highly prevalent and most people experience acute infection during their childhood [5]. One study found 65% of the children between the age of 2.5 and 3.5 years were seropositive for HCoV-229E [6]. The HCoV-229E virus binds to the aminopeptidase N receptor (CD13) [7] and enters the cell after cleavage by TMPRSS2 and fusion [8]. The more pathogenic SARS-CoV-1 and SARS-CoV-2 (COVID-19) viruses belong to the β-genus.

The Betacorona virus SARS-CoV2 is a positive-sense single-stranded ribonucleic acid (ssRNA) of approximately 29,700 nucleotides in length, of about 80% identical to that of SARS-CoV-1 and approximately 96% identical to the bat coronavirus BatCoV RaTG13 [9]. The Spike (S) protein is 1273 amino acid long and S viral envelope protein that has two main subunits (S1 and S2) which protrude outwards with a 'corona' like appearance and binds to the angiotensin-converting enzyme 2 (ACE2) receptors [10,11]. The amino-terminal subunit is responsible for receptor binding and is labeled the S1 domain. The C-terminal part, labeled the S2 domain, contains the fusion machinery. More specifically, amino acids 318–510 of the S1 represent the receptor-binding domain (RBD) that binds to ACE2 [12]. CoV S proteins have two cleavage sites and protease cleavage is required for S2 fusion to the cell membrane. There is an S1/S2 site composed of the amino acids RSVR that is located at the border between the S1 and S2 subunits and an S2′ site, composed of the amino acids RSAR. In SARS-CoV-2, the S2′ site is located at amino acid 815, just upstream of the putative

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https://doi.org/10.1016/j.mehy.2020.110027
Received 8 May 2020; Received in revised form 11 June 2020; Accepted 20 June 2020
Available online 26 June 2020
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fusion loop peptides present within the S2 subunit discussed below. In SARS-CoV-2/COVID-19 the type II transmembrane serine protease (TTSP) TMPRSS2 cleaves the S1–S2 subunits [13]. It is also noteworthy that TMPRSS2 has two calcium-binding domains; a SRCR (scavenger receptor cysteine-rich) domain (aa 149–242) and a LDLRA (LDL receptor class A) domain (aa 113–148) that forms a binding site for -calcium [14]. The SRCR is a conserved calcium-dependent domain in which binding was disrupted by EDTA [15]. Together, the LDLRA and SRCR-like domains that may serve as substrate recognition sites.

Calcium-dependent fusion process required for viral infection

Even now, the actual mechanism of virus membrane fusion is not completely understood. In the case of Coronaviruses (CoVs), it is not a simple two-step process of receptor binding (via the S1 domain) and membrane fusion (via the S2 domain containing the fusion peptide). Viral entry into host cells requires that there is a domain of the S protein that interacts with opposing hydrophobic cellular membranes called a fusion peptide or fusion loop. These fusion peptides (fusion loops, FL) are generally external amino acid domains that insert into the host membranes after major conformational changes of the virus S protein following proteolytic cleavage to initiate the process fusion with the host membrane.

When the S1/S2 site and S2′ are activated by host proteases (e.g., TMPRSS2) there are changes in the cleavage site position relative to the fusion peptide to modulate the fusion loop (FL). This process gives CoVs the unique flexibility to invade different cell types and host species. Additionally, the CoVs fusion process employs a calcium-dependent fusion process that was only recently discovered for Rubella [16] and later described for SARS-CoV-1 infection [17]. While two fusion peptides (FLs) were found with SARS-CoV-1, influenza had no calcium-dependent membrane fusion process. The calcium dependent membrane-ordering results in more effective binding that can penetrate deeper into membranes. There are two FL domains in each SARS-CoV versus a single FL domain found in for HCoV-229E and Rubella shown in Table 2 below. Thus, this calcium-dependent requirement for the FL process may explain both the increased lethality of the beta CoVs and the apparent resistance of asthma patients to SARS-CoV-2 infection due to inhaling medications containing EDTA excipients.

Strategy for utilizing approved drugs

Since the drug development process from discovery of a new drug to an approved drug generally takes over 10 years, it is unrealistic to expect quick development of a novel anti-coronavirus drugs for SARS-CoV-2/COVID-19. The strategies for Coronavirus treatment regimens have mainly relied on combination therapies with drugs known to have acceptable safety profiles include IFNs, ribavirin, and corticosteroids. However, the data from past regimens indicates that the treatments were not effective in treating SARS [18]. Moreover, no perceived benefit, and possible deleterious effects were observed when corticosteroids (methylprednisolone) were given as treatment during the SARS and MERS epidemics [19,20]. Additionally, recent clinical commentary indicates that corticosteroids should not be given routinely for the treatment of COVID-19. Accordingly, the asthma paradox is unlikely due to steroid treatments because recent admonitions against routine systematic corticosteroids for the treatment of COVID-19 and prior reports indicate that systemic steroids for treating SARS-CoV-1 may have been harmful [21].

The safety of EDTA in bronchial dilator solutions

Ethylene Diamine Tetraacetic acid (EDTA) was first synthesized in1935 and EDTA has been employed as an excipient in bronchial dilator solutions for decades (e.g., Albuterol, Metaproterenol). EDTA has been added to nebulized bronchodilator solutions in the United States as both nonsterile and sterile-filled products [22]. Accordingly, Edetate disodium (Na2EDTA) is often present as preservative or stabilizing agents in nebulizer solutions used to treat asthma and chronic obstructive pulmonary disease [23]. Historically, common nebulizer therapies used by asthma and COPD patients have had concentrations of EDTA available in nebulizer solutions that vary from 0.1 to 0.5 mg/mL [24]. For example, Albuterol (manufactured by Dey Laboratories) contained 300 μg of EDTA, which is also far below the threshold dose for bronchoconstriction. Currently, Metaproterenol Inhalation Solution USP is expressly formulated with EDTA (edetate disodium) as a unit-dose bronchodilator to be administered by oral inhalation with the aid of an intermittent positive pressure breathing apparatus (IPPB). It contains 0.4% or 0.6% Metaproterenol sulfate in a sterile, acidic, aqueous solution containing edetate disodium, sodium chloride, hydrochloric acid, and/or sodium hydroxide for pH adjustment [25].

In one study, volunteer subjects received an inhalation challenge with increasing concentrations of EDTA (0.25 to 10.0 mg/mL) in a double-blind fashion [26]. Here, EDTA produced a concentration-dependent bronchoconstriction that did not resolve spontaneously within 1 hour. Mean EDTA PC_{20} FEV_1 was 2.4 mg/mL (range 1.2 to 12.8 mg/mL). This study concluded that there was no significant difference in airway response to EDTA among volunteers receiving Beta2-agonist treatments [27]. To study off-target bronchoconstriction by EDTA, it was found that Albuterol (1 μg/kg IV) significantly attenuated Na2EDTA-induced bronchoconstriction in canines [28]. Additionally, intravenous EDTA chelation therapy has been safely used for more than 50 years [29]. There were an estimated 500,000 visits for chelation therapy in the U.S. for 1993 [30], and 800,000 in 1997 [31]. A Canadian survey found that 8% of patients who had undergone cardiac catheterization had used chelation therapy [32].

Medical hypothesis

Recent medical articles indicate that there is markedly lower reported prevalence of asthma and COPD in patients diagnosed with COVID-19 [33,34].

To explain the unexpected observation that asthma patients and chronic obstructive pulmonary disease patients appear resistant to COVID-19, it is postulated that;

1) SARS-CoV-2/COVID-19 has two calcium-dependent fusion peptide/fusion loop (FL) domains of that is highly homologous to SARS-CoV-1,
2) The substrate recognition site(s) for cleavage by the requisite cell surface protease TMPRSS2 have a conserved SRCR (scavenger receptor cysteine-rich) domain and a LDLRA (LDL receptor class A) domain that utilize calcium to mediate binding to the SARS-CoV-2 (COVID-19) spike protein (i.e., the ligand),
3) SARS-CoV-2 (COVID-19) infection is, and has been disrupted by exposure to calcium chelating agents such as EDTA in nebulizer medications inhaled by asthma patients to either directly interrupt the cleavage of the S protein by TMPS332 and/or impede the calcium-dependent fusion of SARS-CoV-2 (COVID-19) virions with the host membrane via FL1 and FL2 peptide domains, and
4) Replacement of SARS-CoV-2 S protein amino acids 816 to 855 with HC0V-229E amino acids 923 to 928 may lead to a live attenuated SARS-CoV-2 hybrid strain suitable for vaccination to generating protective antibodies.

Evaluation of the hypothesis

Testing of the molecular hypothesis

The NCBI pBlast tool was used to test the hypothesis that SARS-CoV-2 contains a calcium-dependent fusion domain(s) similar to those that were recently discovered in both Rubella and SARS-CoV-1. An amino

Table 1
pBlast Alignment Metrics.

Fusion Loop DomainsAligned	Score	Expect	Method	Identities	Positives
FL1-CoV-1/CoV-2	47.4 bits (111)	7e-13	Composition-Based stats	21/22(95%)	22/22(100%)
FL2-CoV-1/CoV-2	47.4 bits (111)	9e-13	Composition-Based stats	19/22(90%)	20/21(95%)
FL2-CoV-2/Rubella	13.5 bits (23)	0.99	Composition-Based stats	4/7(57%)	5/7(71%)
Fl2-CoV-2/HCoV-229E	14.2 bits (25)	0.48	Composition-Based stats.	4/6(67%)	5/6(83%)
Fl2-CoV-2/Influenza	No similarity	NA	Composition-Based stats.	NA	NA

Table 2
pBlast Alignment Amino Acid Locations.

Source	Fusion Loop Amino Acid Position	Reference Sequence
FL1 CoV-2	816 SFIEDLLFNKVTLADAGFIKQY 837	GenBank: QHD43416.1
pBlast Alignment	SFIEDLLFNKVTLADAGF + KQY	
FL1 CoV	798 SFIEDLLFNKVTLADAGFMKQY 819	NCBI Reference Seq.:NP_828851.1
FL2 CoV-2	835 KQYGDCLGDIAARDLICAQKF 855	GenBank: QHD43416.1
pBlast Alignment	KQYG + CLGDI ARDLICAQKF	
FL2 CoV	817 KQYGECLGDINARDLICAQKF 837	NCBI Reference Seq.:NP_828851.1
FL2 CoV-2	841 GDIAARD 848	GenBank: QHD43416.1
pBlast Alignment	GD + RD	
Rubella	49 GDDSGRD 55	GenBank: ACN50046.1
FL2 CoV-2	835 KQYGDCL 841	GenBank: QHD43416.1
pBlast Alignment	K + YG C	
Fl2 HCoV-229E/CoV-2	923 KRYGFC 928	GenBank: CAA71056.1
Fl2-CoV2/HA Influenza	No significant similarity found	GenBank: AD177360.1

acid comparison of the two relevant amino acid regions in S protein of SARS-CoV-1 representing fusion loop 1 (FL1 = Amino Acids 798–819) and fusion loop 2 (FL2 = Amino Acids 835–855) was conducted using the Protein Blast program from the National Center for Biotechnology Information [35]. Specifically, Table 1 and Table 2 exhibit the data from the Protein Blast Alignment Tool data from the calcium binding fusion domains, labeled FL1 and FL2 respectively, that compare the spike proteins of COVID-19 (SARS-CoV-2) with SARS-COV and Rubella utilizing cited reference data; GenBank: QHD43416.1 (CoV-2), NCBI Reference Sequence: NP_828851.1(CoV-1), GenBank: ACN50046.1. (Rubella), GenBank: CAA71056.1 (Human coronavirus229E) and GenBank: AD177360.1 (hemagglutinin [Influenza A virus (A/Boston/136/2009(H1N1))]).

The results demonstrated a 100% and a 95% correspondence respectively between the postulated FL1 and FL2 domains in SARS-CoV-2 (COVID-19) compared to the known FL regions for SARS-CoV-1 described in 2017. Additionally, the less pathogenic Alpha coronavirus 229E (HCoV-229E) has a solitary FL2 domain and a reduced homology length compared to the SARS-CoV-2 (COVID-19) FL2. Similarly, amino acids 49 to 55 of the Rubella Virus membrane glycoprotein E2 virus also have a smaller, but significant homology with the FL2 domain. In contrast, the Influenza H1N1 hemagglutinin (HA) protein has no significant similarity to any of the CoV FL domains. The reduced homology of HCoV-229E's single calcium-binding domain to SARS-CoV-2 suggests attenuation of HCoV-229E, which is consistent with HC0V-229E having crossed species barriers to infect humans decades or centuries ago [36].

Accordingly, analysis of the different fusion loop homologies suggests replacing SARS-CoV-2 amino acids 816–855 with HCoV-229E amino acids 923–982 may be candidate for an attenuated alpha–beta SARS-CoV-2/COVID-19 vaccine that would be able to generate host immunity, yet lower the pathogenicity to level of HCoV-229E.

Testing of the clinical hypothesis

Technically, the hypothesis can be tested in humans who are COVID-19/SARS-CoV-2 RT-PCR postive by performing a prospective, randomized, placebo-controlled study to compare the effect of admininstering nebulizer treatments with either containing saline solutions containing EDTA exclusively or containing EDTA/B2-agonist (e.g., Albuterol, Metopateronol). The focus of the study is directed towards utilizing objective parameters that distinguish treatment groups. Parameters could include, RT-PCR results, clincal progress, and/or length of hospital stay, etc. Moreover, outpatient studies may be possible. That is, after initial medical supervision, the less clinically imparired COVID-19/SARS-CoV-2 RT-PCR postive patients may be treated as an outpatient if the patient tolerates EDTA. Clinical measurements that may followed in clinic settings include vital signs, FEV1, and pulse oximetry measurements.

Concentrated EDTA solutions suitable for use in nebulizers and mechanical ventilators can be used to directly demonstrate that EDTA can inhibit SARS-CoV-2 infection with relative safety based on prior studies of asthmatics and the long history of adding EDTA [37] to nebulizer treatments. While the lung airways of asthmatics may be more sensitive to the bronchoconstriction effects of EDTA, prior studies on asthmatics indicate that 2.4 mg/ml of EDTA causes, on average a tolerable 20% drop in FEV1 and adding concentrated EDTA to a standard Albuterol nebulizer set-up should mitigate most EDTA induced bronchoconstriction. As a treatment example, assuming the volume from a standard dropper is approximately 0.06 ml and the 0.5 M EDTA concentrate, 2 drops of a concentrated 0.5 M solution, which contains approximately 23.26 mg EDTA/ml, is added to 2.5 ml of nebulizer diluent (saline or saline/Albuterol) would result in approximately 2.8 mg of EDTA per treatment. Nebulizer treatments can be repeated in accord with the chosen B_2-agonist protocol or as tolerated. A local compounding pharmacy can prepare the 0.5 Molar EDTA concentrate as follows; Add 186.1 g of disodium ethylene tetraacetate·$2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. It is noteworthy that the disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH. Additionally, an "Off-Label" use of an FDA approved parenteral EDTA drug for nebulizer use may include dilution of Calcium Disodium Versenate, (200 mg/ml) to achieve a target dose of 2.4 mg per aerosol treatment with or without a Beta2-agonist as tolerated.

HCoV-229E was discovered in 1966 and is a less pathogenic Alpha coronavirus that appears to have crossed species barriers to infect humans decades or centuries ago. Like SARSCoV-2, HCoV-229E enters the cell via TMPRSS2 to infect humans. However, HCoV-229E is missing

the SARS F1 fusion loop. Thus HCoV-229E sequence is comparable to the single FL2 domain in the less pathogenic Rubella virus as shown in Table 2. An attenuated SARS-CoV-2 virus suitable for a vaccine can be created by replacing SARS-CoV-2 amino acids 816–855 with HCoV-229E amino acids 923–982. This replacement will maintain the AEC-2 tissue specificity and host range, yet it will effectively disturb fusion loop mechanism to reduce the pathogenicity of SARS-CoV-2 to the level of HCoV-229E. An alpha-beta hybrid virus may be immunologically necessary, since prior studies have shown that immunity after inoculation to HCoV-229E may disappear within a year. Although surveys for 229E antibodies in adults in the United States range from 19 to 41%, there are many individuals, who despite possessing an anti-HoCV-229E antibody, can subsequently experience reinfection and illness [38]. In fact, one study found a 66% reinfection rate in volunteers re-exposed to 229E after a year [39]. The significant coronavirus 229E reinfection data both underscores the challenges of finding an effective vaccine, and raises serious questions about the underlying clinical premise(s) that justify the use of “immunity” cards [40].

Finally, adding EDTA to alcohol-base hand sanitizers, lotions, sprays and soaps should further reduce COVID-19 virus infectivity. The Cosmetic Ingredient Review Expert Panel found that EDTA ingredients are safe as used in cosmetic formulations. The typical concentration of use of EDTA in cosmetics is less than 2%, and the lowest dose reported to cause a toxic effect in animals was 750 mg/kg/day [41].

Conclusion

Searching for the viral mechanisms to elucidate why asthma patients appear resistant to COVID-19 infection uncovered evidence for the key role of calcium in SARS-CoV-2/COVID-19 infection. First, a new computer sequence analysis of SARS-COV-2/COVID-19 with the NCBI pBlast program revealed two unrecognized calcium-dependent fusion loop domains. Second, the substrate recognition sites for the requisite cell surface protease TMPRSS2 have a conserved SRCR (scavenger receptor cysteine-rich) domain and a LDLRA (LDL receptor class A) that utilize calcium. Analysis of nebulizer solutions typically used by asthma patients, for example Metaproterenol, revealed the presence of an excipient called EDTA, a calcium-chelating agent. Triangulating these, the data converges on the previously unrecognized critical importance calcium for effective SARS-CoV-2/COVID-19 infection, and how calcium chelation by EDTA may prevent infection. Here while EDTA is only a single drug, it can still disrupt 2 key steps in the SARS-CoV-2/COVID-19 infectious process. Accordingly, repurposing EDTA from excipient to therapeutic nebulized drug with or without Beta-2 agonist supplementation logically becomes a new treatment for COVID-19/SARS-CoV-2 patients. The potential to utilize EDTA to both reduce COVID-19 transmission and treat infection through relatively safe modalities that include nebulizer or mechanical ventilator misting of EDTA solutions (in conjunction with Albuterol/Metaproterenol to minimize bronchoconstriction if needed) and adding EDTA to hygienic products warrant further investigation(s). As either an “Off-Label” or formal IRB protocol, the clinician can measure the clinical response of nebulized pharmaceutically sterile Na2EDT dissolved in normal saline at a range up of to 1·2 to 12·8 mg/mL for the treatment of COVID-19 patients on respirators or with stand-alone nebulizers. When administering nebulized EDTA, the clinician should monitor for signs of bronchial constriction, and administer Albuterol/Metaproterenol as needed. Patients not on a respirator can be similarly treated with Na2EDTA solution through a nebulizer facemask under direct medical supervision, and if tolerated, the more stable patients may be treated at home or as outpatient with a portable nebulizer/mask and Na2EDTA solutions. Finally, an alpha–beta hybrid virus replacing F1 and F2 fusion loops with HCoV-229E amino acids 923–982 can maintain the AEC-2 tissue specificity and host range, yet it will effectively disturb fusion loop mechanism reduce the pathogenicity of SARS-CoV-2 to the level of HCoV-229.

Funding source

Self-supported.

Declaration of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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