Algemene gegevens / General Information

Programma / Programme	:	**COVID-19 Programma**
Subsidieronde / Subsidy round	:	**Bottom-up ronde COVID-19 aandachtsgebied 1**
Projecttitel / Project title	:	**ACE2 in COVID-19: Closing the door to an uninvited guest**
Projecttaal / Project language	:	**Nederlands / Dutch**
Geplande startdatum / Planned start date	:	**30-07-2020**
Geplande duur / Planned duration	:	**24 maanden / months**
Datum indienen / Date of application	:	**14-05-2020**
Projecttype / Project type	:	**Toegepast onderzoek**
Vervolg eerder ZonMw-project / Continuation previously funded project ZonMw	:	**Nee / No**

Projectleden / Project members

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Projectgegevens / Project information

Aandachtsgebieden / Focus

1.1 Thema's aandachtsgebied 1
- Behandeling
- Virus, immuniteit, immuunrespons en pathogenese

1.3 Setting
- Anders

Samenvatting / Summary

We will focus on blocking the viral entry pathway i.e. the interaction between Angiotensin Converting Enzyme 2 (ACE2) and CoV-2, as a novel therapy for COVID-19. We previously studied the role of ACE2 in CoV infection.

Overall goal:
Prevent binding of CoV-2 to ACE2 in the early stage of infection. This reduces viral cell entry, replication and viral load, preventing cell damage and (lung) disease.

Hypothesis:
Blocking the binding of CoV-2 to ACE2 using a highly specific small-molecule drug will prevent cell entry, and subsequent viral replication and organ damage.

Aim:
Develop, produce and validate the efficacy of a small-molecule drug which prevents the binding of CoV-2 to ACE2 on target cells, especially in the early phase of infection. Reducing early cell entry and viral replication will allow the patient to build up immunity whereas viral transmission and dissemination is reduced. We have designed a cyclic peptide with three amino acids in the outer ring that binds to ACE2 and thereby prevents virus uptake into key cells.

Approach
- Determine expression of cytoplasmic and membrane-bound ACE2 in airway and lung epithelium (RNAscope, immunohistochemistry and western Blotting)
- Expose cells to the developed peptide (toxicity, viability, ROS production, RNA seq)
- Expose cells to recombinant HIS-tagged spike protein: evaluate ACE2 binding and internalization
- Expose cells to Cov-2: evaluate cellular entry using time-of-addition studies (BSL3 lab)
- Analyze the effects of virus exposure to the different cell types +/- compound -> RNAseq
- Test the peptide in available animal models (ferret and Syrian guinea pig) (BSL3 lab)
- Prepare for nebulization in humans

Perspective:
The first phase of Covid-19 is a clear window of opportunity for the intervention with a SARS-CoV-2 to ACE2 blocking peptide. A potential route of administration will therefore be nasal and pulmonary nebulization in high risk patients who present with mild symptoms.

Trefwoorden / Keywords

Blocking peptide; Pharmacologic intervention; nasal application; pulmonary nebulization

Samenwerking / Collaboration

Samenwerking tussen onderzoek en praktijk / Cooperation between research and practice:

Nee / No

Inhoud / Content

Disciplines / Disciplines

- Infecties, parasitologie, virologie / Infections, parasitology, virology
- Biofarmaceutische wetenschappen, toxicologie / Biopharmaceutical sciences, toxicology
- Microbiologie / Microbiology

Financiële gegevens / Financial data

ZonMw budget

	Jaar / Year								
Kostenpost	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	**Totaal / Total**
Personeel	(10)(1c)								
Materieel									
Implementatie									
Apparatuur									
Overig									
Totaal / Total									

Co-financiering / Cofinancing

Naam co-financier / Name of cofinancier	Bedrag / Amount	Status

Bijzondere gegevens / Additional information

Vergunningen / Permits

	Verklaring nodig / Statement required?		Status verklaring / Statement status		
	Ja / Yes	Nee / No	Verkregen / Acquired	Aangevraagd / Applied	Nog niet aangevraagd / Not applied yet
METC		X			X
DEC	X				X
WBO		X			

Onderschrijvingen / Assents

	Ja / Yes	Nee / No	N.v.t. / N.A.
Code biosecurity / Code Biosecurity	X		
Code openheid dierproeven / Code Transparency of Animal Testing	X		

Andere vergunningen / Other permits

‚AANVRAAGFORMULIER PROJECTIDEE – BOTTOM-UP RONDE

COVID 19 programma

Deadline voor indiening: 14 mei 2020 (14:00 u)

LEES ALSTUBLIEFT ALLE INSTRUCTIES IN BIJLAGE "TOELICHTING INDIENING PROJECTIDEE" VAN DE OPROEPTEKST ZORGVULDIG!

Wanneer u het formulier heeft ingevuld:

1. **Zet het formulier om naar een PDF file en controleer de details**
2. **Upload het complete formulier als een bijlage bij uw indiening in Projectnet (Let op: dit zijn twee verschillende links, gebruik maar 1 van de 2!)**

ProjectNet: Aandachtsgebied 1 (voorspellende diagnostiek en behandeling)
ProjectNet: Aandachtsgebied 2 (zorg en preventie)

BASISGEGEVENS (voorpagina)

NAAM VAN DE HOOFDAANVRAGER:

Prof. Dr. (10)(2e) [10](2e) (10)(2e)

ORGANISATIE:

Universitair Medisch Centrum Groningen

PROJECTTITEL:

ACE2 in COVID-19: Closing the door to an uninvited guest

DATASTEWARD:

Wie is de datasteward die de open science en FAIR data planning in uw project ondersteunt? Zie de webinars op de ZonMw website om de datastewards te informeren en ondersteunen.

☒ Ik betrek een datasteward bij mijn project:
Naam: (10)(2e)
Instituut: Data Manager Informatiemanagement Onderzoek, UMCG
E-mail: (10)(2e) umcg.nl
Was aanwezig bij de webinar: ☒ Ja ☐ Nee

☐ Ik heb nog geen datasteward.

ONDERZOEKSVOORSTEL **max 3 pagina's A4** (inclusief literatuurreferenties)	(voorpagina met basisgegevens niet meegerekend - font type Arial 10 pts)

1. PROBLEEMSTELLING EN DOELSTELLING(EN):

The coronavirus disease COVID-19, caused by SARS-CoV-2 (CoV-2), is hitting the world in an unprecedented manner. No effective intervention is available that prevents or treats the complications of COVID-19. During SARS-CoV-1 outbreak (2002-2004), we gained ample experience and insights into the role of ACE2 in disease pathogenesis and in viral transmission by documenting ACE2 tissue expression in human organs (Hamming et al., 2004). The expression of ACE2 in alveolar epithelium and endothelium contributed to the understanding of the pathogenesis of severe lung damage. ACE2, part of the renin-angiotensin-system (RAAS), is crucially involved in dissemination of CoV-2. We found that these effects are largely independent of RAAS inhibition (Sama et al., 2020, Milne et al., 2020). Membrane-bound ACE2 in combination with activity of protease TMPRSS2 determines disease course by being the port of entry, thereby taking a central role in development of extensive tissue damage and inflammation. Furthermore, COVID-19 patients with co-morbidities (obesity, diabetes mellitus, history of cardiovascular disease) are over represented in the intensive care unit (van der Voort et al., 2020).

Blocking the viral entry into airway cells is an attractive treatment option. In the absence of a vaccine, inhibition of binding of CoV-2 to ACE2 in the early phase of infection is therapeutically the most attractive option. Reducing cell entry and viral replication will then allow the patient to build an adequate immune response to fight off the virus whereas further viral transmission and dissemination throughout the body is prevented. In keeping with this, mapping ACE2 and TMPRSS2 expression in upper (nose) and lower respiratory tract and lung epithelium is of interest to identify the preferential port of entry. We aim to prevent binding of CoV-2 to ACE2 in the early stage of infection using nebulization of a novel small-molecule drug.

The goal of this research proposal is to develop, produce and validate the efficacy of a small-molecule drug which is designed for nebulization to prevent the binding of CoV-2 to ACE2 on different target cells, especially in the early phase following primary infection.

2. PLAN VAN AANPAK:

The proposal is subdivided in the following four work packages (WPs):
WP1: "ACE2 peptide" design and synthesis; **WP2**: SARS-CoV-2 infection of respiratory tract cells in Covid-19 airways and lungs; **WP3**: Efficacy of the "ACE2 peptide" *in vitro*; **WP4**: Efficacy of the "ACE2 peptide" *in vivo*

WP1: "ACE-2 peptide" design and synthesis

Inhibition of protein:protein interactions (PPIs) is a challenge for both small molecule (SME) and antibody (Ab) approaches as SMEs are unable to address large, "featureless" surfaces and economic/ pharmacological issues make antibody approaches challenging. A middle ground can be found through the use of macrocycles/cyclized peptides, which have lower-cost of goods, more straightforward production while still retaining the ability to selectivity antagonize PPIs. This approach is highly successful against a wide range of targets, including IL-17, p53 and VEGFR (Sadre-Momtaz et al, 2019) and a similar approach will provide high potency antagonists of the ACE2:Spike SARS-nCovid-19 Receptor binding domain (SS-RDB). Peptide-based inhibition of the ACE2:SS-RBD interaction was recently shown (Zhang et al, 2020); our integrated approach aims to optimise this and extends the research from bench to bedside.
The crystal structure of the complex between ACE2 and SS-RBD (*6M0J*) reveals that the ACE2 α1-helix fragment 24-42 and segment 419-431 of the ACE2 "hot-loop" are in close contact with SS-RDB. We have designed a peptide 2HN-CQDKHEEDYQMYNKGCKED-COOH (Fig.1) that reproduces key elements of these binding motifs. To drive the peptide towards an alpha helix structure, we incorporated two amino acids with high helical propensity and an integrated disulfide bond connecting the N- and C-termini of the helix. The "hot-loop" element is outside this helical region, generating a distinct binding epitope. A control peptide, containing the same amino acids in a randomised order (2HN-ANEGQYEKQEKDMNDADHK-COOH), has also been synthesized. Based on initial experimental results (Fig. 1), we intend to further optimise the peptide to cover the entire CoV-2 interaction surface of ACE2.

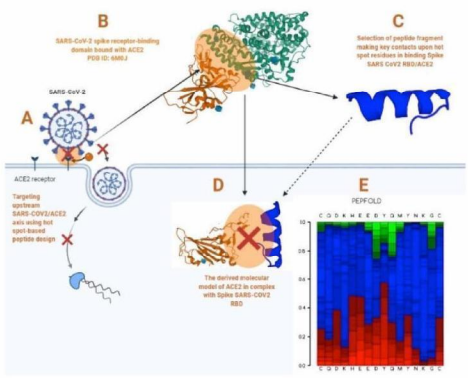

Figure 1: A) Schematic showing targeting the upstream SARS-COV2/ACE2 axis using hot spot-based peptide design, B) Crystal structure of the spike SARS-COV2 receptor-binding domain with ACE2 (ACE2: green, RBD: orange; PDB:6M0J). C) Hot spot regions of ACE2 domain docked against RBD. ACE2 antagonistic peptide generated from the hot-spot residues recognized by Protein Contact Atlas. The peptide is synthesized and binding to glycosylated SARS-CoV-2-RBD is determined by MST. D) Illustration of binding interface between SARS-CoV-2-RBD and peptide. E) Peptide local structure profile predicted by PEPFOLD3.

To verify the ability of the "ACE peptide" to complex with spike SARS-COV2 receptor-binding domain (SS-RBD) *in vitro*, we use MicroScale Thermophoresis (MST) binding assays. Titration of fluorescently labeled SS-RBD against ACE2 peptide will provide a dissociation constant (Kd) of binding.
Deliverables: Obtaining critical information on binding affinity, stoichiometry and thermodynamics of ACE2 peptide"- SS-RBD interaction.

WP2: SARS-CoV-2 infection of respiratory tract cells in Covid-19 airways and lungs
Co-expression of ACE2 and the obligatory protease TMPRSS2 is present in bronchial epithelial cells, alveolar type II pneumocytes and nasal epithelium (Sungnak et al, 2020). We will determine ACE2 and TMPRSS2 expression combined with detection of SARS-CoV-2 infection and replication using multiplex fluorescent *in situ* hybridization (RNAscope) and ACE2 immunofluorescence on FFPE sections from nasal biopsies, airways and alveolar lung tissue obtained during autopsy. Qualitative and quantitative microscopic analysis will be performed using TissueFAXS analysis. Presence of infected cells will be confirmed using validated antibodies reactive with SARS-CoV proteins in immunohistochemistry/IF.
Deliverables: i) Establishment microscopy-based platform to phenotype SARS-CoV-2-infected cells in vivo, and ii) identification key cell populations that will be used as therapeutic target (see below).

WP3: Efficacy of the "ACE2 peptide" *in vitro*
In vitro cell culture will be established using primary respiratory tract cells: airway -and nasal epithelium (Prof. van Baarle, RIVM). ACE2 and TMPRSS2 expression levels will be confirmed (RT-qPCR, immunohistochemistry and western blotting).
Deliverable: Establish the expression levels of cell surface molecules required for SARS-CoV-2 infection.

"ACE2 peptide" toxicity will be tested on Calu-3 cells (lung epithelium) and Vero-E6 cells (gold standard for SARS-CoV-2 infection and drug screening), and on respiratory tract cells by exposing cells to the "ACE2 peptide". Read-out for toxic effects include cell viability, mitochondrial function/proliferation, membrane leakage, and cell function. Efficacy of the "ACE2 peptide" will be determined using SARS-CoV-2 recombinant viral protein uptake as read-out. Cells will be exposed to recombinant HIS-tagged spike protein and "ACE2 peptide" after which binding of HIS-tagged spike protein to membrane-bound ACE2 will be evaluated (IF and western blotting). The efficacy of "ACE2 peptide" in preventing cell entry of infectious SARS-CoV-2 will be tested in Vero-E6 cells and respiratory tract cells. Cells will be inoculated with SARS-CoV-2 in presence of increasing concentrations of "ACE2 peptide". Analyses include: 1) detection antiviral activity [plaque assay, RT-qPCR], 2) measurement cytokine production by legend plex, 3) measurement epithelial cell integrity/activation as a proxy for pathology. Ad2/3 will be done at the highest antiviral, non-toxic dose of the "ACE2 peptide" in the context of primary epithelial cells. The mode-of-action will be determined by time-of-addition studies. The effect on virus-cell binding and cell entry will be evaluated using RT-qPCR and the number of infected cells via flow cytometry, and FISH and immunostaining on fixed and solidified cell paraffin embedded pellets. Durability of the antiviral compound will be assessed in

prolonged virus-cell cultures.
Deliverable: Determining the therapeutic index of the "ACE2 peptide" in vitro.

WP4: Efficacy of the "ACE2 peptide" *in vivo*
To evaluate the efficacy of the "ACE2 peptide" in a preclinical setting, we choose from two potential small animal models which are currently under development by the scientific community (ferret and Syrian hamster). Both species express the ACE2 receptor and are susceptible to infection by SARS-CoV-2. In ferrets, virus is detected in the upper respiratory tract at high titers for at least for 7 days post infection (d.p.i.) (Kim et al., 2020). In Syrian hamsters, virus replication is detected in upper and lower respiratory tract for 4 d.p.i. (Chan et al, 2020). Ferrets develop mild fever after SARS-CoV-2 infection, which represents a large portion of humans that display mild disease. The Syrian hamster develops a more severe disease including viral pneumonia and weight loss (Kim et al., 2020) representing patients hospitalized with COVID-2019. Dependent on knowledge gained in the coming period, either of the models will be used in an infection study in which 2-4 conditions of the "ACE2 peptide" will be examined for the ability to reduce virus replication and clinical symptoms and if applicable pathology of the upper and lower respiratory tract. The cyclic ACE2 peptide will either be administered prior to infection or at defined timepoints after infection and compared to a mock-control group. The RIVM has a lengthy experience with the ferret as an infection model for influenza under BSL-3 housing conditions (de Jong et al, 2016, 2020). Currently the ferret is evaluated as a model for SARS-CoV-2. Assays to detect the live virus (50% tissue culture infectious dose) and viral RNA copies (RT-qPCR) in swabs and tissues are in place. Temperature transponders are used for measuring fever and a pathology scoring system for assessment of viral pneumonia has been developed. These assays and methods can be translated to the Syrian hamster.

Development for human application via nebulization
The widespread use of an ACE2 blocking peptide requires a simple and convenient route of administration and dosage form to improve therapeutic efficacy. The pulmonary route is most optimal for administration of peptides with a molecular weight up to 20 kDa. SARS-CoV-2 is an airborne transmitted disease and pulmonary administration of the peptide would therefore make sense since it delivers the drug immediately at the side of action. This implicates improved efficacy, the option to administer lower doses and a reduction in side effects. At the RuG ample experience in the development of successful inhalers and their formulations (already on the market) is available. This allows for a potentially rapid clinical application. As a final step in this project we will develop the ACE2 peptide for direct clinical use via inhalers.
Deliverable: Determining the therapeutic index of the "ACE2 peptide" in vivo and prepare for human application.

3. HAALBAARHEID VAN HET PROJECT:

Time-line: RNAscope and immunohistochemistry: 3 mo; toxicity and MST: 3 mo; *In vitro* binding and RNA seq: 3 mo. 1-GMP synthesis: 3 mo; animal models: 6 mo. The METC application will run parallel with the above. Most experiments run parallel so we aim to be ready for human application in 12-15 mo.
Feasibility: The RNAscope technology, immunohistochemistry and toxicity techniques are up and running. MST is a standard technique in the lab of Dr. Groves. Prof. Smit has established the BSL3 facility and various *in vitro* experiments using SARS-CoV-2 are ongoing. Animal models are currently running or being validated in the RIVM laboratory by Dr. de Jonge.

4. RELEVANTIE VOOR DE PRAKTIJK:

The clinical consequences of COVID-19 disease vary from asymptomatic disease to organ failure and need for life-saving support in the ICU. The disease burden is large for some patients and for the health care system. ICU hospitalization is expensive and units are overcrowded due to the high number of COVID-19 patients. Preventing cell entry by blocking the interaction with ACE2 is a way to mitigate infection in individual patients, to prevent progression to severe disease and to limit the transmission of virus to others. This will have a positive impact on the return of patients to everyday life and promote economic growth.

5. DEELNAME VAN DE STAKEHOLDER(S) (e.g. patiënten, zorgprofessionals, etc.):

Clinical studies will be the obvious next step but beyond the scope of current proposal. Patient representatives will be involved in definitive proposal.