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Summary of CEAMED compound CM-978, as a potential agent for the treatment of patients with COVID-19.

CEAMED.S.A.

Scientific report

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CONTENIDO

1.1 Scientific Rational: Inhibitors of STAT3 activation as a means to reduce the detrimental effects of cytokine storm in patients with severe COVID-19.

COVID-19, the illness caused by infection with the novel severe acute respiratory syndrome (SARS)-associated coronavirus 2 (SARS-CoV-2), is a rapidly spreading global pandemic in urgent need of effective treatments. The World Health Organization (WHO) has declared coronavirus disease 2019 (COVID-19) a public health emergency of international concern. Neither a vaccine, nor antiviral drugs are yet available with proven efficacy for SARS-CoV-2 prevention or treatment. While most people with COVID-19 develop a mild or controllable form of the disease, approximately 15% develop a severe form of the disease requiring hospitalization and oxygen support, and a mortality rate of 1-5% is currently being observed. In severe cases, COVID-19 can be complicated by acute respiratory distress syndrome (ARDS), sepsis, and/or multi-organ failure (MOF) (see figure 1). Many patients with severe respiratory disease due to COVID-19 have features consistent with cytokine release syndrome (CRS) (also called "cytokine storm").

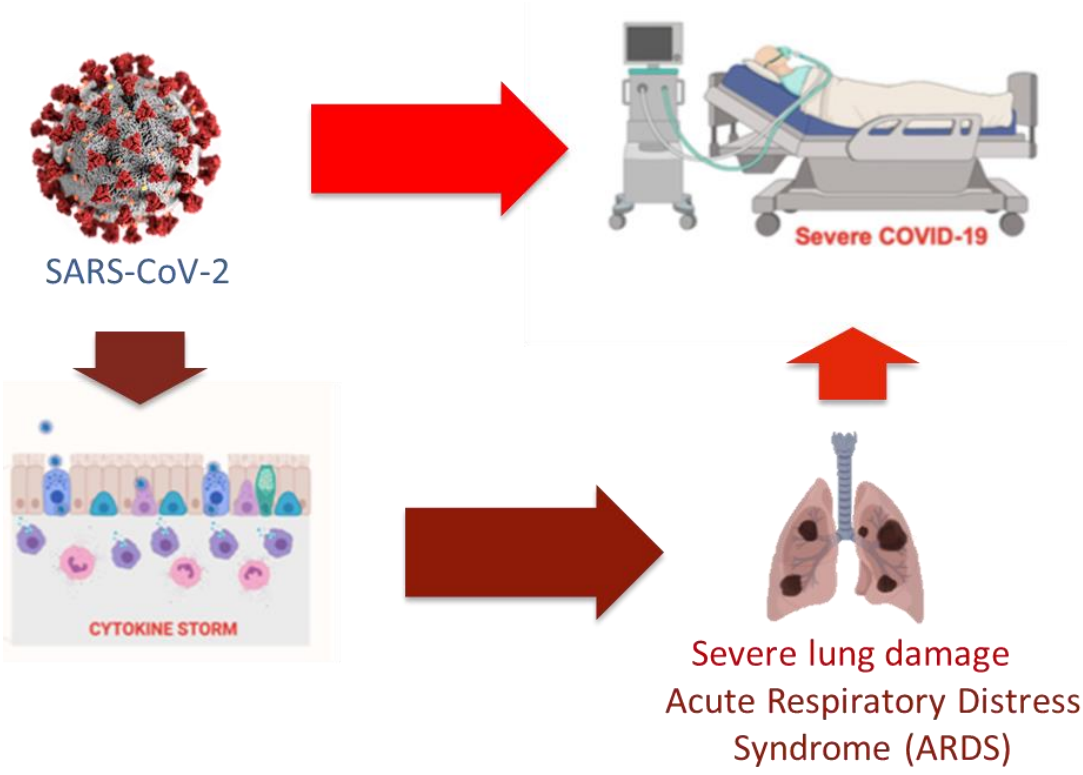

Figure 1: Severe COVID-19 symptoms are generally triggered by a "cytokine storm" that causes ARDS.

Laboratory analysis aiming to distinguish between the severe and mild forms of the disease, suggest that circulatory inflammatory markers, such as interleukin (IL)-6, are closely related to severe COVID-19. SARS-CoV-2 uses the trans-membrane enzyme ACE2 to infiltrate and infect cells. This reduces the enzymatic action of ACE2, causing an increase in the levels of Angiotensin II (AngII). High levels of AngII result in the activation of the IL-6/JAK/STAT3 signalization route that culminates in further amplifying IL-6 (and other ILs), and thus causing the aforementioned cytokine storm **(figure 2).**

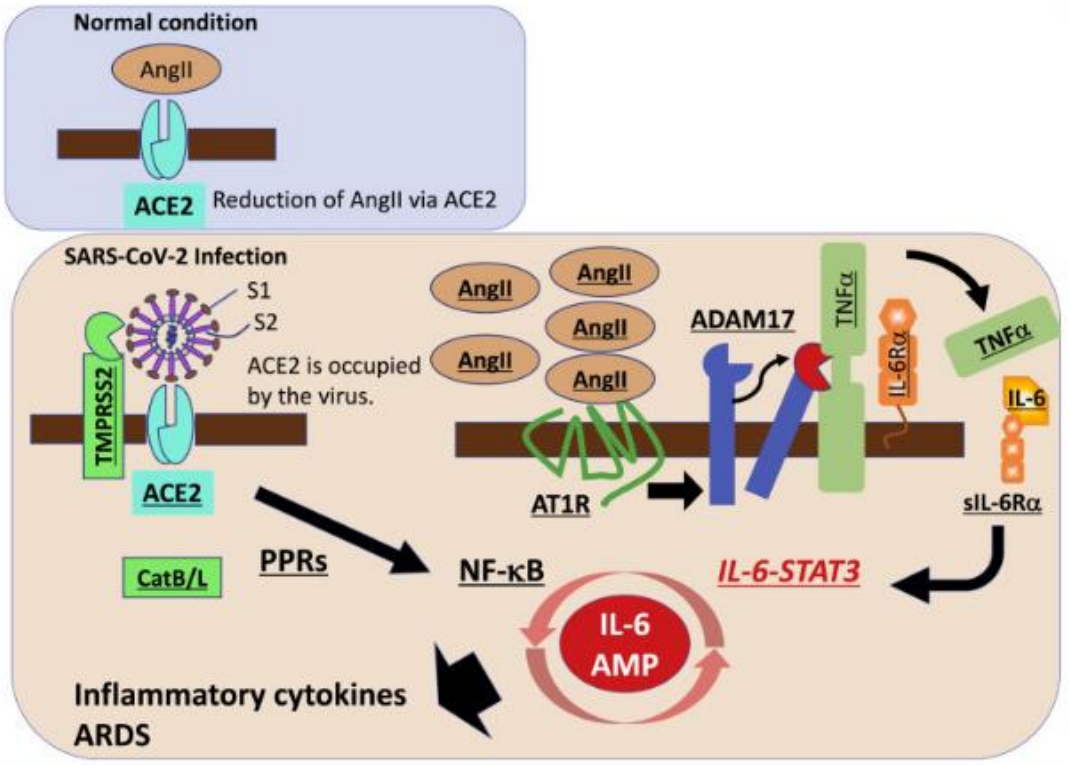

Figure 2. Figure 2: SARS-CoV-2 infection generates higher levels of AngII, resulting in the activation of the IL-6/JAK/STAT3 pathway and eventually cytokine release and ARDS. [1]

Due to increased activation of the IL-6/JAK/STAT pathway, it is postulated that inhibitors of this route might have a useful role in treating these patients (Figure 3). Currently, clinical trials are in progress using an IL-6 antibody (Siltuximab) and a JAK1-2 inhibitor (Ruxolitinib). Recently the compassionate use of both drugs, for patients with severe COVID-19 symptoms, has been authorized by

[1] Hirano, T., & Murakami, M. (2020). COVID-19: A New Virus, but a Familiar Receptor and Cytokine Release Syndrome. *Immunity*, *52*(5), 731–733.

the Spanish health authority. The recruiting of COVID-19 patients for a clinical trial in Spain using the natural product Silibinin, a reported STAT3 inhibitor, has also been reported.

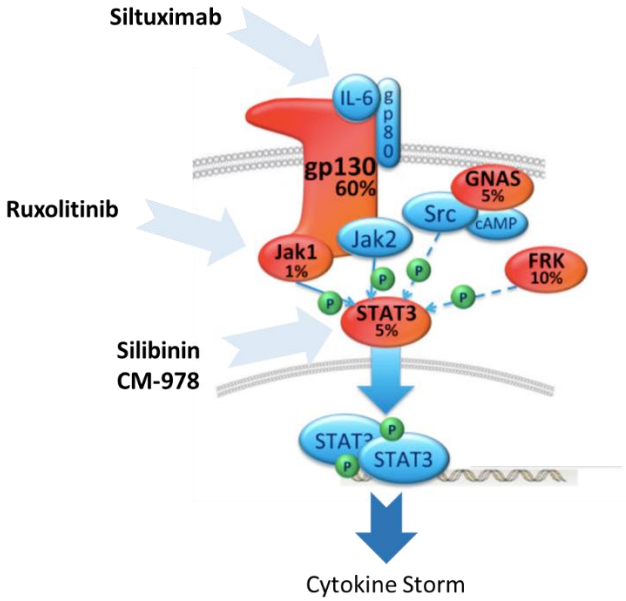

Figure 3: Inhibitors of the IL-6/JAK/STAT signaling route as potential treatments for patients with COVID-19.

CEAMED has been developing small molecule inhibitors of STAT3 as potential treatments for Triple Negative Breast Cancers (TNBCs), whose growth and metastasis are often highly dependent on STAT3 activation. CEAMED has at its disposition several families of compounds that potently reduce IL-6 activated STAT3 levels with IC50 values < 1 μM (for comparison, Silibinin has an IC50 ~ 100 μM). [2]

[2] Bosch-Barrera, J., Martin-Castillo, B., Buxó, M., Brunet, J., Encinar, J. A., & Menendez, J. A. (2020). Silibinin and SARS-CoV-2: Dual Targeting of Host Cytokine Storm and Virus Replication Machinery for Clinical Management of COVID-19 Patients. *Journal of clinical medicine*, *9*(6), E1770

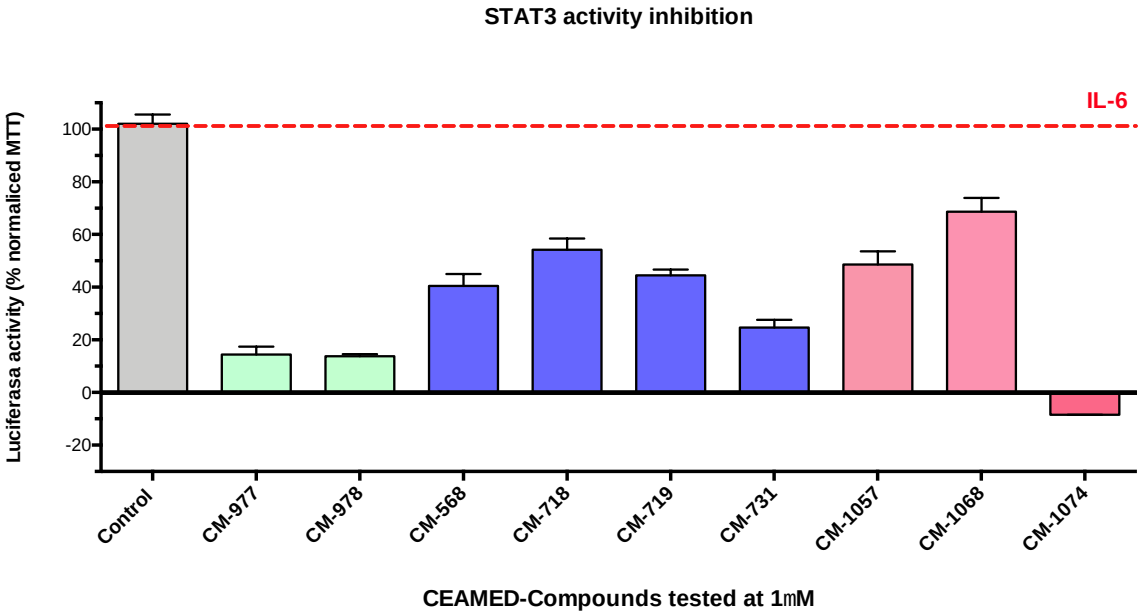

Figure 4: Inhibitors of IL-6 activated STAT3 designed by CEAMED

Of particular note is the *in vitro* and *in vivo* profile of the compound CM-978.

- It is a potent inhibitor of IL-6 induced STAT3 activation (IC50 ~ 0.6 μM)
- It is well tolerated in both mice and rats after oral dosing (50 mg/kg 5-days, 30 mg/kg 21 days)
- Pharmacokinetic studies in both rats and mice indicate it has a good half-life, high volume of distribution and high bioavailability after oral dosing.
- Intrinsic clearance studies using human liver microsomes indicate that it is stable to hepatic metabolism.

Although CM-978 has not yet been tested against human cardiac ion channels, none of the compounds tested from this series have exhibited any inhibition.

Given its potency against STAT3 activation in human cells, its bioavailability in rodents, and the current lack of treatments for patients with COVID-19, we believe CM-978 is a good candidate for further *in vitro* and *in vivo* studies to assess its potential use as a treatment to prevent and/or reduce cytokine storm induced ARDS.

As mentioned above, CEAMED is also developing other families of compounds that inhibit IL-6 induced STAT3 activation. CM-1074 (see figure 4) is possibly the most potent inhibitor of STAT3 activation yet discovered, with an IC50 = 50 nM. Currently this compound is progressing to pharmacokinetic and ADME studies. Further information about this compound will be forthcoming in the near future.

The remainder of this report focuses on the chemical and biological properties of CM-978.

1.2 Compound information: CM-978

1.2.1 Compound specifications

Nombre del compuesto (CEAMED): CM-978

Chemical properties

Mol. Formula: $C_{24}H_{19}NO_4$	MW: 385.4
Polar Surface Area: 73.3	ClogP: 3.20
# H-bond acceptors:: 5	
# H-bond donors: 0	
# rotatable bonds: 1	

In-silico predicted drug-likeness and oral bioavailability measures.

Conforms to Lipinski´s rule of 5: Yes
Conforms to Ghose´s "drug-likeness": Yes
Predicted oral bioavailability (Veber rule): Good
PAINS: 1 alert (quinone)

1.2.2 Chemical synthesis:

- Converging two-step process.
- Reagents are either commercially available or readily prepared on-scale. Reagents are inexpensive.
- Yields are typically between 27-33% (multi-gram quantities).
- CM-978 is a crystalline solid.

- Purification: Crystallization.
- CM-978 is a racemic mixture of enantiomers. To date no studies have been done to prepare, or separate, the two enantiomers.

1.3 Patent Landscape

A European patent contain CM-978 has been granted (EP 2690094) for its use as a medication in the treatment of a disease characterized by the abnormal activity of a JAK-STAT signaling pathway.

1.4 Assesment of CM-978 as an inhibitor of interleukin (IL) activated STAT

1.4.1 Identification of CM-978 as an inhibitor of IL-3 induced STAT5 transcription

Using a luciferase tagged, STAT5-dependent reporter gene assay (in BaF3 cells), we demonstrated that CM-978 rapidly (incubation time = 6 h) reduced STAT5 transcription activity induced by IL-3 (Figure 3). CM-978 did so in a concentration dependent manner, with an IC50 ~ 0.7 μM.

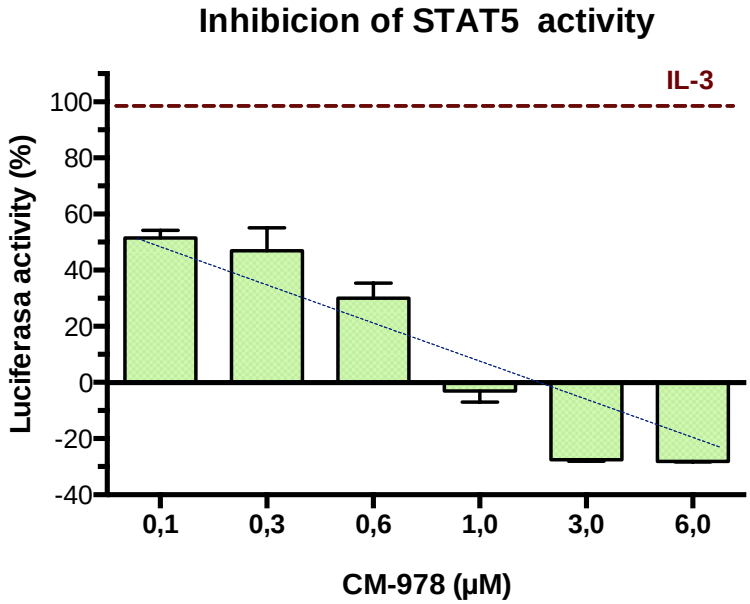

Figure 5: Concentration dependent Inhibition of STAT5 activation by CM-978

1.4.2 Identification of CM-978 as an inhibitor of IL-3 induced STAT5 transcription

Using a luciferase tagged, STAT3-dependent reporter gene assay (in BaF3 cells), we demonstrated that CM-978 rapidly (incubation time = 6 h) reduced STAT3 transcription activity induced by IL-6 (Figure 3). CM-978 did so in a concentration dependent manner, with an IC50 ~ 0.6 μM.

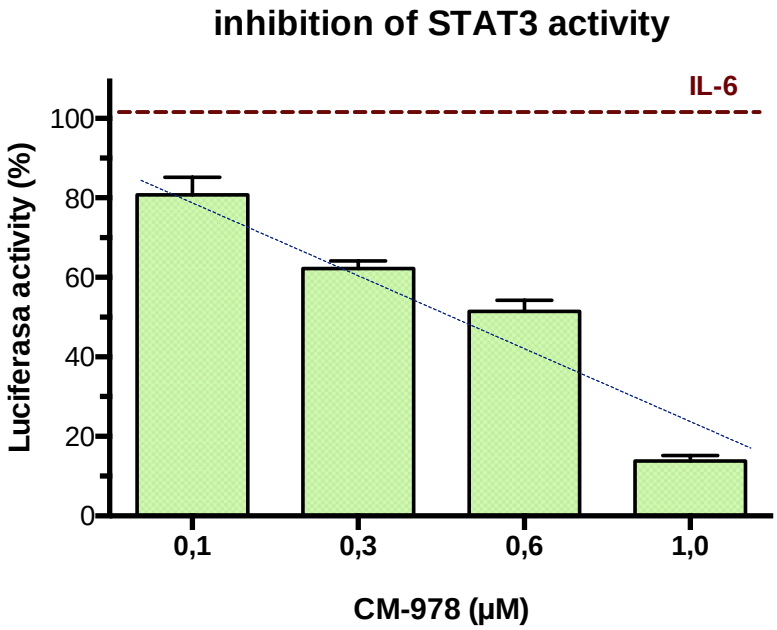

Figure 6: Concentration dependent Inhibition of STAT5 activation by CM-978

1.4.3 Western blot analysis

The human dervied triple negative breast cancer (TNBC) cell line BT549 has STAT3 constitutionally activated (PY-STAT3). After incubating BT549 cells with CM-978 at 5 μM for only 3 h western blot analysis clearly showed a dramatic decrease in STAT3 activation. A similar effect was seen in another TNBC cell line harbouring activated STAT3 (HS578T) (figures 7 & 8).

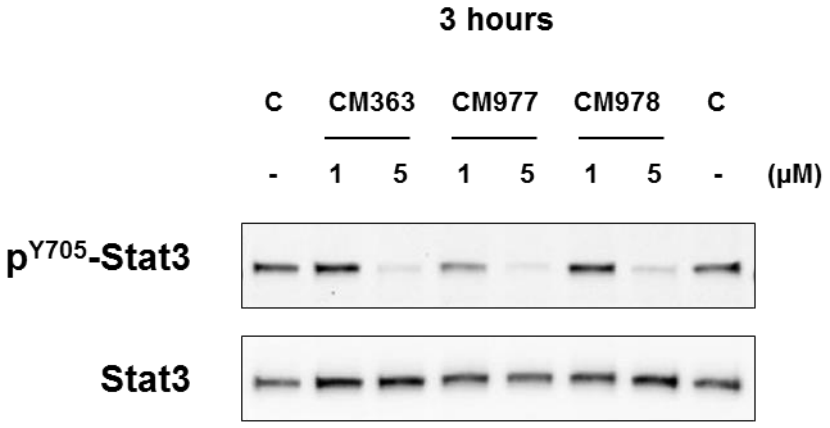

Figura 7. Inhibition of the activation of STAT3 by CM-978 in BT549 triple negative breast cancer cells.

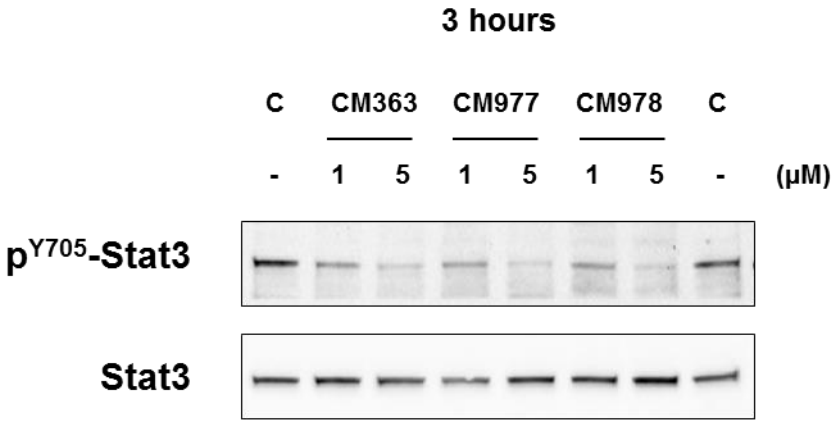

Figura 8. Inhibition of the activation of STAT3 by CM-978 in HS-578T triple negative breast cancer cells.

1.5 Assessment of CM-978 in STAT3-dependent cancers.

CM-978 was shown to reduce the viability of several STAT3-dependent tumor cell lines with IC50 values between 1-4 μM **(Table 1).** These results demonstrate that CM-978 is a potent, cell permeable cytotoxic agent.

Breast		Prostate	Lung	Colon
MDA-MB-231	T47D	DU-145	NCI-1299	HT-29
0.7	0.9	2.0	1.1	4.0

Tabla 1. IC_{50} (μM) data (cell viability assay, MTT, 48 h) for CM-978 in a variety of non-hematological human cancer cell lines.

1.6 In vivo toxicity studies

1.6.1 IRWIN test (IP administration)

Study design:

- Animals: FVB mice (female, 6 weeks old)
- Dose: 20 mg/Kg (IP) mg/kg
- Dosing: 5 consecutive days

Results: IP administration (Figure 9)

External Observations:

- No significant signs of toxicity.
- Corporal weights maintained.
- Piloerrection was the most constant observation, and was generally reversible after 90 minutes.

Internal observations (autopsy):

- No signs of organ damage or changes in internal organs were observed.

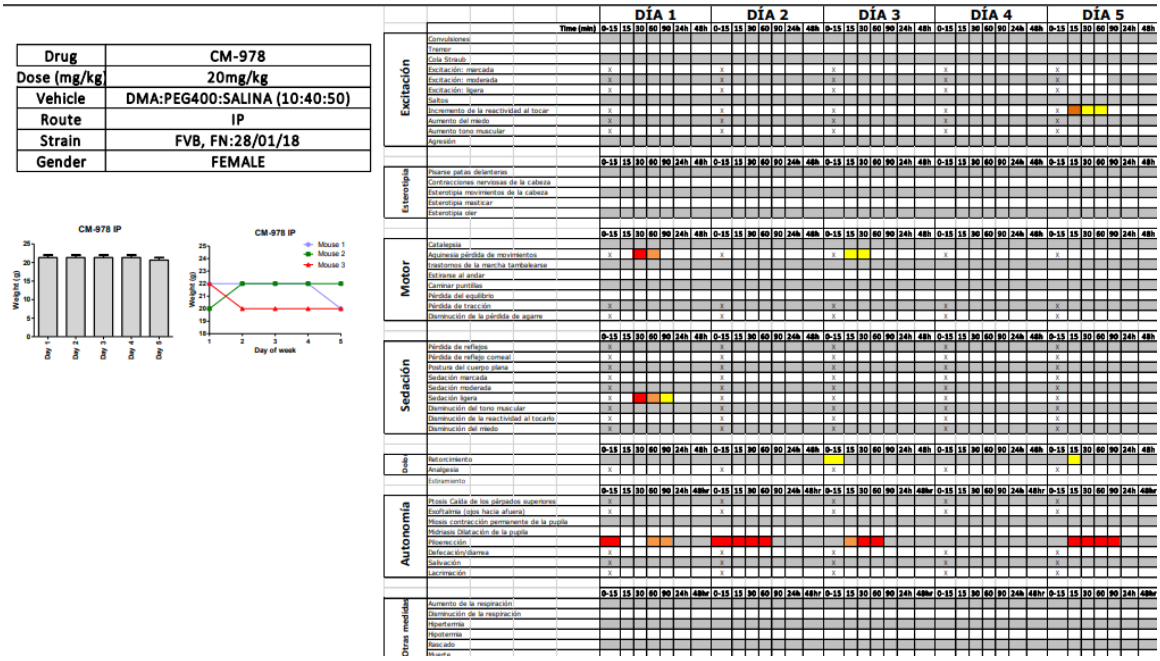

Figura 9. Daily observations after doisng mice with CM-978 (20 mg/kg) IP

1.6.2 IRWIN test (PO administration)

Study design:

- Animals: FVB mice (female, 6 weeks old)

- Dose: 50 mg/Kg (IP) mg/kg
- Dosing: 5 consecutive days

Results: PO administration

External Observations:

- No significant signs of toxicity.
- Corporal weights were either maintained or increased.

Internal observations (autopsy):

- No signs of organ damage, or changes in internal organs were observed.
- Possible signs of compound in stomach.

Conclusions (5-day acute toxicity): CM-978 was well tolerated during a 5 day dosing period, both IP & PO.

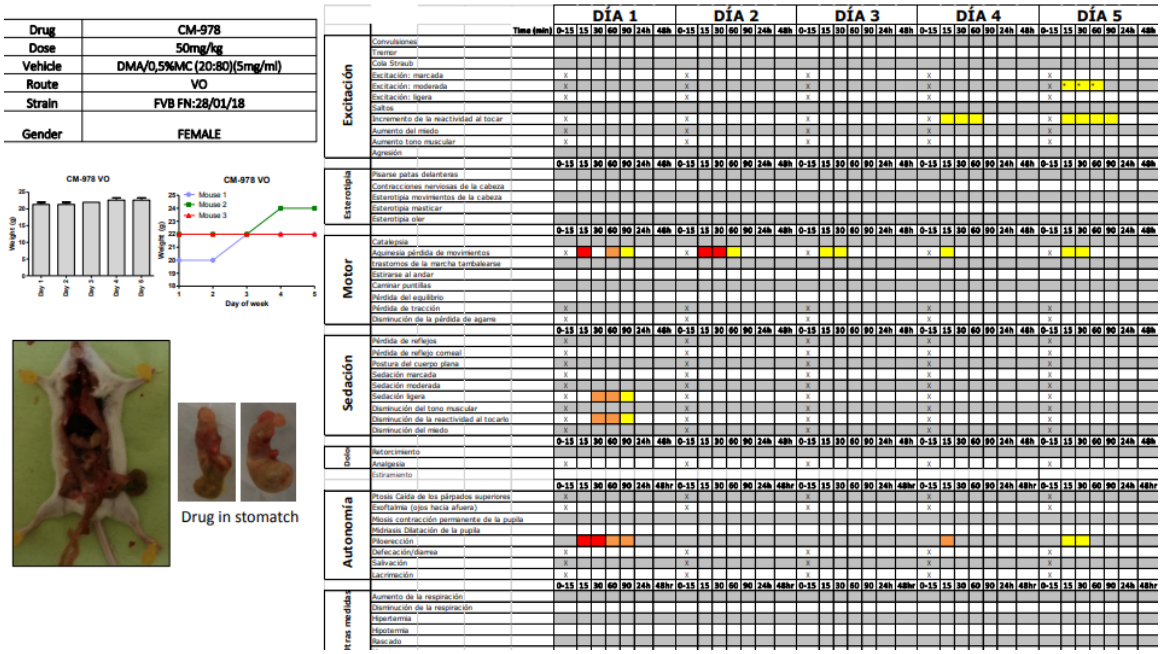

Figura 10. Daily observations after doisng mice with CM-978 (50 mg/kg) PO.

1.7 Pharmacokinetic and ADME studies

1.7.1 Rats PK

Study conducted by Advinus Eurofins.

Study design:

- Animals: Male Sprague-Dawley rats
- Routes of administration: IV and PO

- Doses: IV: 1 mpk; PO: 10 mpk

Results (Figure 11 y table 2):

- CM-978 is bioavailable after PO dosing is high (%F = 95) .
- The IV half-life is good (1.8 h).
- The clearance rate is low, while the level of distribution is high.
- The Cmax obtained after PO (10 mpk) dosing is > 1 μM IC_{50} for 5 h.

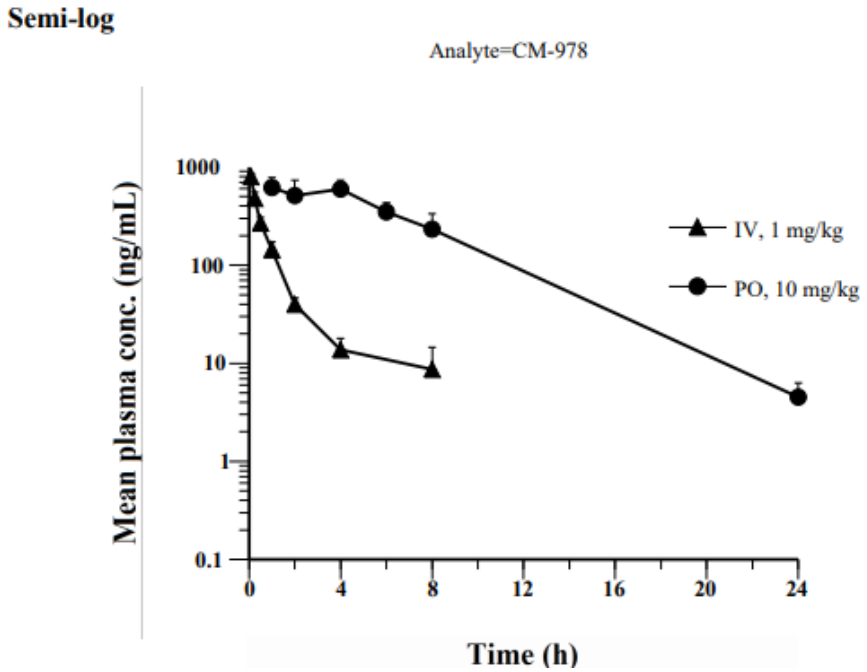

Figura 11. Plasma concentrations of CM363 in rats after dosing via IV, IP or PO.

Treatment	Route/ Dose (mg/kg)	T_{max}[a] (h)	C_0/C_{max} (ng/mL)	AUC_{last} (ng.h/mL)	CL (mL/min/kg)	V_{SS} (L/kg)	$T_{1/2}$ (h)	%F[b]
CM-978	IV / 1	NA	1030 ± 67.6	568 ± 88.4	28.8 ± 4.61	2.38 ± 0.295	1.84 ± 0.482	NA
	PO/ 10	4.00 (2.00-4.00)	632 ± 145	5400 ± 1480	NA		5.29[d] ± 0.643	95

Tabla 2. Calculated Phamacokinetic properties of CM-978 after IV and PO dosing in rats.

1.7.2 Mice PK

Study conducted by Advinus Eurofins.

Study design:

- Animals: Female Balb/c mice
- Routes of administration: PO
- Doses: PO: 5, 10 and 30 mpk

- Frequency: Once per day for 5 consectutive days.

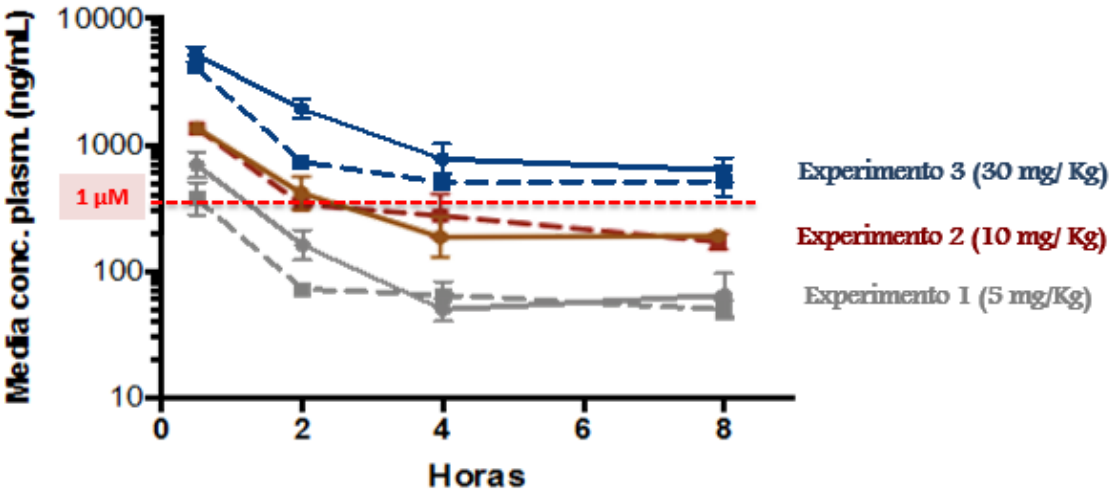

Figura 12. Concentración plasmática de CM-978 en ratones tras la administración por vía oral a distintas dosis. Líneas sólidas (día 1), líneas discontinuas (día 5).

Results:

CM-978 is orally bioavaible in mice. AUC increases with dose, and no significant signs of induction were seen. Plasma concentration levels above 1 micromolar were attained for at least 8 h after oral dosing with 30 mg/kg of CM-978.

1.7.3 Intrinsic clearance (Human liver microsomes)

Study design: CM-978 was incubated at a concentration of 1 x 10^{-7} M with human livermicrosomes and the quantity of CM-978 remaining after T = 0, 15, 30, 45 and 60 minutes were quantified by LC/MS/MS.

Time	% CM-978 remaining n =1	% CM-978 remaining n =2
0	100.0	100.0
15	101.3	101.3
30	96.8	100.0
45	96.2	96.0
60	91.7	92.0

These values result in a mean Half-life of > 60 mins and a Cl_{int} value of <115 µL/min/mg, indicating that CM-978 is stable to metabolism by human liver microsomes.

Conclusions (Pharmacokinetics):

CM-978 is orally bioavailable, has a high level of distribution and a good half-life in rats. In mice, sustained plasma levels of > 1 μM can be achieved for > 8h after dosing orally with only 30 mg/Kg.

1.8 Summary

- CM-978 is an inhibitor of the activation of IL-3 induced STAT5, and IL-6 induced STAT3 with IC_{50} values < 1 μM. This is 100-fold more potent than the natural product silibinin and similar to the effects seen by Ruxolitinib.
- CM-978 significantly reduces the levels of activated STAT3 (PY-STAT3) in cancer cells having STAT3 constitutively active, resulting in their reduced viability.
- CM-978 is highly bioavailable after oral dosing, and possess a good PK profile in both mice and rats.
- Human liver microsome data suggests that it will be stable to hepatic metabolism in humans.

1.9 Perspectives

- CEAMED is hoping to rapidly advance CM-978 into *in vitro* and *in vivo* studies in order to investigate its effect on viral replication and cytokine release due to SARS-CoV-2 infection.

Based on the data currently available, CM-978 possesses favorable characteristics to further elucidate the potentially beneficial role of inhibiting IL-6 induced STAT3 signalization in COVID-19.