

9-Xanthone as a Novel Angiotensin Converting Enzyme 2 Activator: Context-Specific Chemistry in Breast Cancer

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ABSTRACT

Background: Current study evaluates 9-xanthone as an angiotensin-converting enzyme 2 (ACE2) activator and its effect on viability of breast cancer cell lines. Finally, using a chemical narrative review, it formulates a hypothesis for context-specific effects of ACE2 modulators in breast cancer, novel coronavirus, and nephrotoxicity.

Methods: ACE2 activation was confirmed using in vitro activity assay. Next, ACE2 expression after exposure to 9-xanthone was analyzed in the supernatant samples from breast cancer cell lines (MCF-7, MDA-MB-231, and EMT-6). Additionally, ACE2 activity was confirmed in cell line pellets using a fluorometric activity assay kit. Finally, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out at the same concentration ranges.

Results: 9-xanthone is a mild, dose-dependent, ACE2 activator at 50 to 750 μ M. Carnosine, a mild ACE2 inhibitor, partially reverses this effect. ACE2 expression and viability for MCF-7 consistently rose at all doses between 2- and 24-hours post exposure. Similarly, 9-xanthone increased expression of EMT-6. However, the drug showed cytostatic effects on MDA-MB-231 and EMT-6 cell lines. Similar chemical structures consistently affected breast cancer activity, coronavirus infectivity, and nephrotoxicity.

Conclusion: Further exploration of xanthenes and ACE2 in breast cancer, novel coronavirus disease, and nephrotoxicity is much warranted.

Keywords: Breast Cancer; Kidney Failure, Chronic; Chronic Disease; Preventive Medicine; COVID-19.

Introduction

Breast cancer and novel coronavirus disease (COVID-19) seem to have a bidirectional relationship. On one side, COVID-19 increases chemoresistance and, on the other, breast cancer makes patients more vulnerable to the infection (1). Angiotensin-converting enzyme 2 (ACE2) has emerged as a common step in the pathogenesis of both diseases where survival is more likely with its heightened expression (2). However, normal breast tissue has higher ACE2 levels than the cancerous counterparts. To further complicate the matter, Bhari et al found that cancerous subtypes all have lower ACE2 expression than normal cells but the luminal category have both a better prognosis and the lowest ACE2 levels (3). Consistently, another group confirmed that ACE2 confer poor prognostic routes in HER2-enriched tumors (4). While one more article has validated this latter finding and shown that it is mediated by ROS-AKT-HIF-1 α pathway. It is noted that proliferation seems to be an independent process than cancer cytotoxicity (5). Moreover, ACE2 undergoes downregulation post severe acute respiratory syndrome

coronavirus 2 (SARS CoV-2) infection. Subsequently, levels of CD8+ T cells, CD4+ T cells, and neutrophils increase, thus worsening luminal carcinoma outcomes (6). In short, ACE2 level and function may be different in benign hormonal versus aggressive breast cancers.

Current research team have previously shown that carnosine is a mild ACE2 inhibitor at 100 to 300 mM. At the same concentration range, it significantly reduces viability of triple negative breast cancer (i.e., MDA-MB-231) growth with neutral effect on the luminal MCF-7 cell line (7). This is consistent with in-silico investigations by our partners which showed that carnosine has inhibitory interactions with ACE2 and ACE2-spike (8). On the other hand, the small dipeptide interferes with the ROS-AKT-HIF-1 α pathway and thus by ACE2 inhibition it may evade chemoresistance and this complements its reported anticancer effects (9,10). However, carnosine is unstable, and therefore, great efforts in drug delivery were carried out to improve its pharmacokinetic and chemotherapeutic profile (11,12). Our partners also found that LMS1007 designed by

bio isosteric replacements from carnosine and ORE1001 (aka MLN-4760) is 100 times more potent as an ACE2 inhibitor than carnosine (13). LMS1007 showed matching IC₅₀ of 2.3 mM for ACE2-SARS CoV 2 spike interactions in a manner independent of the known S1 subunit. However, it is unclear if ACE2 activators would have the opposite effects on ACE2, breast cancer, and novel coronavirus. Due to our previous research, the representative nature of the breast cancer cell lines of the different types, and the availability in our laboratories, we decided to use MCF-7, MDA-MB-231, EMT-6 and Vero cells in the current new piece of research on this matter.

Xanthenes are a family known for some of its members being among the most ACE2 activators known so far Figure 1. In particular, 9-xanthone, compound A in Figure 1, is the basic structure of xanthenes or xanthenones. This molecule has been shown to both activate ACE2 and interfere with SARS CoV2 induced respiratory distress in mice (14). However, it was not clear in that experiment if the mice would be, as expected, at a greater risk of breast cancer. The current study will serve as an *in vitro* validation of this *in vivo* experiment. Another xanthone derivative, α -mangostin induces apoptosis and arrest DLD-1, colon cancer, cells cycle (15,16). 9-xanthone and its derivatives interfere with various aspects of cancer development including but not limited to; apoptosis, inflammation, cell cycle, cell proliferation, angiogenesis, DNA repair, and immune surveillance (17,18). Reason being that their ability to interact with a wide variety of protein receptors, including topoisomerase, protein kinase, and cyclooxygenase (19). *In silico* docking and modeling anticipated that (1- [(2-(diethylamino)

ethyl] amino)-4-(hydroxymethyl)-7-[[(4-methylphenyl) sulfonyl] oxy]]- 9H- xanthen-9-one), compound B in Figure 1, is going to be one of the two strongest ACE2 activators ever known, the other one is resorcinolphthalein (20). According to this last analysis, these molecules would activate ACE2 through their reported allosteric site. However, 9-xanthone is simpler, cheaper, and readily available with 100% yield in a single synthetic step process (21). According to the last review, xanthenes have great anticancer potential but more experimental validation in animal and clinical assay models is needed. Therefore, further exploration of xanthone ACE2 modulatory and breast cancer cell line metabolic or viability effects are much warranted. However, due to limited funding, these effects will be studied while mechanistic confirmation will largely depend on our extensive literature review. One final noteworthy element in this context is that breast cancer and nephrotoxicity (leading to chronic kidney disease) seem to be linked. While some drugs like curcumin ameliorates drug-induced nephrotoxicity (22). Others like pembrolizumab and palbociclib further compound renal toxicity in breast cancer patients (23,24). Chronic Kidney Disease is implicated in many ways in novel coronavirus disease as well (25). Therefore, it seems that there is a pathologic nexus which may connect these three diseases.

Hence, the current study objective is to validate the hypothesis that 9-xanthone will be a mild ACE2 activator. Moreover, the team postulates that 9-xanthone will have pro-metabolic or viability effects in some or all of the tested breast cancer cell lines. Xanthone mild anticancer efficacy would manifest as a context-specific effect

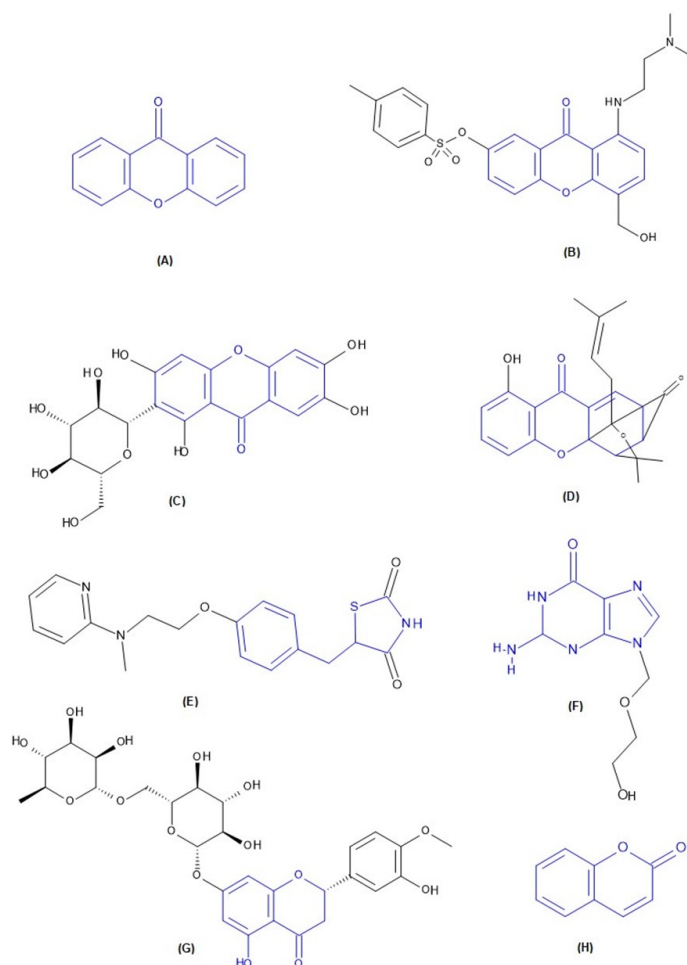


Figure 1. Xanthenes and related-chemical structure examples.

based on type of breast cancer cell line. Furthermore, cancer cell cytotoxicity may be confounded by effects on in vitro cell viability. Finally, chemically similar compounds will be narratively reviewed to elucidate the links of these structures on breast cancer, novel coronavirus and renal toxicity.

Materials and Methods

Chemicals

L-Carnosine was purchased, stored below 25 °C until use, and prepared for the experiments as previously published (7). 9-xanthone 25 g at more than 99% purity was purchased from Cgenomix company (A14812.14, Amman, Jordan). 9-xanthone was prepared by dissolving 10 mg of xanthone with different volumes of solvents including water, DMSO, media, and a mixture of DMSO-Media. A mixture of DMSO and Media was found to be most effective for dissolving higher concentrations. Then the solution was used to treat breast cancer cell lines (MCF-7, MDA-MB231, EMT-6) and Vero cell lines. Current research used all these cell lines for variation in type; luminal versus triple negative, normal versus cancer, and to cover another species in addition to human cells. An in vivo experiment was not carried out with EMT-6 for logistic reasons. All these cell lines were cultured in increasing concentrations of xanthone from 200 to 500 micromolar and incubated for 24 hours in the known 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay previously mentioned in one of research papers (7). In the expression and activity kits we used concentrations ranging from 2.3 to 150 µg/ml (11.8 to 765 micromolar (µM)). For conversion between concentrations in µg/ml and micromolar we used a molecular weight of 196.2 g/mol and the calculator found at the following link URL: <https://www.glpbio.com/xanthone.html>, accessed on December 5th, 2023). Cell lines were taken from Applied Science Private University Cell Culture Lab, Amman, Jordan. All were cultured with the given concentrations for 24 hours for the MTT assay (Genochem world, Spain). We selected these ranges for 9-xanthone on the basis of previously reported ranges by other researchers (14,15,16,17,18,19) as well as in anticipation of mild ACE2 activity for 9-xanthone.

ACE2 activity screening kit

ACE2 activity was evaluated using a fluorometric assay kit (Abcam, Cat. No. ab273297, Abcam plc, Cambridge, MA, USA) following the manufacturer's instructions with minor modification. First, we generated the control method graph for the kit which shows the activity of ACE2 in the presence and absence of the kit ACE2 inhibitor. In this kit, a synthetic peptide substrate based on 7-Methoxycoumarin-4-acetyl (MCA) was cleaved by an active ACE2 to liberate a free fluorophore. The quantification of the released MCA can be conveniently achieved by the utilization of a fluorescent microplate reader capable of measuring fluorescence at Excitation/Emission (Ex/Em) = 320/420 nm. Additionally, an ACE2-specific inhibitor (officially not disclosed but through verbal communications seem to be ORE1001 also known as MLN-4760 with an IC50 of 33 nM) was used that possesses the ability to distinguish ACE2 activity from other proteolytic activities. The kit had the capability to detect concentrations as low as 0.4 milliunits/ml. For our experiment, we used the pellets of the cell line cultures that remained after we used the supernatant for the expression experiment below. Cells were washed with PBS, harvested and lysed using

the buffer included in the assay kit. Lysates were centrifuged at 12,000 rpm for 10 minutes and supernatants were collected for protein analysis. We measured ACE2 activity in pellets of all three breast cancer cell lines in the presence of xanthone at concentrations ranging from 9.4 to 150 µg/ml (48 to 765 µM) +/- in the presence of l-carnosine at concentrations ranging from 6.25 to 100 mg/ml (27 to 434 mM). All measurements were made in triplicates. Unfortunately, we did not evaluate 9-xanthone in the presence and absence of inhibitors in supernatants. In terms of the ACE2 protein concentration in the culture cell lines we used the Bradford assay instead of the BCA assay recommended by the protocol. The assay kit was characterized by its simplicity and suitability for high-throughput applications. This experiment was conducted by a generous supervision of professors from the Hashemite University – College of Pharmacy. Instrumental devices used were previously published and the same (7). Protein concentration was measured as it is part of the activity assay protocol and to give a quality check by quantifying the amount of protein in the cell lines which enabled us to compare them to what is already known about this content in the literature.

Bradford assay

To perform the well-known Bradford assay, first, we prepared the Bradford reagent by dissolving Coomassie Brilliant Blue G-250 in a mixture of phosphoric acid and ethanol. Next, prepare protein standards by diluting BSA to create a range of concentrations (e.g., 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml). Then, we mix the Bradford reagent with protein samples and standards in a 1:5 ratio and incubate the mixtures at room temperature for 5 minutes to facilitate dye-protein binding. After incubation, 50 microliters of substrate solution were added to initiate the reaction. Subsequently, use a spectrophotometer to measure absorbance at 595 nm for both samples of cells lines and standards. Construct a standard curve by plotting the absorbance of standards against their known concentrations. Finally, determine the protein concentrations of our samples by measuring their absorbance and referencing the standard curve.

Finally, the readings were read as RFU values and the approximate conversion to ACE2 levels and optical density on a protein assay would approximately be calculated as follows:

$$1 \text{ mU of ACE2 activity} = 10,000 \text{ RFU} = 0.05 \text{ OD at } 595 \text{ nm} \quad (2).$$

Stat Tools (version 8.5.2, Palisade Corp., Ithaca, NY, USA) was then used to generate the final graphs of the ACE2 activity in all cell lines and in the presence and absence of carnosine at the various concentrations.

ACE2 Expression Kits

We have presented the details of ACE2 expression experiments in our previous research (7). This was performed to measure the ACE2 expression in the human breast cancer cell lines with or without exposure to Xanthone at various concentrations from 2.3 µg/ml up to 150 µg/ml and at two time points 2 and 24 hours of exposure. Xanthone stock was prepared under sterile conditions by dissolving 15 mg of the compound in DMSO and vortexing thoroughly to achieve full solubilization. For the experiments, breast cancer cell lines including MCF-7, MDA-MB-231 and EMT-6 were

cultured and treated with xanthone across the range (2.3 to 150 µg/ml) The procedure was carried out in the cell lines culture lab at Applied Science Private University – College of Pharmacy under the kind supervision of lab Professors and Research Assistants. Cell culture preparation was previously detailed for both MCF-7 and MDA-MB-231. Rigorous procedures ensure accurate handling of both cell lines for subsequent analyses.

ACE2 Expression in Murine Breast Cancer Cell Line

The expression levels of mouse ACE2 protein were measured in EMT-6 murine breast cancer cells using a sandwich ELISA kit, following similar kit to our previous research (7). This experiment was carried out at the Applied Science Private University (ASU) – Faculty of Pharmacy (FOP), the study examined concentrations ranging from 2.3 µg/ml to 150 µg/ml with triplicate measurements at 2 hours and 24 hours post-exposure. To construct the standard curve, the original standard (1080 pg/mL) was serially diluted with the provided diluent to produce final concentrations of 720, 480, 320, 213.3, and 142.2 pg/mL. Fifty microliters of each standard solution were added to the ELISA plate in duplicate.

Experimental samples included cell lysates prepared from EMT-6 cultures that had been exposed to 9-xanthone (150 µg/mL) for either 2 or 24 hours. Untreated lysates served as negative controls. For each well, 40 µL of sample dilution buffer was mixed with 10 µL of diluted lysate (1:5 dilution). One well was designated as a blank.

Plates were sealed with adhesive film and incubated at 37°C for 30 minutes. Following incubation, wells were washed five times using a 1:30 dilution of wash buffer in distilled water. Next, 50 µL of HRP-conjugated detection reagent was added to each well (excluding the blank), followed by a second incubation at 37°C and another wash cycle to ensure specificity.

For color development, 50 µL of Chromogen Solutions A and B were added to each well. The plate was incubated in the dark at 37°C for 15 minutes. The reaction was stopped by adding 50 µL of stop solution, resulting in a color change from blue to yellow. Absorbance was read at 450 nm using a microplate reader. Protein expression levels were calculated by interpolating the absorbance values from the standard curve.

This experimental setup allowed us to assess ACE2 expression changes in response to 9-xanthone exposure over short- and longer-term durations in EMT-6 cells, helping to establish a clearer understanding of its regulatory effect in a murine cancer model.

ACE2 Expression in Human Cell Lines

This experiment was Carried out at the ASU – FOP, the study examined concentrations ranging from 2.3 µg/ml to 150 µg/ml with triplicate measurements at 2 hours and 24 hours post-exposure to generate a standard curve, the original ACE2 standard was serially diluted with standard solvent to yield final concentration of 180, 120, 80, 53.3, and 35.5 pg/ml Fifty microliters of each dilution were added to designated wells in duplicate. Experimental samples consisted of lysates obtained from MCF-7 and MDA-MB-231 cells previously treated with 9-xanthone at a concentration of 150 µg/mL for either 2 or 24 hours. Untreated samples served as controls. Each test well received 40 µL of sample dilution buffer

followed by 10 µL of the diluted cell lysate (1:5 dilution). A blank control well was also included in each assay plate. After sealing the plate with adhesive film, samples were incubated at 37°C for 30 minutes. The wells were then washed five times using a 1:30 dilution of wash buffer in distilled water. Subsequently, 50 µL of horseradish peroxidase (HRP)-conjugated ACE2 detection antibody was added to each well, excluding the blank. This was followed by another incubation at 37°C for 30 minutes and repeated washing steps to ensure specificity.

Chromogenic detection was initiated by adding 50 µL each of Chromogen A and Chromogen B to every well. The plate was incubated at 37°C in the dark for 15 minutes. The enzymatic reaction was halted by the addition of 50 µL of stop solution, resulting in a color change from blue to yellow. Absorbance was measured at 450 nm using a microplate reader. ACE2 concentrations were interpolated from the standard curve, and all sample measurements were performed in triplicate to ensure accuracy and reproducibility.

The design and methodology of this experiment closely followed the previous research⁷. The range of 9-xanthone concentrations tested in the broader study spanned from 2.3 µg/mL up to 150 µg/mL to assess short-term and prolonged effects of exposure at 2 and 24 hours, respectively.

Cell line, Subculture, and Cellular Conditions

We have followed the same protocol from our previous published research (7).

MTT assay

The metabolic effects of 9-xanthone were evaluated using the MTT assay, following similar methods to our previous research (7). Breast cancer cell lines (MCF-7, MDA-MB-231, and EMT-6) were treated with varying concentrations of 9-xanthone, ranging up to 500 µM, for 24 hours.

Post-treatment, culture medium was carefully aspirated from each well, and MTT reagent (5 mg/mL in PBS) was added to assess cell viability based on mitochondrial activity. Plates were incubated at 37°C for 3 hours to allow viable cells to reduce the yellow MTT reagent into purple, insoluble formazan crystals. After incubation, the MTT solution was removed, and dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan product. Plates were incubated for an additional 60 minutes to ensure complete dissolution of the crystals.

Absorbance was measured at 550 nm using a microplate reader. The optical density values were used to determine the percentage of viable cells relative to untreated controls, allowing for quantification of 9-xanthone's antiproliferative activity across the different breast cancer cell lines.

Statistical analyses

Experiments all in vitro were done in three or four replicates. Similar to our previous re-search a sample size of 3 measurements would give us a power of 80% to detect a statistically significant difference at the 0.05 alpha level of significance between groups. Stat Tools (version 8.5.2, Palisade Corp., Ithaca, NY, USA) was then used to graph data and generate error bars with 95% confidence intervals (95% CI) and/or standard errors of the mean (SEM).

Effective concentration (EC50) and inhibitory concentration (IC50) were calculated as previously mentioned in our past research (7). MTT survival sigmoidal curve graphs were generated likewise as previously mentioned.

Results

ACE2 Activity and Inhibition

9-xanthone had a dose-dependent rise in ACE2 activity as can be seen in RFU values (Table 1) and ACE2 activity in mU (Figure 2). At the tested 9-xanthone concentrations, ACE2 activity was about 3x to 10x in contrast to the tested l-carnosine ACE2 activity levels. Figure 3 shows the activity levels in all three cell lines with the presence of 9-xanthone or l-carnosine across the board of concentrations. It is clear that there is about 60 to 70% difference in ACE2 activity levels for MCF-7 and MDA-MB-231 breast cancer

cell lines. Obviously, it is not clear why the EMT-6 cell line does not show this difference, it could be absent or it could be a technical error in the experiment done on this cell line. Building on our previous research work on carnosine which showed the average % of carnosine inhibition from baseline would be around 30%. The average baseline ACE2 activity in the absence of l-carnosine and 9-xanthone would be calculated as follows:

ACE2 activity baseline = ACE2 activity (in presence of l-carnosine) / 0. (1).

Therefore, the baseline ACE2 activity occur at an average RFU reading of 684. As a result, average ACE2 activity in the presence of 9-xanthone which can be calculated across all concentrations tested to occur at around RFU of 911. This means that 9-xanthone in the concentration range tested account for about 33% increased ACE2 activity.

Table 1. RFU readings ($\times 1000$) for the various concentrations of 9-xanthone and l-carnosine in the presence of homogenized tissues of the three breast cancer cell lines.

Drug	Concentration	MCF-7	MDA-MB-231	EMT-6
9-Xanthone	150 ($\mu\text{g/ml}$)	1034 ± 38	1087 ± 15	1019 ± 59
	75	916 ± 62	1014 ± 47	987 ± 101
	37.5	932 ± 48	856 ± 166	946 ± 42
	18.75	812 ± 62	806 ± 57	857 ± 84
	9.375	860 ± 24	915 ± 119	819 ± 39
l-Carnosine*	100 (mg/ml)	340 ± 33	575 ± 51	2353 ± 267
	50	111 ± 11	394 ± 36	476 ± 38
	25	621 ± 61	212 ± 11	491 ± 35
	12.5	287 ± 63	100 ± 4	2351 ± 204
	6.25	83 ± 12	37 ± 6	377 ± 40

* Projected Baseline ACE2 Activity from Previous Twin Work⁷ is calculated as follows: ACE2 activity in the presence of l-carnosine is about 30% of its baseline activity. Therefore, in the absence of l-carnosine the average ACE2 activity would be around an RFU reading of 684 units. This means that the % average activation by the range of 9-xanthone concentrations would be around 33% of the baseline ACE2 activity.

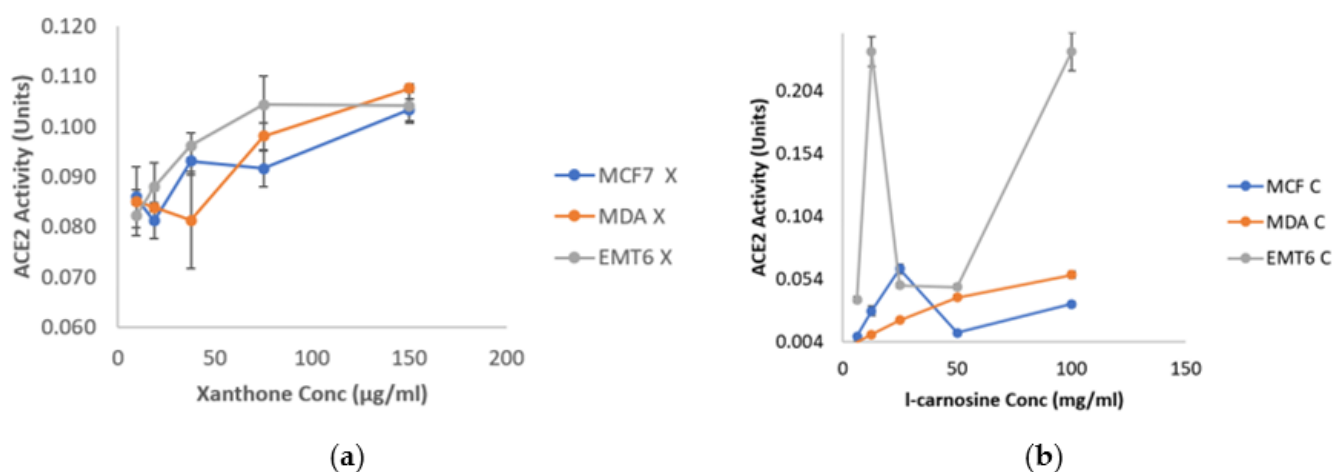


Figure 2. Dose-dependent ACE2 activity in the pellets of three different breast cancer cell lines; MCF-7, MDA-MB-231, and EMT-6. Each data-point is an average of triplicate. Error bars are Standard Errors of the Mean. (a) 9-xanthone; (b) l-carnosine.

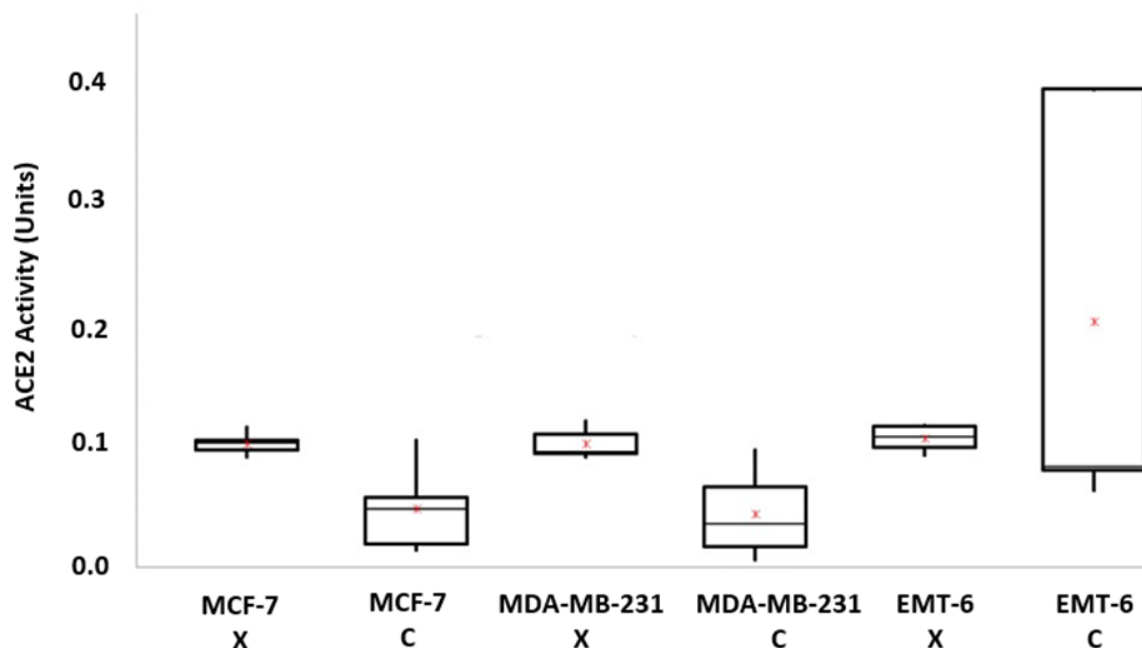


Figure 3. ACE2 activity levels in three breast cancer cell lines; MCF-7, MDA-MB-231, and EMT-6, in the presence of either 9-xanthone (X) or l-carnosine (C). Note: ACE2 Activity in Units, 9-xanthone at concentrations ranging from 9.4 to 150 $\mu\text{g/ml}$ (48 to 765 μM) +/- in the presence of l-carnosine at concentrations ranging from 6.25 to 100 mg/ml (27 to 434 mM).

Breast Cancer Cell and Total Protein Content

Figure 4 shows the total protein content in all three breast cancer cell lines at the conclusion of the Bradford assay. As expected, both the MCF-7 and MDA-MB-231 cell lines had a higher protein content than EMT-6. However, it is not clear how the MCF-7 protein content is higher than the MDA-MB-231 which is a finding inconsistent with previous reports. Yet, the ACE2 activity level in the presence of 9-xanthone is around an optical density of about 5 which is far above the optical density seen for the total protein concentration as our Bradford assay show that a total protein content of about 10 $\mu\text{g/ml}$ result in an optical density of around 0.5.

Therefore, this finding confirms that 9-xanthone significantly activate ACE2 in the activity kit. (Table 1) and ACE2 activity The text continues here (Figure 2 and Table 2).

ACE2 Expression

Table 2 shows that ACE2 expression is barely changed with 9-xanthone except for the highest two concentrations with the MCF-7 breast cancer cell line. Therefore, it seems that the ACE2 activation is more likely due to functional allosteric activation of the enzyme and not due to upregulation of the enzyme. Upregulation may partially modify the ACE2 activity, especially in some cell lines; namely MCF-7, but not all.

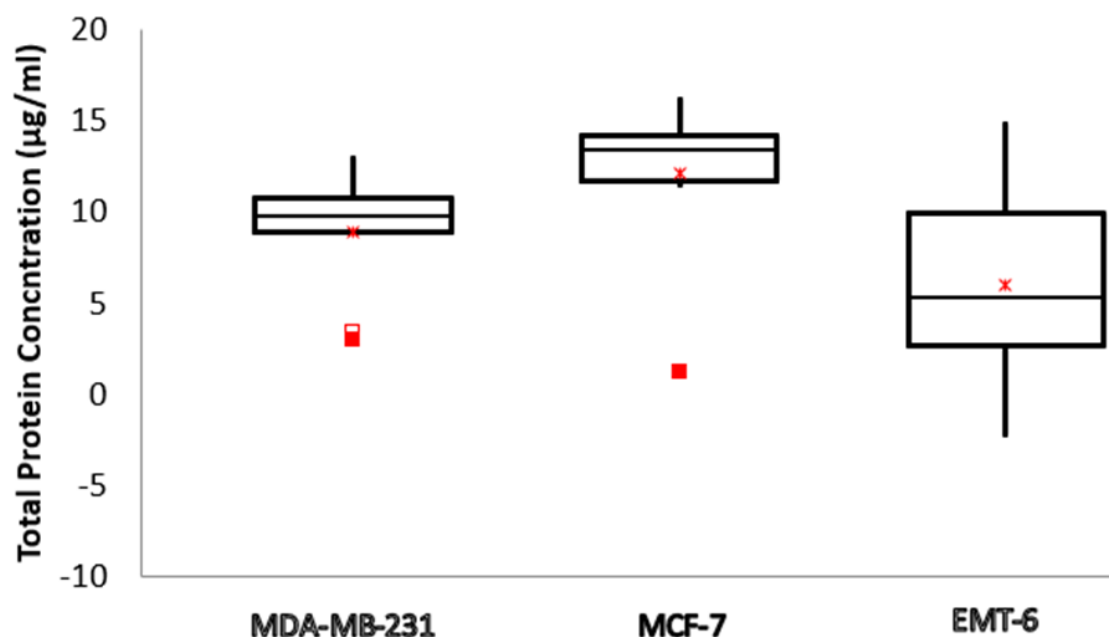


Figure 4. Determination of extracted total protein concentration of EMT-6, MCF-7, and MDA-MB-231 cell lines by Bradford assay.

Table 2. Effect of various concentrations of 9-xanthone ($\mu\text{g/ml}$) on ACE2 expressed in absorbance or optical density (OD) following 2 and 24 hours of exposure in one each of a luminal (MCF-7), triple-negative (MDA-MB-231) and mouse (EMT-6) breast cancer cell lines.

Cell Line	Test Condition ($\mu\text{g/ml}$)	2 Hours	24 Hours
MCF-7	150	3.67 ± 0.29	4.79 ± 0.11
	75	3.68 ± 0.16	4.05 ± 0.09
	37.5	3.72 ± 0.18	3.92 ± 0.09
	18.75	3.60 ± 0.06	3.43 ± 0.16
	9.38	3.75 ± 0.12	–
	4.56	3.74 ± 0.07	–
	2.28	3.33 ± 0.11	–
	Cell Line	3.66 ± 0.04	3.67 ± 0.04
	Background	0.23 ± 0.05	0.22 ± 0.04
MDA-MB-231	150	3.42 ± 0.12	3.76 ± 0.09
	75	3.59 ± 0.13	3.59 ± 0.11
	37.5	3.65 ± 0.09	3.74 ± 0.09
	18.75	3.70 ± 0.10	3.69 ± 0.14
	9.38	3.67 ± 0.07	3.68 ± 0.07
	4.56	–	–
	2.28	–	–
	Cell Line	3.74 ± 0.27	3.76 ± 0.07
	Background	0.37 ± 0.09	0.40 ± 0.10
EMT-6	150	2.55 ± 0.16	3.42 ± 0.22
	75	1.63 ± 0.20	3.49 ± 0.19
	37.5	1.43 ± 0.17	3.52 ± 0.10
	18.75	1.40 ± 0.05	3.52 ± 0.12
	9.38	1.58 ± 0.12	3.32 ± 0.15
	Cell Line	3.06 ± 0.08	3.03 ± 0.14
	Background	0.26 ± 0.12	0.20 ± 0.03

[†]Test conditions were 9-xanthone in the given concentrations from 2.3 to 150 $\mu\text{g/ml}$ or the cell line or background level of ACE2 expression.

[‡]There were no readings or measurements at the concentrations with the hyphen.

MTT Assay

9-xanthone seem to have cytotoxic effects on MDA-MB-231 and EMT-6 cell lines as the % survival was lightly reduced at values below 50% of baseline (Table 3 and Figure 5). So, it was not possible to calculate an IC₅₀ for the MDA-MB-231 cell line. A theoretical IC₅₀ of about 6 μM was calculated for EMT-6. For the vero cell line, 9-xanthone seemed to have negligible effect on survival in an MTT assay with an EC₅₀ of about 63.9 μM and this is close to the

results seen with MCF-7. However, for the MCF-7 there was massive metabolic activity in this cell line as evident in Figure 4. An EC₅₀ could be calculated as about 75 μM . Therefore, in contrast to our previous research with ACE2 inhibitors which showed marked reductions in survival for the triple negative cell lines. Here we report for the first time a massive, seemingly proliferative, effect for 9-xanthone, a mild ACE2 activator on luminal breast cancer cell. Again, the EC₅₀ was consistent in the Vero (or normal) cell line as well.

Table 3. % Survival in MTT assay for four breast cancer cell lines at 9-xanthone concentrations ranging from ~ 1.6 μM to ~500 μM (Average $\pm$ Standard Deviation).

Concentration (μM)	MCF-7	MDA-MB-231	EMT-6	Vero
1.5625	71.1 ± 7.8	70.5 ± 9.3	57.1 ± 7.4	52.7 ± 12.7
3.125	79.4 ± 4.8	64.2 ± 8	55.6 ± 3.7	47.1 ± 17.2
3.90625	87.3 ± 4.9	49.4 ± 10	94.6 ± 26.3	41.1 ± 2.1
6.25	85.3 ± 8.5	62.2 ± 5.3	51.3 ± 5.8	42.2 ± 5.8
7.8125	98.9 ± 20.2	48.3 ± 14.3	83.5 ± 12.6	37.3 ± 1
12.5	111.2 ± 2.6	53.1 ± 5.2	43.8 ± 1.7	52.8 ± 2.5
15.625	150.6 ± 11.9	47.3 ± 11.9	61.1 ± 13.8	41.7 ± 1.4
25	157.2 ± 18.3	61.2 ± 6.1	40.6 ± 7.3	61 ± 3.1
31.25	157.2 ± 17.4	47.9 ± 2.4	49.4 ± 14.8	52.5 ± 4
50	229.3 ± 9	67.1 ± 5	32.6 ± 5.6	75 ± 7.4
62.5	210.2 ± 17.7	53.4 ± 2.2	41.5 ± 2	67.1 ± 8.8
100	243.4 ± 9.8	61.6 ± 2.9	30.2 ± 1.1	85.6 ± 11
125	264.5 ± 13.6	60.2 ± 5.7	39.3 ± 9.2	106.2 ± 3
200	277.2 ± 31.9	63.9 ± 12.5	39.3 ± 3.9	64 ± 4
250	325.6 ± 17.1	51.6 ± 3.6	32.8 ± 11.4	100.8 ± 10.8
500	495.4 ± 280.5	42.4 ± 4.6	36.4 ± 7.6	101 ± 5

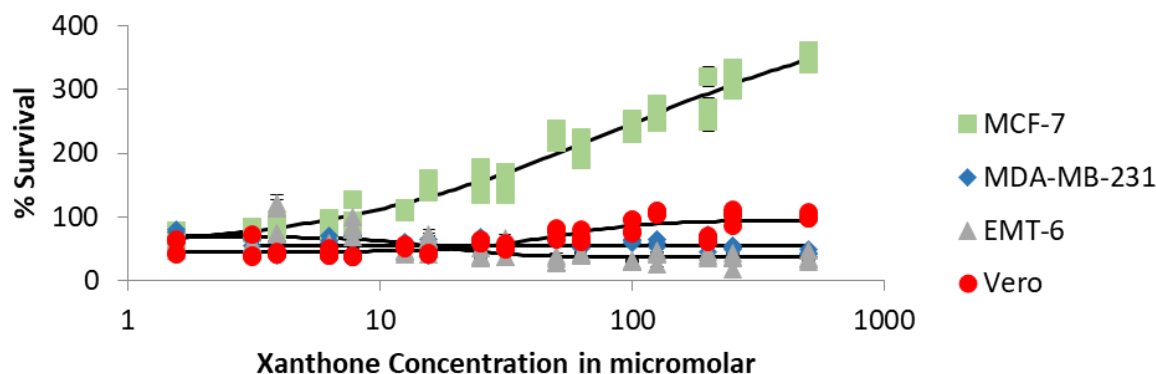


Figure 5. Average % survival in MTT assay at 9-xanthone concentrations ranging from 48 to 765 μ M for MCF-7 (Green Squares), MDA-MB-231 (Blue Diamonds), EMT-6 (Grey Triangles), and Vero Cells (Red Circles). Standard error of the means for triplicate measurements is shown as error bars.

Discussion

Xanthenes antitumor activity is well reported in the literature. For example, mangiferin, compound C in Figure 1, had significant cytotoxic effects on MDA-MB-231. However, the mechanistic pathways analyzed did not explain these effects. Therefore, ACE2 could be further explored as a potential key to understand xanthenes activity (26). Another study on a caged xanthone MAD28, compound D in Figure 1 have been consistent with our findings as well (27). Authors found that the drug has more prominent cytotoxic effects on triple negative breast cancer cell lines tested (including MDA-MB-231) compared to luminal ones (including MCF-7). It was not clear, though, why the luminal cell lines behaved less favorably compared to the more aggressive cell lines. Wang et al also showed similar results for another xanthone where IC₅₀ ranged from about 13 to 19 μ M for aggressive cancer cell lines tested including MDA-MB-231 (28). These IC₅₀ values compare well with our IC₅₀ for EMT-6, a well-known aggressive cell line in mice. Although it was difficult in our study to calculate an IC₅₀ for 9-xanthone against the MDA-MB-231 cell line, our results point approximately to the same IC₅₀ range mentioned. Giannouli et al have shown in an old study that various novel xanthone derivatives had IC₅₀ in the range from 9 to 50 μ M against MDA-MB-231 (29). They also concluded that it was more of a cytostatic than a real cytotoxic effect. This again compares well with our MTT assay results. However, our study was unique in showing that 9-xanthone possess paradoxical effects on breast cancer cell lines. At one end it compares with Giannouli in producing kind of a static effect on MDA-MB-231 viability. At the other, it shows paradoxically increased metabolic effect on MCF-7. Therefore, we conclude that 9-xanthone may indeed have rivaling cytotoxic and possibly proliferative effects in aggressive breast cancer cell lines. One noteworthy statement here is that the high micromolar concentrations in our study are strictly used to support the concept of the study. However, translation into clinical practice for xanthone itself remain largely restricted.

As far as ACE2 is concerned, Ibrahim et al has previously shown that xanthenone, although not clear which particular molecule, increases ACE2 activity and levels which result in interruption of leptin-induced hypertension, proteinuria, and endothelial activation in pregnant Sprague-Dawley rats (30). Another group have

also shown that xanthenone induced Angiotensin (1-7) (Ag1-7) and ACE2 expression, as well as exhibited anti-inflammatory and antioxidant effects, thus counteracting gentamicin-induced nephrotoxicity (31). They found that this was associated with TNF- α , NF- κ B and IL-6 reduction. These results were echoed with tacrolimus induced nephrotoxicity where again xanthenone upregulated ACE2 and minimized inflammation and oxidative stress (32). Here, p-ERK/ERK and p-p38 MAPK/p38 MAPK inflammatory signals were downregulated. Azouz et al also found the same mechanistic pathway was modulated such that xanthenone reduced the amphotericin B induced nephrotoxicity in rats (33). Our study showed ACE2 upregulation and activation in EMT-6 at 24 hours post exposure to 9-xanthone. This followed an initial reduction in expression at 2 hours. These findings are consistent with the last two research groups (32,33). ACE2 is linked to p-ERK/ERK and p-p38 MAPK/p38 MAPK in all the forementioned studies. Therefore, it seems that ACE2 plays an important role in the anti-inflammatory and anti-oxidant effects of xanthenones. Context specificity is evident where another group have shown three xanthenone ACE2 activators are associated with PI3k/Akt/mTOR Pathway inhibition in the triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 (34). Therefore, it seems that ACE2 activation may result in different downstream pathway effects in different in vivo models and cell lines.

Now pivotal related molecules potentiated anti-breast cancer effects while either stimulate or reduce nephrotoxicity of others such as Rosiglitazone, compound E in Figure 1 combined with cisplatin (35). Acyclovir, compound F in Figure 1 is a another substrate for breast cancer cells and its intracellular uptake is increased while at the same time nephrotoxicity is reduced in breast cancer patients taking it (36). Cisplatin is one of the most potent breast cancer drugs that suffer from the curse of nephrotoxicity as well (37). Nordihydroguaiaretic acid which has two aromatic benzene rings like 9-xanthone, yet they are connected with a flexible link rather than a cyclic structure, potentiated anti breast cancer effects of cisplatin (38). At the same time, it did reduce its nephrotoxic effects in animal models. As we have previously mentioned, curcumin increases cisplatin chemotherapeutic effects in breast cancer rats while reducing cisplatin-induced nephrotoxicity (22). Pembrolizumab has a similar nephrotoxicity curse like cisplatin (23). Even CDK 4/6 inhibitors approved for HR+/HER2-breast cancer;

including Palbociclib, ribociclib, abemaciclib, and dalpiciclib had increased nephrotoxicity risk as well (24). Hesperidin, compound G in Figure 1 a molecule, quite close to xanthone molecule in basic chemical structure, has been shown again to improve cisplatin anti-tumor effects against MDA-MB-231 while at the same time alleviating cisplatin induced-nephrotoxicity (39). Therefore, it seems that closer we are to 9-xanthone there seem to be a fingerprint for breast antitumor effect while at the same time reducing the related nephrotoxicity. Researchers will only need further explore these structures by looking more into it. ACE2 seems to be an important protein that may give us clues as to the optimal chemistry needed to produce cure for breast cancer yet reduce the myriads of related side effects. These findings are fully consistent with previous studies that have shown that ACE2 inhibition is associated with reduced proliferation of the normal and luminal breast cancer cell lines (7). Making ACE2 inhibition seem more helpful across the board of all breast cancer cell culture tissue types. It also explains the cross talk that exists between breast cancer and novel coronavirus infection where another ACE2 inhibitor; namely, LMS1007, inhibited ACE2-viral spike interaction with potentially saving these infected patients from worse breast cancer outcomes (13). This LMS1007 has been published as a patent by our colleagues (40).

Now, can we anticipate a role for ACE2 in Nephrotoxicity? Abbas et al have shown that prophylactic administration of LCZ696 which is a well-known new medication which increases heart failure survival, also protect against cisplatin-induced nephrotoxicity through upregulating ACE2 (41). This is the known drug sacubitril/valsartan which is a patent of another Jordanian colleague and a breakthrough in heart failure therapy (42). The complete path which is upregulated being apelin-13/ACE2, miR-200, versus down-regulating TGF- β /SMAD 2/3 and miR-192. Going a bit further in the chemical structure to Chenodeoxycholic acid have shown that this molecule effectively reduces cyclosporin induced nephrotoxicity through upregulating ACE2 but without any evidence so far on it being potentially effective for breast cancer (43). Coumarins compound H in Figure 1, which are close chemical relatives of 9-xanthone, possess significant ACE2 modulation. These compounds have proven anti-novel coronavirus activity, potent breast cancer inhibition, and attenuation of potassium bromate-induced nephrotoxicity (44,45,46,47). Therefore, as the reader can see this is an emerging novel, where ACE2 may indeed be very crucial in deciphering the quite related yet divergent biological paths between coronavirus infections, breast cancer and renal failure. Therefore, the current work adds an important block. 9-xanthone, a basic structure of ACE2 activators, increase ACE2 activity and may up-regulate it as well at the same concentrations that demonstrate increased viability in hormonal breast cancer cell line MCF-7. 9-xanthone also possesses a cytostatic effect on aggressive breast cancer cell lines while seem to increase proliferation of normal- and luminal breast cancer cell lines through contrasting downstream pathway effects. Finally, these relations of ACE2 activation, COVID-19 outcomes, and nephrotoxicity, are mere hypotheses and await experimental proof in future investigations.

Limitations of the study

The study did not investigate all the biochemical aspects of ACE2 involvement in breast cancer proliferation. However, this can

be further investigated by the research community to try and make up the complete puzzle image.

One noteworthy limitation is the high micromolar concentrations of xanthone in our study. These are strictly used to support the concept of the study. However, translation into clinical practice for xanthone itself remain largely restricted. Further validation of the hypotheses generated by this study are largely warranted.

Conclusions

Researchers need to put more effort on understanding ACE2 enzyme and its role in breast cancer, novel coronavirus, and renal disease. Scientific community worth try and find chemicals that can target breast cancer and novel coronavirus while reducing related side effects.

Patents

This work followed a series of articles that started with an article in *Molecules* and a subsequent patent publication (8) (40).

Author Contributions

Conceptualization, L.S.; methodology, Z.A. and I.M.; software, L.S. and I.M.; validation, Z.A., I.M. and S.H.; formal analysis, Z.A. and L.S.; investigation, Z.A., S.M., I.M. and S.H.; resources, I.M., S.H. and W.T.; data curation, Z.A. and L.S.; writing—original draft preparation, L.S.; writing—review and editing, Z.A., S.M., I.M., S.H. and W.T.; visualization, L.S.; supervision, S.H. and W.T.; project administration, L.S.; funding acquisition, Z.A., L.S. and W.T. All authors have read and agreed to the published version of the manuscript.

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Data is presented in tables and figures. Raw data are available upon a reasonable request.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

No supplementary material.

Abbreviations

The following abbreviations are used in this manuscript:

ACE2: Angiotensin Converting Enzyme 2

COVID-19: Coronavirus disease of 2019

SARS CoV 2: Severe acute respiratory syndrome coronavirus 2

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