**Sex and age hormones as host factors in modulating Coronaviruses infection: *Invitro* and *in-silico* analysis**

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Abstract

keywords

**Introduction**

In many viral infections, gender plays a role in susceptibility to viral infections, pathogenicity and mortality. These differences are in most part related to the exposure rates and to the access to medical treatments and health care facilities **(**[**Organization, 2007**](#_ENREF_42)**)**. However, males were proven to be more susceptible to most viral infections. As in case of Dengue virus, hantaviruses, hepatitis B virus (HBV) and hepatitis C virus (HCV), that are more detected in male than female **(**[**Balogun *et al.*, 2009**](#_ENREF_3)**;** [**Jacobsen and Klein, 2021**](#_ENREF_27)**;** [**Klein *et al.*, 2011**](#_ENREF_33)**;** [**Tsay *et al.*, 2009**](#_ENREF_57)**)**. Not just due to male higher exposure, some studies linked the bias in HBV infection toward male due to presence of testosterone and androgen receptors used to activate HBV genome transcription **(**[**Breidbart *et al.*, 1993**](#_ENREF_6)**)**. Males also show more sever diseases when infected with Epstein Barr virus, HBV, HCV, and West Nile virus **(**[**Jacobsen and Klein, 2021**](#_ENREF_27)**)**. The Macrophage cells in males are more susceptible to HIV infection than female macrophages **(**[**Szaniawski *et al.*, 2019**](#_ENREF_55)**)**.

on the other hand, other viruses are more dominant and causes more severe infections in females than in males as in case of herpes simplex virus type 2 (HSV2), Ebola hemorrhagic fever (EHF), cytomegalovirus, and human T-cell leukemia virus type 1 **(**[**Balogun *et al.*, 2009**](#_ENREF_3)**;** [**Cobra *et al.*, 1995**](#_ENREF_9)**;** [**Eshima *et al.*, 2009**](#_ENREF_16)**;** [**Tsay *et al.*, 2009**](#_ENREF_57)**;** [**Villacres *et al.*, 2004**](#_ENREF_59)**)**. Despite that most reported cases of dengue infections are in males, dengue infection shows more severe symptoms in female cases. A study from Puerto Rico reported that females show more severe pain and other studies reported menorrhagia **(**[**Cobra *et al.*, 1995**](#_ENREF_9)**;** [**Gubler, 1998**](#_ENREF_19)**;** [**Organization, 2007**](#_ENREF_42)**)**. Males are supposed to be more susceptible to getting Ebola hemorrhagic fever (EHF) due to higher exposure rates, however female infection rates are often higher than male infections **(**[**Organization, 2007**](#_ENREF_42)**)**. Also, during Asian flu pandemic with A/H2N2 virus, the mortality rate of males was less than females at the same age group. **(**[**Serfling *et al.*, 1967**](#_ENREF_49)**)**, Hence it could be assumed that gender may be a factor in many diseases, and not just due to the exposure rates.

Regarding coronaviruses, the outbreak of SARS that occurred in 2003 infection rates were slightly higher in females, however, females had lower mortality rates from SARS than males even when correlated with age **(**[**Karlberg *et al.*, 2004**](#_ENREF_32)**;** [**Organization, 2007**](#_ENREF_42)**)**. Middle east respiratory syndrome MERS-CoV infections were also reported to bias toward males **(**[**Hajjar *et al.*, 2013**](#_ENREF_22)**;** [**Jansen *et al.*, 2015**](#_ENREF_28)**)**. And in case of the new severe acute respiratory syndrome-2 SARS-CoV-2 pandemic, which was first reported in late 2019 and still possess a great threat to human health **(**[**Guo *et al.*, 2020**](#_ENREF_20)**)**, The primary data in China till February 2020 documented it 2,442 death case, most of them were at elder age, while two-thirds of them were males **(**[**Jin *et al.*, 2020**](#_ENREF_29)**)**. [**Scully *et al.* (2020)**](#_ENREF_47) reported that the infection rate is higher in male than female by 1.7 time from the data analysis of the infection and mortality rates in 38 countries . Data from many countries supports the hypothesis that severe COVID19 is linked to male more than female infections, and also that the ICU administration is twice in male as it in females **(**[**Bischof *et al.*, 2020**](#_ENREF_5)**;** [**Islam *et al.*, 2021**](#_ENREF_26)**;** [**Jacobsen and Klein, 2021**](#_ENREF_27)**)**. The available data illustrated that the infection and death rate by COVID-19 are higher in male rather than female in all age groups **(**[**Abduljalil and Abduljalil, 2020**](#_ENREF_1)**;** [**Nguyen and Chinn, 2021**](#_ENREF_41)**)**. Other studies showed that difference between genders in the rate of SARS-CoV-2 infections is present, but is non-significant **(**[**Gomaa *et al.*, 2021**](#_ENREF_18)**)**.

The mystery question we address here is if the infection by SARS-CoV-2 is really gender biased or not, and what could be the factors causing this bias. So far, there are many hypotheses explaining why gender could be linked to infection by SARS-COV-2. the bias could be linked to whether it is due to the receptor activity difference linked with gender, if it is linked to the direct effect of sex steroids on the innate and adaptive immune functions, or if sex hormones play role in viral infection steps.

One example of the direct interaction of hormones to viral particles is the HCV, which acquire a progesterone response element and could be under the regulation of progesterone receptor **(**[**Chan *et al.*, 1989**](#_ENREF_7)**)**. Another example of the direct link between sex hormones and viral infections is that female mice infected with SARS-CoV virus and treated with estrogen receptor antagonist showed higher mortality rates, as compared to non-treated infected female mice **(**[**Channappanavar and Fett, 2017**](#_ENREF_8)**)**. In a randomized controlled trial, oral administration of estradiol in a post menopause COVID19 women patents was positively correlated with an improvement in the clinical signs, a significant reduction in inflammatory biomarkers, and Significantly less viral clearance time **(**[**Seth *et al.*, 2021**](#_ENREF_50)**)**. And SARS-CoV-2 spike protein showed high affinity to the human estrogen receptor alpha (ERα), and is positively correlated with increased cytoplasmic accumulation of the human estrogen (Erα) . SARS-CoV-2 infected hamsters and cells showed higher accumulation of the human estrogen (ERα) and ACE2 in the lung tissue **(**[**Solis *et al.*, 2022**](#_ENREF_52)**)**.

Regarding the viral specific host receptors, both the angiotensin-converting enzyme 2 (ACE2) and the transmembrane protease serine 2 (TMPRESS2) play a key role for SARS-COV-2 to enter the cell and start its replication cycle **(**[**Majdic, 2020**](#_ENREF_36)**;** [**Zhang *et al.*, 2020a**](#_ENREF_62)**)**. SARS-COV-2 Virus binds specifically and tightly to the ACE2 receptor of the host cell using the spike protein (the main surface protein) to facilitate the entry of cell. **(**[**Tai *et al.*, 2020**](#_ENREF_56)**;** [**Zhang *et al.*, 2020a**](#_ENREF_62)**)**. Thus leads to the downregulation of ACE2 receptor expression, which leads to reduction in availability of the receptor **(**[**Mostafa-Hedeab, 2020**](#_ENREF_38)**)**. so far, severe acute lung failure could be happened **(**[**Fu *et al.*, 2020**](#_ENREF_17)**;** [**Imai *et al.*, 2005**](#_ENREF_24)**)**.

In our body, The main role of ACE2 is the regulation of fluid homeostasis in the body **(**[**Reckelhoff, 2001**](#_ENREF_45)**)**. The expression of ACE2 receptor was proven to be regulated by estrogen, through the estrogen response element (ERE) in the promotor of ACE2 gene **(**[**Stelzig *et al.*, 2020**](#_ENREF_54)**;** [**Wang *et al.*, 2021**](#_ENREF_60)**;** [**Wang *et al.*, 2015**](#_ENREF_61)**)**, and thus There is a link between female sex hormone estrogen and the lower prevalence of cardiovascular diseases in Premenopausal women **(**[**Channappanavar and Fett, 2017**](#_ENREF_8)**)**. Transmembrane serine protease 2 (TMPRSS2) also showed an important role in cleavage of S glycoprotein which consider as activation of SARS-COV-2 to facilitate cell entry **(**[**Mollica and Rizzo, 2020**](#_ENREF_37)**)**. TMPRESS2 gene expression is known to regulated by androgen in male prostate**(**[**Qiao *et al.*, 2021**](#_ENREF_44)**)**. It was found that bioavailability of TMPRSS2 and ACE2 higher in male’s pneumocytes I/II compared to female’s cells **(**[**Song *et al.*, 2020**](#_ENREF_53)**)**

Normal human bronchial epithelial cells NHEB (fully differentiated) when treated with estradiol (E2) showed significant reduction in the ACE2 expression **(**[**Stelzig *et al.*, 2020**](#_ENREF_54)**)**. A549 lung epithelial cells **(**[**Baristaite and Gurwitz, 2022**](#_ENREF_4)**)** also showed a reduced expression of ACE2 and TMPRESS2 expression when treated with estradiol . Higher activity (but not expression) of ACE2 in male mouse kidneys and adipose tissue was reported in comparison to female mice, which may explain the higher infection rates in male vs. female **(**[**Gupte *et al.*, 2012**](#_ENREF_21)**)**. In a retrospective study of hormone therapy in female COVID-19 patients, post menopause females receiving estradiol therapy showed a reduction in fatality rates by more than 50% **(**[**Seeland *et al.*, 2020**](#_ENREF_48)**)**.

Age has been a determinant in many viral disease’s infection. Growth hormones were found to stimulate the production of healthy CD4+ T cells in HIV patients **(**[**Napolitano *et al.*, 2008**](#_ENREF_39)**)**. Many viruses (as flu and CMV) utilize the epidermal growth factor receptor (EGFR) to activate cell mediated endocytosis and stimulate the viral infection. **(**[**Eierhoff *et al.*, 2010**](#_ENREF_10)**;** [**Nguyen and Kamil, 2018**](#_ENREF_40)**;** [**Zheng *et al.*, 2014**](#_ENREF_65)**)**. For many infectious diseases transmitted by communication, young adults and children are at greater risk, as in case of 1918 flu pandemic **(**[**Reid, 2005**](#_ENREF_46)**)**. However, this is not the case for COVID-19. SARS-CoV-2 infections among population under 19 years old were less compared to other age groups . the severity and hospitalization rates were also minimal **(**[**Verity *et al.*, 2020**](#_ENREF_58)**)**. Cells treated with either the growth hormone or the estradiol hormone showed elevated proinflammatory cytokines response, compared to cells treated with testosterone hormone **(**[**Zhu *et al.*, 2022**](#_ENREF_66)**)**. And in severely ill patient, it is not favored to administrate GH as this would elevate the cytokines

Here in this study we aim to address the possible antiviral activity of female vs. male sex hormones, and the possible factors causing the possible higher prevalence and pathogenicity of two beta-corona viruses ( SARS-CoV-2 and MERS-COV) and one alpha (229E) viruses in males versus female patients.

**Methodology**

**Drugs and viruses**

Raw materials from commercially available test hormones and receptor regulating agents The eight examined hormones namely; estradiol valerate, ethinyl estradiol, testosterone, testosterone enanthate, progesterone, levonorgestrel, tamoxifen, and somatropin were obtained from the Egyptian Holding Company for Pharmaceuticals and Chemicals, and the Egyptian National Organization for Drug Control and Research in Egypt. Two forms of estradiol (estradiol valerate and ethinyl estradiol),

The hCoV-19/Egypt/NRC-3/2020 SARS-CoV-2 “NRC-03-nhCoV” virus, 229E HCoV and the MERS-CoV isolate NRCE-HKU27021 were propagated in Vero-E6 cells in infection medium (DMEM with 1% pen/strep, 0.2% Bovine Serum Albumin (BSA), and 1 µg/ml TPCK-treated trypsin) at a multiplicity of infection (MOI) of 0.1. viruses were harvested at 72 hpi and stored at -80 oC. the 3 viruses were then titrated using TCID50 titration assay.

**Determination of CC50 and IC50**

In 96-well tissue culture plates, 2.4×104 VeroE6 cells were distributed in each well and incubated overnight at a humidified 37°C incubator under 5%CO2 condition. The cell monolayers were then washed once with PBS and subjected to virus adsorption for 1 h at room temperature (RT). The cell monolayers were further overlaid with 100 μl of DMEM containing varying concentrations of the tested drugs. Following incubation for 72 h, the cells were fixed with 100 μl with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 15 min at RT. The crystal violet dye was then dissolved using 100 μl absolute methanol per well and the optical density was measured at λ:570 nm using Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). Concentrations that kills half of treated cells (CC50) relative to the cell control and The IC50 required to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control were measured [[20](#_ENREF_20)].

**Docking studies**

Six different molecular docking processes were performed for the eight examined hormones (**1-8**) using the MOE 2019.012 suite **(**[**El-Demerdash *et al.*, 2021**](#_ENREF_11)**;** [**Inc., 2016**](#_ENREF_25)**)** towards HCoV-229E, MERS-CoV, and SARS-CoV-2 spike (S) and main protease (CLpro) receptors. It is worth mentioning that, the co-crystallized inhibitor for each CLpro receptor was used as a reference standard in order to compare their binding scores and interactions.

**2.1 Preparation of the examined eight hormones (1-8):**

The eight examined hormones namely; estradiol valerate (**1**), ethinyl estradiol (**2**), testosterone (**3**), testosterone enanthate (**4**), progesterone (**5**), levonorgestrel (**6**), tamoxifen (**7**), and somatropin (**8**) were downloaded from the PubChem database. Each hormone was imported into the MOE program, adjusted for partial charges, and energy minimized as described before in detail **(**[**Al-Karmalawy *et al.*, 2021**](#_ENREF_2)**;** [**El Gizawy *et al.*, 2021**](#_ENREF_12)**)**. The prepared eight hormones (**1-8**) were imported into the same database to be used in the three docking processes against the S receptors of HCoV-229E, MERS-CoV, and SARS-CoV-2. On the other hand, three different databases including the prepared eight hormones (**1-8**) were created with the addition of the co-crystallized inhibitor of HCoV-229E, MERS-CoV, and SARS-CoV-2 CLpro in each case, respectively.

**2.2 Preparation of the S and CLpro targets of** **HCoV-229E, MERS-CoV, and SARS-CoV-2:**

The S and CLpro X-ray structures of HCoV-229E, MERS-CoV, and SARS-CoV-2 were extracted from the Protein Data Bank (PDB ID: 6U7H **(**[**Li *et al.*, 2019**](#_ENREF_34)**)**, 2J98 **(**[**Ponnusamy *et al.*, 2008**](#_ENREF_43)**)**, 5YY5 **(**[**Zhang *et al.*, 2018**](#_ENREF_64)**)**, 5WKJ **(**[**Kankanamalage *et al.*, 2018**](#_ENREF_31)**)**, 6VW1 **(**[**Shang *et al.*, 2020**](#_ENREF_51)**)**, and 6Y2G **(**[**Zhang *et al.*, 2020b**](#_ENREF_63)**)**, respectively). Each downloaded protein was corrected, 3D protonated, and subjected to energy minimization processes to be ready for docking as described earlier **(**[**Elebeedy *et al.*, 2021a**](#_ENREF_13)**;** [**Elebeedy *et al.*, 2021b**](#_ENREF_14)**)**.

**2.3. Docking of the tested hormones (1-8) to the S and CLpro receptors of** **HCoV-229E, MERS-CoV, and SARS-CoV-2:**

Using the previously prepared databases, six different docking processes were performed accordingly. Each suitable database was inserted in the place of the ligand and subjected to a general docking process. Notably, each co-crystallized inhibitor of CLpro receptor was inserted in the corresponding database to act as a reference standard. Also, all of the MOE program specifications were adjusted as previously mentioned **(**[**Elmaaty *et al.*, 2021**](#_ENREF_15)**;** [**Hazem *et al.*, 2021**](#_ENREF_23)**)** before starting the docking process.

Furthermore, it is worth mentioning that three different validation processes for the MOE program were carried out. The co-crystallized inhibitor of the CLpro receptor of HCoV-229E, MERS-CoV, and SARS-CoV-2 was subjected to a separate redocking process, respectively. The valid performance in each case was confirmed by obtaining a low RMSD value (< 2) indicating the close superimposition of both the native co-crystallized and redocked inhibitor molecules **(**[**Kandeil *et al.*, 2021**](#_ENREF_30)**;** [**Mahmoud *et al.*, 2021**](#_ENREF_35)**)**.

**3. Results and discussion**

Cytotoxicity of estradiol valerate, ethinyl estradiol, testosterone, testosterone enanthate, progesterone, levonorgestrel, tamoxifen, and somatropin on VeroE6 cells were determined separately using crystal violet assay (**Fig. 1**). Results of half maximal cytotoxic concentration (CC50) of tested compounds were…… ……… respectively.

Half maximal inhibitory concentration (IC50) and selectivity index (SI=CC50/IC50) of ….. against … .

Based on these results, …..,…..,…. exhibited a potent antiviral activity against the tested …..,…..



**Table of SI :**

**3.2. Docking studies**

Based on the biological data of the eight examined hormones (**1-8**) against HCoV-229E, MERS-CoV, and SARS-CoV-2, we decided to give more in deep explanations regarding their expected binding modes and affinities using molecular docking studies. Moreover, it is worth mentioning that both the S and CLpro pockets of each virus (HCoV-229E, MERS-CoV, and SARS-CoV-2) were targeted as recommended possible mechanisms of action for the tested hormones. Therefore, estradiol valerate (**1**) and ethinyl estradiol (**2**) as the most promising members on HCoV-229E; progesterone (**5**) and tamoxifen (**7**) as the most active ones on MERS-CoV; besides ethinyl estradiol (**2**), progesterone (**5**), and tamoxifen (**7**) as the most active hormones on SARS-CoV-2 were selected for further investigations.

Regarding HCoV-229E, it was found that estradiol valerate (**1**) bound its S protein binding pocket with a binding score of -6.00 Kcal/mol (RMSD = 1.84). Although it formed no interactions its binding score indicated its promising affinity towards the HCoV-229E S protein without the aid of interacting amino acids. Moreover, it got a binding score of -5.33 Kcal/mol (RMSD = 2.24) towards the CLpro binding pocket of HCoV-229E, compared to that of the co-crystallized inhibitor which achieved a score of -5.42 Kcal/mol (RMSD = 0.90). Also, it formed one H-bond interaction with Glu25 amino acid residue, **Table 1**.

At the same time, ethinyl estradiol (**2**) was found to be able to bind with the S protein of HCoV-229E with a binding score of -5.02 Kcal/mol (RMSD = 1.62) with the formation of one H-bond with Asn450 and one pi-H interaction with Gly424 amino acids. Furthermore, it got a binding score of -4.49 Kcal/mol (RMSD = 2.05) in the binding pocket of HCoV-229E CLpro, compared to the co-crystallized inhibitor (score = -5.42 Kcal/mol and RMSD = 0.90). Notably, it formed two pi-H bonds with Glu25 and Gly26 amino acids as well (**Table 1**).

Based on the above, we can conclude that estradiol valerate (**1**) and ethinyl estradiol (**2**) have comparable binding affinities to the co-crystallized inhibitor of HCoV-229E CLpro. Besides, their binding affinities to the S protein of HCoV-229E were very promising.

**Table 1.** 3D pictures describing the receptor interactions and positioning for the most active hormonal members (estradiol valerate (**1**) and ethinyl estradiol (**2**)) inside the S (**6U7H**) and CLpro (**2J98**) binding pockets of HCoV-229E.

The **red** dash represents H-bonds and the **black** dash represents H-pi interactions.

| **Hormone** | **Receptor** | **3D pocket binding** | **3D pocket positioning** |
| --- | --- | --- | --- |
| Estradiol valerate (**1**) | **S** |  |  |
| **CLpro** | Shape, arrow  Description automatically generated |  |
| Ethinyl estradiol (**2**) | **S** |  |  |
| **CLpro** |  |  |

Regarding the MERS-CoV docking, it was clear that progesterone (**5**) formed one H-bond with Ser426 amino acid of the S pocket (score = -5.45 Kcal/mol, RMSD = 1.63). On the other hand, it formed one H-bond with Gln192 and one H-pi interaction with His166 amino acids of the CLpro binding pocket. Its binding score was found to be -5.86 Kcal/mol (RMSD = 1.61), compared to that of the co-crystallized inhibitor (score = -7.48 Kcal/mol, RMSD = 1.70), **Table 2**.

However, tamoxifen (**7**) achieved a binding score of -6.53 Kcal/mol (RMSD = 2.11) with the formation of two pi-H bonds with Lys470 amino acid of the S protein of MERS-CoV. On the other hand, it formed two H-bonds with Gly149 and Cys148 amino acids of the CLpro pocket and achieved a binding score of -7.51 Kcal/mol (RMSD = 1.40), **Table 2**.

Notably, tamoxifen (**7**) (score = -7.51 Kcal/mol) was found to be superior to that of the co-crystallized inhibitor of MERS-CoV CLpro (score = -7.48 Kcal/mol).

**Table 2.** 3D pictures describing the receptor interactions and positioning for the most active hormonal members (progesterone (**5**) and tamoxifen (**7**)) inside the S (**5YY5**) and CLpro (**5WKJ**) binding pockets of MERS-CoV.

The **red** dash represents H-bonds and the **black** dash represents H-pi interactions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Hormone** | **Receptor** | **3D pocket binding** | **3D pocket positioning** |
| Progesterone (**5**) | **S** |  |  |
| **CLpro** | A picture containing diagram  Description automatically generated | A close-up of a crystal  Description automatically generated with low confidence |
| Tamoxifen (**7**) | **S** |  |  |
| **CLpro** | A picture containing diagram  Description automatically generated |  |

Regarding the SARS-CoV-2 docking, it was obvious that ethinyl estradiol (**2**) formed two H-bonds with Asp367 and Glu375 amino acids of the S protein (score = -6.77 Kcal/mol, RMSD = 2.16). On the other hand, it formed one H-bond with Glu166 amino acid (score = -6.03 Kcal/mol, RMSD = 1.21), compared to the co-crystallized inhibitor (score = -8.29 Kcal/mol, RMSD = 1.84), **Table 3**.

However, the progesterone (**5**) binding score in the S protein of SARS-CoV-2 was found to be -6.29 Kcal/mol (RMSD = 2.09) with the formation of one H-pi bond with His374 amino acid of the pocket. Also, it showed the formation of three H-bonds with Gly143, Ser144, and Cys145 amino acids of the CLpro pocket and its binding score was -6.20 Kcal/mol (RMSD = 1.68), **Table 3**.

Finally, tamoxifen (**7**) formed one H-bond with Glu375 and one pi-pi bond with His345 amino acids of the S protein of SARS-CoV-2 (score = -6.87 Kcal/mol, RMSD = 1.41). Furthermore, it was able to interact with the binding pocket of SARS-CoV-2 CLpro through the formation of two H-bonds with Gly143 and Asn142 amino acids, besides, one H-pi bond with His41 amino acid. Its binding score was found to be -6.76 Kcal/mol at an RMSD value of 1.67, **Table 3**.

Based on the aforementioned results, we can conclude the promising inhibitory activities of ethinyl estradiol (**2**), progesterone (**5**), and tamoxifen (**7**) towards both the S and CLpro targets of SARS-CoV-2.

**Table 3.** 3D pictures describing the receptor interactions and positioning for the most active hormonal members (ethinyl estradiol (**2**), progesterone (**5**), and tamoxifen (**7**)) inside the S (**6VW1**) and CLpro (**6Y2G**) binding pockets of SARS-CoV-2.

The **red** dash represents H-bonds and the **black** dash represents H-pi interactions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Hormone** | **Receptor** | **3D pocket binding** | **3D pocket positioning** |
| Ethinyl estradiol (**2**) | **S** |  |  |
| **CLpro** |  |  |
| Progesterone (**5**) | **S** |  |  |
| **CLpro** |  |  |
| Tamoxifen (**7**) | **S** |  |  |
| **CLpro** |  |  |

**Conclusion**

Molecular docking studies recommended the possible mechanism of action for the examined hormones (**1-8**) towards both the S and CLpro targets of HCoV-229E, MERS-CoV, and SARS-CoV-2. It was clear that especially ethinyl estradiol (**2**), progesterone (**5**), and tamoxifen (**7**) -as the most active hormones on SARS-CoV-2- are further recommended for more advanced *in vitro* and *in vivo* studies to be used in current form or even optimized to be more active drug candidates.

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