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Molecular insights on potential combination of MAPK inhibitors together with HCQ and HCQs analogs in viral infection

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Abstract

The outbreak of coronavirus disease-19 (COVID-19) has infected more than 11 million people and has claimed more than 530.000 deaths world-wide. In July 2020, still, there is no specific treatment for disease caused by the novel coronavirus. In the search to curb the global pandemic COVID-19, some eastern and developing countries have approved various treatment with controversial efficacy, among that the use of the antimalarial Hydroxychloroquine (HCQ), so far with inconclusive clinical evidence of effectiveness. On the other hand, computer-based screening suggest that HCQs analog are promising molecules, to impair viral replication in vitro[1]. Therefore, what is emerging from this complex background, is the need to understand molecular mechanism beyond drugs that can be helpful against viral infection for this and future pandemic. The intent of this Brief Report is to highlight: i) the involvement of the Mitogen Activated Protein Kinase (MAPK) cascade in viral infection and ii) the urgent need to have molecular data on the effectiveness of the combination of MAPK inhibitors together with HCQ and HCQs analogs in curbing viral infection. We are convinced that a better understanding of the patterns of elicited molecular mechanisms will be critical for new molecular approaches to this severe disease.

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Introduction

Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2)-mediated CoVid19 is a global pandemic that has infected more than 11 million people and has claimed more than 530.000 lives world-wide. During the global crisis such as that due to CoVid19, the need to discover new drugs or to repositioning old medicines for new uses is urgent. In June 2020, still, there is no specific treatment for disease caused by the novel coronavirus, but many specific treatments are under investigation, among those Remdesivir, a nucleotide analogue of adenosine 5-monophosphate with antiviral activity against hepatitis B virus (HBV) and human immunodeficiency virus (HIV), Tocilizumab (TCZ) a humanized monoclonal antibody anti interleukin-6-receptor (IL-6R), or convalescent plasma or immunoglobulins.

MAPKs are highly conserved serine-threonine protein kinases that link cell-surface receptors to transcription factors, transducing extracellular signals into various outputs, which may also impact on host defence and apoptosis. The MAPK cascade includes Extracellular Signal-Regulated Kinase (ERK1/2), p38, and c-Jun NH2-terminal kinase (JNK), with each MAPK signalling pathway consisting of at least three components, a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK[2]. The membrane proximal upstream kinase Raf-1 is activated by the small GTPase Ras, which is triggered by the canonical receptor tyrosine kinase (RTK)-Grb2-SOS signalling scheme[3]. Members of the Ras family of proteins, including K-Ras, H-Ras, and N-Ras, play a key role in transmission of extracellular signals into thecells[4]. Since decades, intense work is under way to develop and evaluate compounds that target components of MAPK pathways, for the treatment of inflammatory and neurodegenerative diseases, and of cancer[5],[6],[7].

CQ (N4-(7-Chloro-4-quinolinyl)-N1,N1-diethyl-1,4-pentanediamine) has long been used to treat malaria and amebiasis. Hydroxychloroquine (HCQ) sulfate is one of its derivatives, demonstrated to be at least 40% less toxic than CQ in animals[8], and it is still widely available for treatment of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. HCQ has shown potential to modulate COVID19 disease[9],[10], suggesting its prophylactic use against coronavirus[11]. Very recently, mefloquine, a quinoline-derivative belonging to the same class of antimalaria agents of CQ and HCQ, has been reported for their effects on ACE2 maturation[12]. Computer-based screening suggest that HCQs analogues are promising molecules to impair viral replication in vitro[1],[12]. However, to date, there are no clear data indicating that HCQ has a favourable effect on outcomes in patients with COVID-

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19. Several randomized controlled trials under way use both chloroquine and HCQ as prophylaxis, postexposure pro phylaxis, and in treatment regimens. Increasing caution should be taken about the application of CQ/HCQ in COVID-19 before conclusive findings are obtained by well-designed, multi-center, randomized, controlled studies. Informative data from the COVID-19 Global Rheumatology Registry, a registry of COVID-19 rheumatology patients, will also help understand characteristics and outcomes in patients already taking disease-modifying and immunosuppressive medication [13]. Overall, in pour opinion, at least two main issues to be rapidly solved are: i) in the clinic, due to the absence of a commonly agreed dosing protocol based on pharmacokinetic considerations, the dose and treatment duration for hydroxychloroquine (HCQ) COVID-19 disease currently vary across national guidelines and clinical study protocols) [14] (ii) in the basic science, the urgent need of detailed molecular and biological studies on HCQ and derivatives in the context of COVID-19 infection by in vitro and in vivo assays. Therefore, the intent of this Brief Report is to highlight the impact on the Mitogen Activated Protein Kinase (MAPK) cascade on the viral infection, to propose that combinatorial approaches targeting MAPK cascade together with HCQ derivatives can represent new generation antivirals. Basic molecular studies deserve further investigation, in that they may lead to new strategies controlling the replication of several viruses.

The MAPK cascade in viral infections

It is already well known that the MAPK pathways may be altered/involved in viral infection, as a cellular signalling pathways exploited by viruses for their own effective replication, translation, transport across nuclear membrane as well as capsid assembly, and spreading, as well reactivation of virus latency[15]. Interestingly, MAPK cascade also participate in regulating immune response[16]and apoptosis[17] in virus infected cells. A variety of DNA and RNA viruses induce signalling through MAP kinase cascades in infected host cells[18],[19]. MAPK signalling may either act as positive or negative regulator of viral replication, exploiting exogenous activator of the MAPK pathway, such as G-protein linked or tyrosine kinase receptors to promote their own replication[20]. MAPK pathways can be active upon both live or inactivated virus cell contact[20], and even viral secretary proteins can trigger ERK1/2 activation, likely due to the homology of viral proteins with Epidermal Growth Factor (EGF) and Transforming Growth Factor (TGF) [21]. Host factors may either support (proviral effect) or inhibit (antiviral effect) viral replication. The proviral factors may serve as targets for development of antiviral therapeutics[15]. Indeed, such widespread use of MAPK cascade by viruses suggests that this pathway may be targetable for developing broad-spectrum

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antiviral drugs. Several viruses can be sensitive to MAPK kinase inhibitor, that may have potential to act as antiviral agents, as summarized in[15]. Interestingly, drugs targeting the Raf-MAPK signalling pathways through the use of the p38 MAPK inhibitor, SB203580, inhibited effectively phosphorylation of HSP-27, CREB, and eIF4E in SARS-CoV-infected cells, as a promising new class of antiviral agents [22].

Influenza A viruses, a relevant world-wide pathogen group both in humans and different animals, causes a biphasic activation of the Raf/MEK/ERK1/2 cascade inhibited by treatment with the MEK inhibitor U0126. Inhibition of Raf signalling causes nuclear retention of viral ribonucleoprotein complexes (RNPs), impaired function of the nuclear-export protein (NEP/NS2), and concomitant inhibition of virus production. Overall, signalling through the MAPK pathway may play a key role in virus production and RNP export from the nucleus during the viral life cycle[23]. U0126 also interferes with spreading of Borna Disease Virus (BDV) to neighbouring cells, impacting on BDV-host cell interaction[24]. MAPKs including ERK1/2, JNK, and p38, play a crucial role in infection of coronaviruses, such as mouse hepatitis virus and SARS-CoV[25],[26], while the ER-stress caused by Japanese Encephalitis virus infection induces the activation of p38 mitogen-activated protein kinase (MAPK) and host cell apoptosis [27].

Moreover, the inhibition of ERK1/2 and of p38 MAPK pathway is also associated with the signalling cascade that leads to the decline in the Varicella Zoster viral progeny[28]. ERK1/2 cascade is partially activated during Varicella Zoster virus infection and contributes to the induction of cell-survival signals. In this example, for instance, c-Raf was not active, whereas its downstream kinases MEK1/2 and ERK1/2 were transiently phosphorylated. Inhibition of this signal cascade impaired virus replication and increased the apoptotic response, through the suppressed phosphorylation of Bad, a cell-death regulator and a cytosolic indirect target of ERK1/2. New insights into the molecular basis of viral hepatitis reveal that three of these agents – the hepatitis B, which infects more than 300 million people world-wide and is a common cause of liver disease and liver hepatocellular carcinoma (HCC), C, and E viruses (HBV, HCV and HEV) modulate the mitogen-activated protein kinase (MAPK) signalling pathway[28]. Dysregulation of signalling mechanisms such as ERK1/2 are known to promote several different stages of HBV infection. Among them, the HBxAg protein exerts multiple functions, including signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation. HBxAg directly activates the MAPK-ERK pathway through the activation of Ras, leading to cell progression into the S phase through the upregulation of cyclin D1[28]. Overall,

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these data open new avenues with respect to antiviral drug development. On the other hand, inhibition of cyclin dependent kinase (CDK1, CDK2, and CDK5) significantly reduces the replication of viruses as well[29] and the higher expression of CDK1 and CDK2 leads to the reactivation of the herpes virus [30]. Interestingly, inhibiting Raf associated MEK-ERK1/2 results in lowering the reactivation of Kaposi's sarcoma associated herpes virus [31],[32],[33].

Can CQ and HCQ impact on MAPK activation?

So far, there are sufficient data in the literature about the possible mechanisms of action of both CQ and HCQ drugs in human cells, that can be exploited for addressing their action against viral infection. A very recent update on human coronavirus receptors/co-receptors as possible targets for CQ-induced inhibition of the virus replication cycle has been recently published [34]. Both CQ and HCQ are weak bases that can elevate the pH of acidic intracellular organelles, such as endosomes/lysosomes, essential for membrane fusion. Indeed, CQ mainly impairs autophagy by interfering with autophagosome fusion with lysosomes rather than affecting the acidity and/or degradative activity of this organelle[35]. In addition, CQ could inhibit SARS-CoV-2 entry through changing the glycosylation of angiotensin-converting enzyme 2 (ACE2) receptor and spike protein[36], as also suggested for HCQ analogues[1].

Digging into the previously published literature of mechanism of action of HCQ revealed its possible role through the action of MAPK pathway[37]. Addressing the CQ and HCQ mechanisms on the MAPK cascade, CQ can affect the activation of p38 MAPK and cytokine production caused by bacterial CpG DNA [38], as well as ERK1/2 is also affected by virus infection and CQ[23],[37]. In peripheral blood PMBC, CQ reduced the phosphorylation event of ERK1/2 through blocking the phosphorylation event of mitogen activated protein/ERK kinase (MEK)[37]. Interestingly, the CQ inhibitory mechanism was different from that of the classical MEK pathway inhibitor PD98059, as CQ treatment resulted in deactivation of the upstream regulatory activator Raf. Moreover, they also showed that CQ treatment restored HeLa cells sensitivity to anti-Fas-mediated apoptosis in a manner similar to PD98059 sensitivity that is lost when ERK activation is interrupted in this cell line[39]. Consistent with these data, a correlation between CQ and the impaired activation of p38 MAPK, and ERK1/2 in human coronavirus 229E (HCoV-229E) infection of human epithelial lung cells, have been shown, demonstrating their involvement in the replication of HCoV-229E[40].

The MAPK pathways is also required for the production of tumour necrosis factor alpha (TNF-alpha)[37]. The application of CQ, as well as MAPK inhibitor PD98059 leads to inhibition of

lipopolysaccharide (LPS)-induced MAPK activation and TNF-alpha expression in the human THP-1 and murine AMJ2C-8 macrophage cell lines, but not inRAW264.7 cells, that does not require MEK-ERK signalling for TNF-alpha production[37],[15]. These were the first hints on the ability of CQ of profoundly affecting both signalling and expression of inflammatory cytokines. CQ was also effective in reducing lipopolysaccharide (LPS)-induced IL-1 beta release in THP-1 cells at transcriptional level and strongly inhibited phosphorylation of p38 MAPK, and to a lesser extent c-Jun N-terminal kinase and ERK1/2[41].Overall, these results show that therapeutic concentrations of CQ may interfere with MAPK pathways activation at different levels through a still partially unexplored mechanism, which can also impact, at least in part, on the observed anti-inflammatory response to this drug.

The complexity of the mechanisms of action of MAPK cascade associated with the viral activation and progeny can be highly correlated with the mechanism of action of CQ. As shown above, in the model of HCoV-229 coronavirus, CQ-induced virus inhibition occurs through inhibition of p38 MAPK[40]. Therefore, HCQ treatment can directly impact the replication of viral progeny through the inhibition of MAPK pathway. Simultaneously, the reactivation of viral infection can also be inhibited by HCQ through the inhibition of ERK-MEK1/2 signalling cascade. Mice studies are still controversial. The only viral disease where CQ was effective so far before COVID-19 era was chronic hepatitis C, suggesting an increased virological response to pegylated interferon plus ribavirin[42]. However, these drugs are of low cost, reasonably safe, and widely available in low-income countries where other pathologies like malaria are endemic[15], to be proposed as a prophylactic treatment.

MAPK activation in severe COVID-19 disease

The epidemiology of COVID19 has not yet been fully understood. It has been estimated that the number of positive people can be many times higher than the numbers available to us, with countries, such as Italy, with 10 times the estimated positive compared to the real ones. A continuous stream of new information arrives each day about COVID-19 statistics and data to date suggest that 80% of the cases are mild or asymptomatic, 15% are severe infection, requiring oxygen and 5% are critical infections, requiring ventilation (WHO report 46).

Moreover, COVID19 severe illness is more common in people who have other health problems, particularly the elderly, those with cardiovascular disease, chronic lung disease and hypertension[43]. People with diabetes are among those high-risk categories that can have serious illness if they get the virus[44]. In diabetic mellitus (DM), the MAPK cascade exerts a

regulatory role in pancreatic islet beta-cell in mediating the cellular responses to excessive generation of intracellular ROS (oxidative stress). Additionally, novel pharmacological agents targeting MAPK have potential to improve beta-cell function in diabetes[45]. Moreover, upon insulin uptake by the endothelial barrier that mediate insulin's actions in muscle, heart, fat, and the brain, the MAPK pathway enhances the expression of ET-1 and PAI-1 and migration and proliferation of contractile cells, which have proatherogenic actions. In insulin resistance or deficiency and in diabetes, there is a loss of insulin's antiatherogenic actions via IRS/PI3K/Akt cascade, all of which leads to the acceleration of atherosclerosis. In contrast the activated MAPK pathway still has pro-atherosclerotic actions[46]. MAPK is also essential in mediating the pathogenesis of renal growth seen in early DM. In a model of DM rats, Erk1/2 MAPK level and activity in glomeruli is highly increased, also due to the loss of MAPK phosphatase-1, a dual specificity phosphatase that inactivates MAPK[47], with a strong implication in the pathogenesis of DM nephropathy. it is thus tempting to speculate that diabetic patients are more prone to CoVid19 infection and subsequent death also due to the activation of MAPK pathway upon viral activation and progeny [48],[33],[31]. Moreover, the existing data on the down-regulation of MAPK pathway activity upon CQ and HCQ treatment, can be taken in account, mainly on the severity of the COVID19 disease in patients with additional health problems. Application of CQ can control the glucose metabolism in DM patient as well[49],[50]. Thus, application of CQ in DM patient can improve the glucose metabolism and down regulate the MAPK pathway which could contribute to dampen the viral activation and progeny.

The MAPK cascade is a master regulator of cell proliferation in cancer. Application of CQ and HCQ has long been studied against cancer[51], [52],[52]. A combination of ERK and autophagy inhibition was useful towards treatment of the pancreatic cancer[53]. Inhibition of ROS mediated MEK/ERK pathway induces breast cancer cell death and autophagy[54]. Elicitation of autophagy via inhibition of MEK/ERK pathway proven to be strategy for treatment of RAS-driven cancers[55]. CQ increases antioestrogen responsiveness in resistant tumour cells through the inhibition of autophagy and it has suggested to use CQ as an adjuvant to anti- cancer chemotherapy[56],[57]. It is also well known that lymphocytes play an important role in maintaining immune homeostasis and inflammatory response in our body, particularly relevant in cancer patients. It has also shown that ACE2 expression is high in lymphocytes in oral mucosa[58]. In the recent work, it is shown that the percentage of blood lymphocyte showed the most significant and consistent reduction trend among the COVID19 positive patients, suggesting that lymphopenia could be a predictor of prognosis among

COVID-19 patients[59]. Since it has been well known that CQ inhibit the MEK/ERK pathway in human, the inhibition of MEK/ERK1/2 pathway via CQ-mediated treatment can be further investigated as a boon and double benefit for the CoVid19 patients suffering with cancer problems.

Many patients around the world have been already treated with these drugs in this pandemic situation. The data presented here outline that the MAPK cascade, which, in turn, is a key pathway in regulating different steps of viral replication, transport, translation, capsid assembly, and spreading, may be affected at some levels by CQ and HCQ treatment. Moreover, MAPK may also control inflammatory cytokine production and cellular apoptosis upon viral infection, integrating signals from complex intracellular networks in performing cellular functions, thus being a rather obvious target to fight against viral infection. Due to the initial discovery of the core elements of the MAPK pathways nearly four decades ago, considerable effort has been focused on development of MAPK inhibitors which have shown promising clinical responses in cancer patients, both as single inhibitors or in combination[7]. The pandemic crisis imposes to think about high-throughput molecular approaches to moving from operational to scientifically-driven pharmacovigilance. Common challenges, including advancements in modern technologies and in data quality to strengthen translational science are needed. Therefore, it is tempting to speculate that combinatorial approaches with low dose of both MAPK inhibitors and CQ or HCQ are needed to set optimal conditions, i.e. computing the Combination Index (CI) values or the different combinations of drugs in all the experimental settings. Moreover, more basic science is required for a better understanding of each individual drug's mechanism of action, and to identify the key molecular signalling mechanisms involved. To this end, both automatized platforms for cell viability/apoptosis assays, and genome-wide siRNA screens and/or CRISPR-CAS9 technology are largely available. Of note, genome wide siRNA screens conducted on SARSCoV6[60] have identified MNK1 as one of the cellular factors that regulate virus replication. More detailed functional analyses of these host genes (identified in genome-wide screens) will provide insights for development of novel antiviral therapeutics.

Conclusions

Overall, the number of COVID-19 cases represents only the point of an iceberg, since apparently from 60-80% of the infected people are asymptomatic. The epidemiological classical measures of social distancing in all the world have reduced the overload of hospitals

and intensive therapies but the numbers are still confirmed that the infection curve is not at all under control and the risk of a new surge of cases in the next months is always lurking. Therefore, understanding the MAPK cascade mechanisms in sustaining infection may be relevant for this pandemic. Moreover, any knowledge we can add to the correlation between MAPK cascade and HCQ could pave the way to new molecular approaches to this severe disease, keeping always in mind that a systematic strategy is needed for pre-clinical optimization of drugs to serve at risk population.

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Conflict of interest

There is no competing of interest to declare

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