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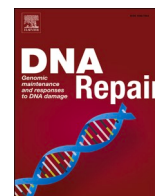
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# Hydroxychloroquine induces oxidative DNA damage and mutation in mammalian cells

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## ABSTRACT

Since the early stages of the pandemic, hydroxychloroquine (HCQ), a widely used drug with good safety profile in clinic, has come to the forefront of research on drug repurposing for COVID-19 treatment/prevention. Despite the decades-long use of HCQ in the treatment of diseases, such as malaria and autoimmune disorders, the exact mechanisms of action of this drug are only beginning to be understood. To date, no data are available on the genotoxic potential of HCQ *in vitro* or *in vivo*. The present study is the first investigation of the DNA damaging- and mutagenic effects of HCQ in mammalian cells *in vitro*, at concentrations that are comparable to clinically achievable doses in patient populations. We demonstrate significant induction of a representative oxidative DNA damage (8-oxodG) in primary mouse embryonic fibroblasts (MEFs) treated with HCQ at 5 and 25  $\mu\text{M}$  concentrations ( $P = 0.020$  and  $P = 0.029$ , respectively), as determined by enzyme-linked immunosorbent assay. Furthermore, we show significant mutagenicity of HCQ, manifest as 2.2- and 1.8-fold increases in relative *cII* mutant frequency in primary and spontaneously immortalized Big Blue<sup>®</sup> MEFs, respectively, treated with 25  $\mu\text{M}$  dose of this drug ( $P = 0.005$  and  $P = 0.012$ , respectively). The observed genotoxic effects of HCQ *in vitro*, achievable at clinically relevant doses, are novel and important, and may have significant implications for safety monitoring in patient populations. Given the substantial number of the world's population receiving HCQ for the treatment of various chronic diseases or in the context of clinical trials for COVID-19, our findings warrant further investigations into the biological consequences of therapeutic/preventive use of this drug.

## 1. Introduction

The global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1,2], has spurred interest in repurposing historically safe drugs for COVID-19 treatment/prevention [3–6]. Of these, hydroxychloroquine (HCQ) has gained special attention due to the promising results from early *in vitro* studies and public endorsement by prominent political leaders [7]. Initial *in vitro* findings suggested that HCQ limits entry of SARS-CoV-2 into human cells by inhibiting glycosylation of cell receptors targeted by coronaviruses and raising endosomal pH, thereby reducing endosome-mediated viral entry [8–10]. Additionally, HCQ was shown to lower the production of several pro-inflammatory cytokines involved in the development of acute respiratory distress syndrome

storm [11], a severe manifestation of COVID-19 [3,12]. On March 28, 2020, the Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for HCQ to treat adults hospitalized with COVID-19 [13]. On June 15, 2020, however, the FDA revoked the EUA for HCQ [14] as multiple randomized clinical trials demonstrated that this otherwise versatile drug is not efficacious in treating COVID-19 patients [15–18].

Despite the decades-long use of HCQ, originally as an antimalarial drug and later as a therapeutic for autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, and other inflammatory rheumatic diseases, the exact mechanisms of action of this drug are only beginning to be elucidated (reviewed in refs. [19–21]). HCQ is a derivative of 4-aminoquinoline, which has a characteristic flat aromatic core structure, with a 'basic' side chain (Fig. 1). The lipophilic and weak

**Abbreviations:** 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; EUA, Emergency Use Authorization; FDA, Food and Drug Administration; DMEM, Dulbecco's Modified Eagle's Medium; *E. coli*, *Escherichia coli*; FBS, fetal calf serum; HCQ, hydroxychloroquine; MEFs, mouse embryonic fibroblasts; MHC, major histocompatibility complex; PBS, phosphate buffered saline; ROS, reactive oxygen species; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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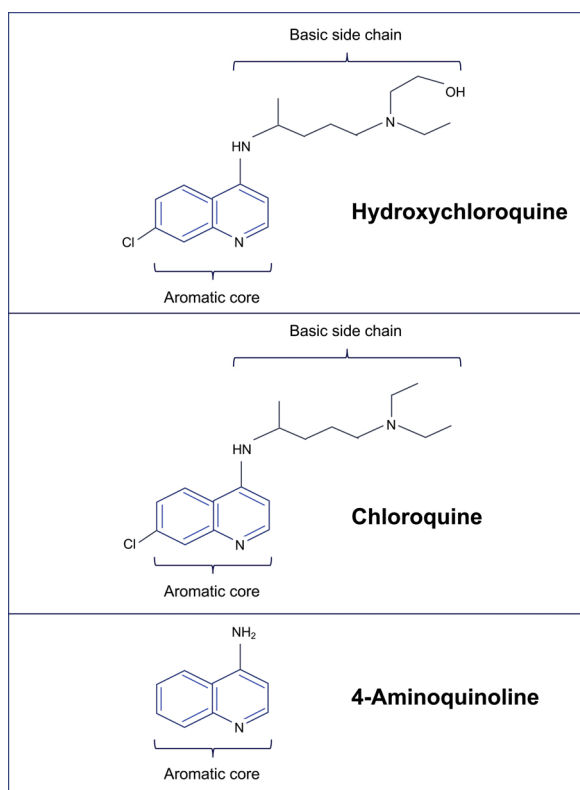
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**Fig. 1.** Chemical structures of the 4-aminoquinoline family of drugs. The parent compound, 4-aminoquinoline, and its synthetic derivatives, chloroquine and hydroxychloroquine (HCQ) are shown.

basic properties of HCQ enable this chemical to easily pass through the cell membrane and accumulate in acidic intracellular compartments, such as lysosomes [22,23], as well as interact with other molecular targets, such as nucleic acids [19]. HCQ interferes with lysosomal activity and autophagy, disrupts membrane stability, and alters signaling pathways and transcriptional activity, which can lead to inhibition of production of various pro-inflammatory cytokines, and modulation of specific co-stimulatory molecules [19,21,24]. More recently, the utility of HCQ, as an anti-neoplastic drug, has also been demonstrated in various types of cancer [25,26]. The anti-cancer properties of HCQ are mostly ascribed to its ability to modulate autophagy [20,27]. As an evolutionarily conserved self-defense mechanism for the degradation of cytoplasmic components, autophagy is essential for the maintenance of intracellular homeostasis and cellular remodeling [23,28], while also playing paradoxical roles in hyper-proliferating, hypoxic, and metabolically stressed cancerous cells [26,29].

While investigations of the mechanisms of action of HCQ have mainly centered on its intracellular effects, specifically on lysosomal function and activity [19,21,24], very little is known about the interaction of HCQ with DNA, including its DNA damaging- and mutagenic potentials [30]. This is an important gap in knowledge considering that HCQ can produce reactive oxygen species (ROS) [31–35] that are known to cause DNA damage and mutation [36]. The present study is the first investigation of the genotoxicity of HCQ whereby both the DNA damaging- and mutagenic effects of this drug are tested in mammalian cells, at concentrations that are comparable to clinically achievable doses in patient populations. More specifically, we have performed the  $\lambda$  Select-*cII* assay on both primary and spontaneously immortalized Big Blue<sup>®</sup> mouse embryonic fibroblasts (MEFs) treated *in vitro* with increasing concentrations of HCQ. Fast-proliferating spontaneously immortalized MEFs, which are more resistant to genotoxins than primary MEFs [37], were chosen to model conditions in which rapidly dividing and highly stressed cancer cells in patients are treated with

HCQ. To examine the formation of ROS-induced DNA damage in the HCQ-treated cells, we have also measured the levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), the most extensively studied oxidative base lesion [38,39], using an enzyme-linked immunosorbent assay (ELISA).

## 2. Materials and methods

### 2.1. Generation and treatment of mouse embryonic fibroblasts

Generation of primary and spontaneously immortalized Big Blue<sup>®</sup> mouse embryonic fibroblasts (MEFs) was done according to our published protocols [37,40]. Briefly, cultures of primary and spontaneously immortalized MEFs (C57BL/6 genetic background) were prepared for HCQ cytotoxicity examination, prior to DNA damage and mutagenicity experiments, as described previously [37]. For the cytotoxicity experiments, primary and spontaneously immortalized Big Blue<sup>®</sup> MEFs were grown as monolayers at ~50–60 % confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum (FBS) (Irvine Scientific, Santa Ana, CA). Prior to treatment, the medium was removed, and cells were washed thrice with phosphate buffered saline (PBS). Freshly prepared hydroxychloroquine sulfate (Sigma-Aldrich Corporation, St. Louis, MO) dissolved in double-distilled water (stock solution: 4.6 mM) was added to aliquots of DMEM at final concentrations of 1, 5, 25, 125, and 625  $\mu$ M. The DMEM-aliquots, containing HCQ or control solvent (ddH<sub>2</sub>O), were added to pre-washed MEFs culture dishes, in triplicate. The culture dishes, containing increasing concentrations of HCQ as compared to control, were then incubated at 37 °C for 24 h in the dark. Subsequently, the HCQ-treated and control cultures were harvested by trypsinization and subjected to trypan blue dye exclusion for cytotoxicity testing.

Upon completion of the cytotoxicity experiments and establishing a relevant dose range for DNA-damage and mutagenicity experiments, similarly prepared MEFs cultures (in triplicate) were treated with HCQ (at three different concentrations: 1, 5, and 25  $\mu$ M) as compared to control. For DNA damage experiments, treatment lasted for 3, 6, and 24 h. The treatment durations were chosen to examine kinetics of the formation and repair of DNA damage, while avoiding attenuation of the induced lesions due to cell division. For mutagenicity experiments, treatment was performed for 24 h to allow integration of the effects of DNA damage/repair and mutation. In our hands, primary and spontaneously immortalized MEFs undergo cell division, approximately every 28 and 22 h, respectively. At the end of all treatments for DNA damage analysis, HCQ-treated- and control cultures were harvested by trypsinization, pelleted by centrifugation, and preserved at –80 °C until further analysis. Following the treatments for mutation analysis, all cultures were thoroughly washed with PBS, fed with complete growth medium, and grown in standard cell culture conditions (20 % O<sub>2</sub>, 5% CO<sub>2</sub>, and 37 °C). The cultures were passed (1:3) when cells reached ~90 % confluence. At eight days post-treatment, the treated- and control cultures, undergone multiple rounds of cell division, were harvested, and stored at –80 °C until further analysis.

### 2.2. DNA isolation

High molecular weight genomic DNA was isolated from MEFs, treated with HCQ or control, using a standard protocol for mutation assay, as described previously [40]. For DNA damage analysis, we used a salt-based DNA extraction method that is proven to minimize artifactual oxidation of DNA during isolation procedure [41]. DNA was dissolved in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and stored at –80 °C until further analysis.

### 2.3. DNA damage detection

To detect 8-oxodG, a typical mutagenic oxidative DNA damage, we

used the EpiQuik 8-OHdG DNA Damage Quantification Direct Kit (Colorimetric) according to the manufacturer's instructions (Epigentek Group Inc., Farmingdale, NY). Briefly, upon measurement by PicoGreen, 300 ng of genomic DNA from each sample (in duplicate), and standards (including positive controls with known quantities of 8-oxodG and negative control) were added to 80  $\mu$ L binding solution in strip wells of a microplate. Incubation was performed for 90 min at 37 °C. The unbound DNA was removed from the wells by three times washing with the provided wash buffer (150  $\mu$ L/wash), and subsequently a capture antibody (50  $\mu$ L, patented Ab) was added to each well. After one-hour incubation at room temperature, the wells were washed with the wash buffer (as above), and detection antibody (50  $\mu$ L/well, patented Ab) was added, afterwards. Following 30 min incubation at room temperature, the wells were washed multiple times with the wash buffer, and enhancer solution (50  $\mu$ L/well) was added for an incubation period of 30 min at room temperature. After thoroughly washing the wells with the wash buffer, 150  $\mu$ L of developer solution were added to each well. Incubation was performed at room temperature in the dark, while monitoring for color change, from clear to medium blue, in the sample wells and control wells. Once a color change was observed in the positive control wells, stop solution (50  $\mu$ L/well) was added, and absorbance was read at 450 nm, using a SpectraMax® i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA).

#### 2.4. Determination of the *cII* mutant frequency

Genomic DNA isolated from Big Blue® MEFs treated with HCQ or control was screened for *cII* mutations by the Transpack Packaging Extract kit according to the manufacturer's instructions (Stratagene Corp., Acquired by Agilent Technologies Inc., Sigma-Aldrich Corp., and BioReliance, Darmstadt, Germany). Using the packaging extracts, we recovered the  $\lambda$ LIZ shuttle vectors from the genomic DNA and packaged them individually into viable phage particles. The packaged lambda phages were then used to infect cultures of G1250 host *E. coli*. Subsequently, the phage-infected G1250 cultures were plated onto TB1 agar plates and incubated at 24 °C for 48 h (*i.e.*, selective conditions) to screen for *cII* mutant plaques. To enumerate the total number of plaques screened, dilutions of the phage-infected G1250 cultures were plated similarly and incubated at 37 °C overnight (*i.e.*, non-selective conditions). For verification, all putative *cII* mutant plaques were re-plated at low density under selective conditions, as described previously [40]. Mutant frequency of the *cII* gene, which represents the mutagenic potency of the tested chemical, was computed by dividing the number of verified mutant plaques (*i.e.*, formed in selective conditions after re-plating) by the total number of plaques screened (*i.e.*, formed in non-selective conditions) [42]. All samples were assayed for a minimum of three times.

#### 2.5. Statistical analysis

Distribution of data was evaluated by the Shapiro-Wilk test. Results are expressed as means  $\pm$  SD. Comparison of variables between two groups or multiple groups was done using the Student's *t*-test or one-way analysis of variance (ANOVA), respectively. All statistical tests were two-sided. Values of  $P < 0.05$  were considered statistically significant. All statistical analyses were performed using the R environment for statistical computing, available at RStudio (<https://rstudio.com/>), which is a free and open source software.

### 3. Results

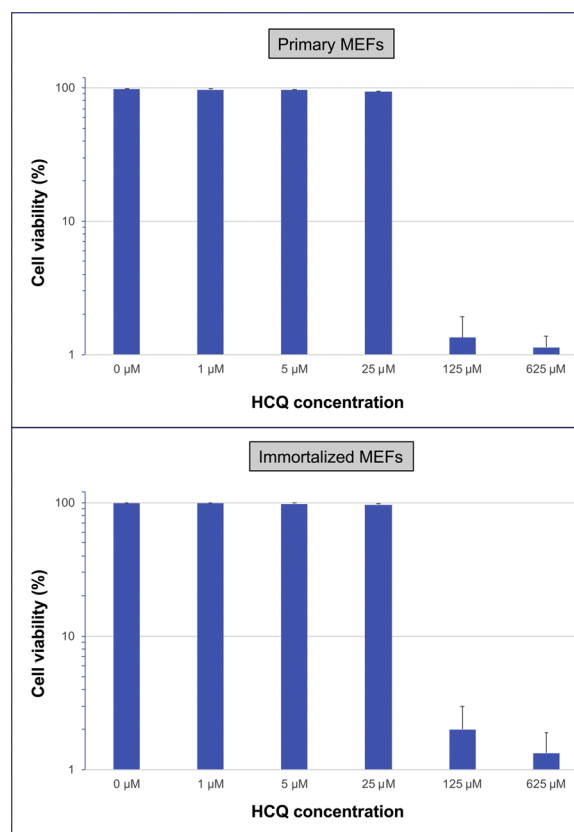
#### 3.1. Cytotoxicity examination

In mutagenicity studies, cytotoxicity testing is performed to establish a physiologically-relevant dose at which a given treatment exerts detectable genotoxic effects without causing excessive cell death [42].

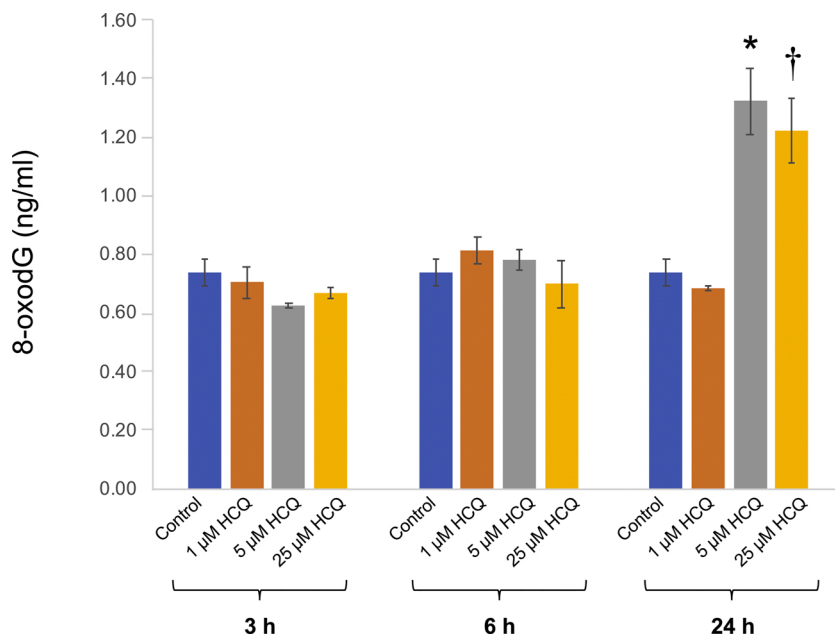
This is important because DNA damage induced by *in vitro* treatment can only be translated into mutation if the treated cells remain viable and proliferate, afterwards [42,43]. The induced DNA damage, if not repaired properly and in time, may miscode during translesion synthesis, and upon DNA replication, cause mutation [42,43]. We examined the cytotoxicity of HCQ in primary and spontaneously immortalized MEFs at concentrations ranging from 1  $\mu$ M to 625  $\mu$ M (at 5-fold increments). As shown in Fig. 2, HCQ at concentrations up to 25  $\mu$ M was not appreciably cytotoxic in primary or spontaneously immortalized MEFs. Treatment of the cells with HCQ at higher concentrations tested (*i.e.*, 125  $\mu$ M and 625  $\mu$ M) obliterated both the primary and immortalized MEFs, causing nearly absolute cytotoxicity. In all cases, the immortalized MEFs were slightly more resistant than primary MEFs to the cytotoxic effects of HCQ treatment (Fig. 2). Because of the prohibitive cytotoxicity of HCQ at the last two tested concentrations (*i.e.*, 125  $\mu$ M and 625  $\mu$ M), we selected doses of 1, 5, 25  $\mu$ M HCQ as compared to control (*i.e.*, solvent treatment) for all DNA damage and mutagenesis experiments (*see*, below).

#### 3.2. DNA damage analysis

We quantified 8-oxodG levels in primary MEFs treated with HCQ for 3, 6, and 24 h at concentrations of 1, 5, 25  $\mu$ M in comparison to control. As illustrated in Fig. 3, detectable and statistically significantly higher levels of 8-oxodG relative to background were observed in cells treated with concentrations of 5 or 25  $\mu$ M HCQ at 24 h post-treatment ( $P =$



**Fig. 2.** Cytotoxicity examination in Big Blue® MEFs treated with HCQ and control. Cytotoxicity examination was performed by the trypan blue dye exclusion technique in primary and spontaneously immortalized Big Blue® MEFs treated with increasing concentrations of HCQ as compared to control solvent, as described in the text. The percentage of viable cells was determined in triplicate MEF cultures for each treatment condition, and the results were expressed as mean  $\pm$  SD. Y-axis is in log scale. Upper and lower panels represent results for primary MEFs and spontaneously immortalized MEFs, respectively.



**Fig. 3.** DNA damage analysis in Big Blue® MEFs treated with HCQ and control. 8-oxodG, a representative oxidative base damage [38,39], was quantified in primary MEFs treated with increasing concentrations of HCQ for 3, 6, and 24 h as compared to control by ELISA, as described in the text. Quantification results from duplicate MEF cultures for each treatment condition were averaged and expressed as mean  $\pm$  SD.

\* Statistically significant as compared to control;  $P = 0.020$ .

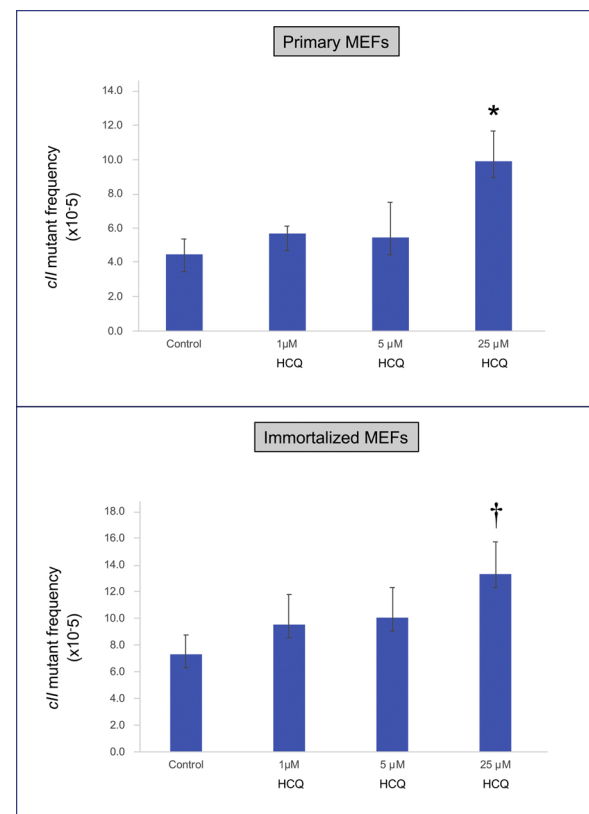
† Statistically significant as compared to control;  $P = 0.029$ .

0.020 and  $P = 0.029$ ). Shorter treatment times (*i.e.*, 3 and 6 h) or lower concentration of HCQ (1  $\mu$ M) did not result in discernable change in 8-oxodG levels in the treated cells relative to control. There were 1.8- and 1.7-fold increases in 8-oxodG levels relative to background in cells treated with 5 and 25  $\mu$ M HCQ, respectively, for 24 h; these fold-changes are not statistically significantly different from one another ( $P = 0.463$ ). Together with the cytotoxicity data (above), the results of DNA damage quantification in HCQ-treated MEFs indicate that a 24-hour treatment at the last two tested concentrations is sufficient to produce detectable promutagenic DNA damage without causing excessive cell death. These observations validate the appropriateness of the selected dose range for our mutagenicity experiments.

We note that due to limited supply of HCQ during the early months of the pandemic (*i.e.*, when our experiments were initiated), we had to prioritize the use of test compound in our studies. As such, we performed the cytotoxicity and mutation experiments in both primary and immortalized MEFs, while DNA damage experiments were conducted using the primary MEFs, which are more sensitive to genotoxins as compared to spontaneously immortalized MEFs, as demonstrated in our recent publication [37].

### 3.3. Mutation analysis

We determined the *cII* mutant frequency, as an indicator of the mutagenic effect of HCQ *in vitro*, in primary and spontaneously immortalized Big Blue® MEFs treated with 1, 5, and 25  $\mu$ M HCQ in comparison to control. The genome of Big Blue® MEFs contains multiple copies of the chromosomally integrated- and easily recoverable  $\lambda$ LIZ shuttle vector, which carries two mutation reporter genes (*i.e.*, *cII* and *Lacl*) [40], that are widely used for mutagenesis studies [42,43]. As shown in Fig. 4, while HCQ treatment resulted in slight, but non-significant, elevation of the relative *cII* mutant frequency at doses of 1 and 5  $\mu$ M in primary MEFs and immortalized MEFs, it caused significant mutagenicity at a dose of 25  $\mu$ M in both cell types ( $P = 0.005$  and  $P = 0.012$ , respectively). Specifically, there were 2.2- and 1.8-fold increases in relative *cII* mutant frequency in the primary and immortalized MEFs, respectively, treated with 25  $\mu$ M HCQ. We note that the background *cII* mutant frequency in control primary MEFs was significantly lower than that in the immortalized MEFs ( $4.5 \pm 0.9$  vs.  $7.3 \pm 1.5 \times 10^{-5}$ ,  $P = 0.049$ ). The latter is consistent with accumulation of endogenously derived promutagenic lesions (*e.g.*, due to oxidative stress) in



**Fig. 4.** Determination of *cII* mutant frequency in Big Blue® MEFs treated with HCQ and control. The frequency of *cII* mutants was determined in primary and spontaneously immortalized Big Blue® MEFs treated with increasing concentrations of HCQ as compared to control by the  $\lambda$  Select-*cII*™ Mutation Detection System for Big Blue® Rodents, as described in the text. All samples were assayed for a minimum of three times, and the results were averaged and expressed as mean  $\pm$  SD. Upper and lower panels represent results for primary MEFs and spontaneously immortalized MEFs, respectively.

\* Statistically significant as compared to control;  $P = 0.005$ .

† Statistically significant as compared to control;  $P = 0.012$ .

the spontaneously immortalized MEFs, as demonstrated in our recent publication [37].

#### 4. Discussion

Since the early months of the pandemic, HCQ, a widely used drug with good safety profile in clinic [19–21], has been extensively researched for repurposing in COVID-19 treatment/prevention [3–6]. HCQ is commonly used in the treatment of a variety of diseases, including malaria, autoimmune disorders, and more recently cancer [19–21]. Current knowledge on the mechanisms of action of HCQ is mainly deduced from its inhibitory effects on lysosomal activity and interference with autophagy [19]. These effects are also known to result in impairment of antigen processing and major histocompatibility complex (MHC) class II presentation [44–46], which are critical for immunity and inflammatory response [44–46]. Notwithstanding the known properties of HCQ to produce ROS [19,31–35], no data are available on the genotoxic mode of action of this drug *in vitro* or *in vivo* [30]. Also, there are limited data on the genotoxic effects of chloroquine, the parent compound of HCQ (see, Fig. 1), in experimental model systems (reviewed in ref. [30]).

In the present study, we have investigated, for the first time, the potential of HCQ to induce DNA damage and mutation in mammalian cells *in vitro*. Using a highly validated and extensively utilized model system, namely transgenic Big Blue® MEFs [42,43], we have demonstrated that HCQ, at a clinically achievable dose range, exhibits significant DNA-damaging- and mutagenic effects. More specifically, we observed significant induction of 8-oxodG, a representative oxidative DNA damage [38,39], in primary Big Blue® MEFs treated with HCQ, and significant increase in relative *cII* mutant frequency in both primary and spontaneously immortalized Big Blue® MEFs treated with this drug. We note that while the induced levels of 8-oxodG were approximately equal after treatment with 5 and 25  $\mu$ M HCQ, respectively (Fig. 3), only the latter treatment did cause statistically significant rise in the relative *cII* mutant frequency (Fig. 4). The latter might have arisen from the formation of additional types of mutagenic DNA lesion at the higher tested concentration of HCQ. Such lesions would go undetected under the herein applied assay, which is specific for 8-oxodG (ELISA). The additional lesions may include, but not limited to, oxidation products of 8-oxodG, such as hydantoin-type DNA adducts, which are orders of magnitude more mutagenic than the original oxidized base lesion [38, 47,48]. Follow up analytical chemistry studies are needed to characterize the types and quantities of DNA lesion induced by HCQ at various concentrations. Owing to high specificity and sensitivity, mass spectrometry detection of HCQ-DNA adducts will likely be a method of choice for future follow up studies.

The over 2.2- and 1.8-fold increases in relative *cII* mutant frequency in HCQ-treated primary and spontaneously immortalized MEFs, respectively, indicate that this drug has a significant, yet, weak mutagenic ‘potency’ in this mammalian model system. Historic data from experiments performed in our laboratory and others (reviewed in [42, 43]) have shown that at equitoxic doses, moderate mutagens, such as aflatoxin B<sub>1</sub> [49], *N*-Hydroxy-4-acetylamino-biphenyl [50], photo-activated riboflavin [51], and tamoxifen [52], and potent mutagens (e. g., sunlight ultraviolet radiation [53] and benzo[*a*]pyrene diol epoxide [54]) raise the *cII* mutant frequency several-fold and more than 50-fold, respectively, over the background in this same model system. The observed mutagenic potency of HCQ in the present study is comparable to those of other significant but weak mutagens, such as acrylamide [55], photoactivated methylene blue [37], and secondhand smoke [56], in the Big Blue® mouse cells.

Altogether, the observed genotoxic effects of HCQ, manifest within a dose range that is therapeutically relevant [25], are novel and important. In addition, these findings may have significant implications for safety monitoring in patient populations. The peak concentration of HCQ in blood of rheumatoid arthritis patients chronically treated with

this drug can reach ~10 mmol/L [57]. As an anti-neoplastic drug, HCQ is administered at higher doses (e.g., 1200 mg/daily) [58] than the therapeutic doses of this drug used for long-term treatment of patients with autoimmune diseases (i.e., 400 mg/day for >5 years) [59]. The recommended dosage of HCQ in COVID-19 patients is generally higher than that in autoimmune disease but in shorter duration, e.g., an initial loading dose (for up to a few days), followed by several days of maintenance dosing [60–62]. The administered doses of HCQ in our study are comparable to the therapeutic doses used in patient populations [25,57, 63–65], as described above.

HCQ has a large volume distribution and a long half-life (>40-days in blood), consistent with its slow onset of action and prolonged effects after drug discontinuation [22,66–68]. The use of HCQ is associated with cardiotoxicity, and ophthalmologic (retinopathy) and gastrointestinal complications [69–74]. In this study, the observed genotoxicity of HCQ *in vitro* suggests the possibility of additional side-effects, other than cardiac, ocular, and gastrointestinal toxicities, which might impact patient populations. Being cognizant of this possibility, we emphasize, however, the importance of not over-interpreting or generalizing our *in vitro* findings before they are validated in follow up *ex vivo/in vivo* studies. If validated, these findings may add a new layer of complexity to the risk-benefit analysis of HCQ, especially in the context of clinical trials. The widespread use of HCQ in the treatment of a great variety of diseases [19], and the renewed interest in the use of this drug for COVID-19 treatment/prevention [15–18], underscore the need for such validation studies. As of May 10, 2021, there are 247 clinical trials on HCQ, entered into “ClinicalTrials.gov”, which is a database of privately and publicly funded clinical studies conducted around the world. Supplementary Fig. S1 summarizes the status of clinical trials on HCQ, worldwide, and distribution of the geographic regions/countries wherein the studies are performed.

While we highlight the novelty and significance of our findings, we also acknowledge the limitations of our study. As for all studies using various model systems, inter-species differences between, e.g., mouse and human, and *in vitro* vs. *in vivo* conditions may varyingly impact the pharmacokinetics and pharmacodynamics of drugs, such as HCQ. The use of transgenes, as mutational reporter genes, as compared to endogenous genes, should also be taken into consideration. Nonetheless, growing evidence supports that the high copy number of transgenes is likely to result in higher sensitivity for mutation detection, when compared to single copy endogenous genes [42,43]. Notwithstanding the added complexities, mutation analysis in transgenes have been shown to faithfully capture many aspects of mutagenesis in endogenous genes, although discordant results have also been reported [42,43]. Furthermore, we stress that we have measured 8-oxodG, as a representative oxidative DNA damage [38,39], in HCQ-treated cells. Similarly, we have quantified *cII* mutant frequency, as an indicator of the mutagenic potency of HCQ *in vitro*. Although 8-oxodG predominantly induces G:C→T:A transversion, in addition to G:C→A:T transition and G:C→C:G transversion [36], other types of DNA damage, potentially induced by HCQ but not measured in this study, may also have similar mutational signature [38,47,48]. Thus, we have not attributed (explicitly or implicitly) the increased relative *cII* mutant frequency in HCQ-treated cells directly to 8-oxodG because other types of damage (not measured in this study) may also be produced by HCQ treatment. Unfortunately, the pandemic-related disruption of research and closure of core facilities, including DNA-sequencing core, prevented us from sequencing the HCQ-induced *cII* mutants in this study. Future follow up studies are needed to expand the scope of research presented in this study. Of particular interest will be to perform DNA sequencing and mutation spectrometry analysis to establish the type of mutations induced by HCQ *in vitro/in vivo*. Obviously, the unprecedented challenges caused by the pandemic have, and continue to force researchers across the globe, including our group, to be extra-selective in conducting experiments and choosing techniques for their studies. As we adopt to a post-COVID-19 world, we continuously learn how to navigate research under many

newfound restrictions and mandates.

In summary, we have demonstrated significant genotoxicity of HCQ, including its ability to induce oxidative DNA damage and mutation in mammalian cells *in vitro*. Notably, the observed genotoxic effects of HCQ are manifest at clinically achievable doses. Given the substantial number of the world's population receiving HCQ for the treatment of various chronic diseases [19] or in the context of clinical trials for COVID-19 [3–6,63–65], our findings warrant further investigations into the biological consequences of therapeutic/preventive use of this drug.

#### Author contributions

AB: Conceived and designed the study, Performed experiments and collected data, Analyzed data and interpreted the results, Wrote the manuscript; AWC: Performed experiments and collected data, Analyzed data, Reviewed the manuscript; ST: Analyzed data and interpreted the results, Reviewed & edited the manuscript.

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#### Declaration of Competing Interest

All the authors declare no conflict of interest.

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.dnarep.2021.103180>.

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