Original Article

Molnupiravir Metabolite--N^4-hydroxycytidine Causes Cytotoxicity and DNA Damage in Mammalian Cells *in vitro*

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Abstract

N^4-hydroxycytidine (NHC) is the active metabolite of molnupiravir—a new drug for COVID-19 treatment. NHC exerts antiviral activity by incorporating into SAR-CoV-2 RNA leading to false base-paring and lethal mutations to the virus. However, the risk of non-specific mutagenesis to host cells has been a concern. The goal of this study is to detect cytotoxic activity and DNA damage induced by NHC in rapid-growing cells including human keratinocyte (HaCaT), and human adenocarcinomic alveolar basal epithelial (A549) cells *in vitro* by using sulforhodamine B (SRB) colorimetric and comet assays. NHC induced cytotoxicity in a concentration-dependent manner (0.1-30μM) in HaCaT and A549 cells. Half-maximal inhibitory concentration (IC_{50}) values of NHC were lower in HaCaT compared to A549 cells after 3, 5, 10 days of exposure (4.40 ± 0.09 *vs* 23.21 ± 3.42, 5.82 ± 0.91 vs 16.35 ± 2.04, and 5.41 ± 0.88 *vs* 13.83 ± 2.05 μM, respectively), suggesting that the cytotoxic effect of NHC is more potent in HaCaT cells than in A549 cells. Significant increase in DNA damage parameters were observed in comet assay for HaCaT and A549 cells after exposure to NHC. NHC-induced DNA damage in HaCaT cells was concentration-dependent (1-10 μM), and time-dependent (3-10 days). NHC-induced DNA damage in A549 cells was concentration-dependent (1-10 μM), but not time-dependent (3-10 days). Within the limitations of this *in vitro* study, we conclude that NHC could induce cytotoxic and DNA damage in mammalian cells at therapeutic and supratherapeutic concentrations. We propose caution in the use and supervision of molnupiravir, especially in patients with impaired xenobiotic clearance.

Keywords: N^4-hydroxycytidine, Molnupiravir, Cytotoxicity, DNA amage, Comet assay

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Introduction

Molnupiravir is a new cost-effective oral antiviral agent classified as anti-ribonucleoside analog currently in phase III clinical trials.[1] Although molnupiravir has not been officially approved owing to low safety database, and mutagenesis concern, it is authorized for emergency use for COVID-19 treatment worldwide due to the pandemic crisis and high demand for antiviral drug treatment.[2,3] Molnupiravir is a prodrug that is hydrolyzed by esterase to form an active metabolite N^4-hydroxy cytidine (NHC).[4] NHC in systemic circulation is up-taken into host cells and phosphorylated to form NHC-triphosphate.[4] This compound competes with cytidine triphosphate (CTP) or uridine triphosphate (UTP) as a substrate for the viral RNA-dependent RNA polymerase leading to lethal mutations to the SARV-CoV-2.[5-7]

Molnupiravir is contraindicated in patients younger than 18 years of age and in pregnancy, as it inhibits myeloid and erythroid proliferation, and is associated with embryofetal lethality and teratogenicity in animals at high concentration.[8] A recent study from the Zhou and colleagues showed that NHC is mutagenic in Chinese hamster ovary cells *in vitro*.[9] The missense and frame-shift deletion mutations occur in cells that are exposed to NHC.[9] In contrast, Githaka group showed that the use of molnupiravir at therapeutic doses for 5 days efficiently eliminates SARS-CoV-2 without altering the number of gene missense and frameshift variants in lung cells of golden hamsters.[10] Troth and colleagues showed that molnupiravir treatment did not increase mutation in Pig-a mutagenicity assay and Big Blue transgenic rodent assay in 5-day exposure.[11] Accumulation of toxic NHC may occur in individuals with impaired xenobiotic clearance. Genotoxicity study of supratherapeutic concentration or prolonged exposure was not performed in these studies. Here, we assessed cytotoxic and DNA damage effects of NHC in HaCaT, and A549 cells *in vitro* by using SRB and comet assay. This work could in part fulfill the safety profiles of molnupiravir.

Methods

Cell culture

The cells in this experiment consisted of two cell types: adenocarcinomic human alveolar basal epithelial cells A549 (ATCC CCL-185), and human keratinocytes HaCaT (CLS 300493-SF) cells. The A459 cells were grown in RPMI 1640 medium, which was supplemented with 10% heated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The HaCaT cells were grown in DMEM medium supplemented with 10% heated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). The cell lines were cultured at a temperature of 37ºC with 5% CO_2 and 95% humidity.

Drug preparation

N^4-hydroxycytidine (TCI, Oregon, USA) was dissolved in DMSO making stock solution at 10 mM in small aliquots and kept at -20ºC. Working solutions of NHC contains <0.3% DMSO v/v. Final solutions were diluted in culture medium on the day of experiment.

In vitro assay for cytotoxic activity

Cytotoxic activity could be measured by using SRB assay as described previously.[12-14] The plating densities of cell lines were identified based on their growth patterns, expressed as cells per well. The plating densities of cell lines A594 were 5.5×10^3, 2.5×10^3, and 5×10^2 cells and HaCaT were 10×10^3, 8×10^3 and 3×10^3 cells for 3, 5, and 10 days of NHC exposure, respectively. These densities were chosen to encourage exponential growth during the experiment and to find a linear relationship between absorbance at 492 nm and cell count in the SRB assay. Firstly, the cell cultures were cultivated as monolayers in a 75 cm^2 flask and afterwards rinsed with phosphate buffer saline (PBS) (Sigma-Aldrich® in Saint Louis, USA). After that, PBS was removed and 0.15% trypsin-EDTA (Gibco BRL, Grand Island, NY, USA) was added to obtain a suspension of single cells in the medium. The cells were incubated for 5 to 10 minutes and then washed with 5 mL of medium in a 75 cm^2 flask to stop the trypsin-EDTA activity. The count density of cell viability was determined with trypan blue reagent using a hemocytometer. The A549 and HaCaT cell suspensions were diluted in their suitable medium to achieve their plating densities, then seeded in 96-well microplates at a volume of 100 µL per well. The microplates were then placed in an incubator at 37 ºC, with 5% CO_2 and 95% humidity for 24 hours. Subsequently, NHC was added to treat cells at various

doses ranging from 0.1 to 30 μM. The exposure times were 3, 5 and 10 days. The concentration of each NHC was added at a volume of 100 μL per well in 96-well microplates. The control medium was added to 100 μL of cell culture medium, while the control solvent was mixed with a 100 μL solution of 2% DMSO. The cell culture plates were incubated at 37°C and 5% CO_2 for 72 hours. In the case of 5 or 10 days of exposure treatment, the medium was removed after 72 hours of incubation, and the new NHC was added at various doses to each well and incubated, a process that was repeated every 72 hours until fixation time. For the fixation time, 100 μL of cold 40% trichloroacetic acid (TCA) was added directly to the medium supernatant in each well and incubate at 4ºC for 1 hour. Then, the culture plates were washed four times with slow-running tap water and gently tapped onto a paper towel to remove excess water. The plate was allowed to air dry at room temperature. 50 μL of SRB solution (0.4% w/v in 1% glacial acetic acid) was added to each well for 30 min, then washed four times with 1% acetic acid and excess water removed. The plate was allowed to dry completely at room temperature. 100 μL of 10 mM Tris base (Tris (hydroxymethyl) aminomethane, pH 10.5) was added to each well to dissolve the dry product. The absorbance (OD) was read on a microplate reader at 492 nm. The survival percentage and IC_{50} values were analyzed.[13,15]

Comet assay for detection of DNA damage

DNA strand breaks can be measured by a comet assay, a relatively simple, and sensitive method, as described previously.[16-18] The assay is based on measurements of fragmented DNA migrating out of the nucleus during electrophoresis.[17,19]

Cell treatment and isolation

The plating densities in 24-well culture plates of cell lines A594 were $1.65x10^4$, $7.5x10^3$, and $1.5x10^3$, and HaCaT were $3x10^4$, $2.4x10^4$ and $9x10^3$ for 3, 5, and 10 days of NHC exposure, respectively. A594 and HaCaT cells were treated with NHC (1 or 10 μM) at exposure time of 3, 5 and 10 days. In the case of 5 or 10 days of exposure, the medium and NHC were replaced every 72 hours of incubation until the cells were harvested by using 0.05% trypsin-EDTA (Sigma). Untreated cells were used to confirm that background damage was low. Negative controls included untreated cells cultured in normal media (control media, CM), and in normal media with DMSO (control solvent, CS with 0.3% DMSO v/v). Positive control was performed by exposing untreated cells at day-5 cultured with 20 mM hydrogen peroxide.[20,21] Cell density was adjusted to about 2×10^4 cells/mL in phosphate-buffered saline lacking divalent cations by using hemocytometer. Cell suspension of 0.4 mL were transferred into a 5-mL tube for combining with low-melting-point agarose in the next step.

Slide preparation and gel electrophoresis

Comet assay was performed under alkaline conditions according to Singh and coworkers with a few modifications.[22] Briefly, clear-glass microscopic slides (25.4×76.2×1.0 mm) were precoated with 1.0% (w/v) normal melting point (NMP) agarose (Thermo Scientific, Massachusetts, USA.), then the treated cells (~10,000 cells) were mixed with 0.5% (w/v) low melting point (LMP) agarose (Thermo Scientific, Massachusetts, USA.) and pipetted over the first layer. After drying, the third layer of LMP was pipetted over the second layer. The glass slide (24×60 mm) was then applied to cover a layered-agarose gel and allowed to solidify. Then the slides were immersed in a cold lysing solution for 2 hours at 4ºC. After lysis, DNA was allowed to unwind for 20 minutes in alkaline electrophoresis solution pH>13. Electrophoresis was performed at 4°C, 24 V/cm, and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with ethidium bromide. Slides were scored using the Comet Imager program (Metasystems, Germany) attached to a fluorescent microscope (Olympus BX50). Comets were scored at 100x magnification. Images from at least 100 cells (50 cells from each replicate slide) were analyzed. The parameter taken to assess cell line DNA damage was tail length (μm) and % DNA in tail.

Data analysis

Data were analyzed by using Prism9 (GraphPad Software, San Diego). The data presented as mean ± SD, and the significance difference is indicated when the P-value is less than 0.05. Group data were analyzed by unpaired student t-test or ANOVA followed by multiple comparisons against control.

Results

Effects of N4-hydroxycytidine (NHC) on cell viability

NHC decreased cell viability in a concentration (0.1-30 μM)-dependent manner to HaCaT and A549 cells ($p = 0.0001$ and $p<0.0001$, respectively). Furthermore, the cytotoxicity effects remained consistent across exposure duration of 3, 5, and 10 days of exposure in both HaCaT ($p = 0.0794$, Fig.1A) and A549 cells ($p = 0.3852$, Fig.1B).

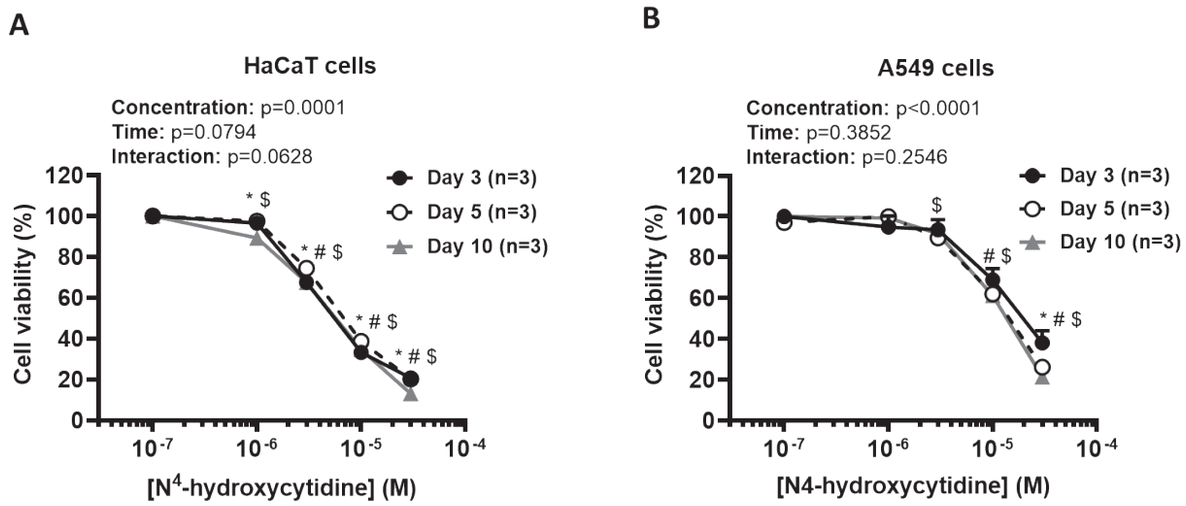

Figure 1 Cytotoxicity in response to N^4-hydroxycytidine (NHC) exposure in HaCaT (A) and A549 cells (B). Data are presented as mean ± SD; n indicated number of replicates; *, $^{\#}$, $^{\$}$ significant differences compared to the cytotoxic effect of 0.1μM NHC at 3, 5, and 10 days, respectively, as analyzed by two-way ANOVA with Dunnett's multiple comparison test.

Differential cytotoxic response to NHC of HaCaT and A549

Concentration-response curves (CRCs) of NHC (0.1-30 μM) were generated, and IC_{50} values were analyzed to compare NHC-induced cytotoxicity in HaCAT and A549 cells. The CRCs of NHC in HaCaT showed a leftward shift compared to A549 at 3, 5, and 10 days of exposure (Fig. 2A-C). The IC_{50} values of NHC in HaCaT were lower than those in A549 cells at 3, 5, 10 days of exposure (Fig. 2D). Furthermore, the IC_{50} values of A549, but not HaCaT exhibited a time-dependent decrease from 3 to 10 days (Fig. 2D). The IC_{50} values of NHC at various exposure durations in HaCaT and A549 were summarized in Table 1.

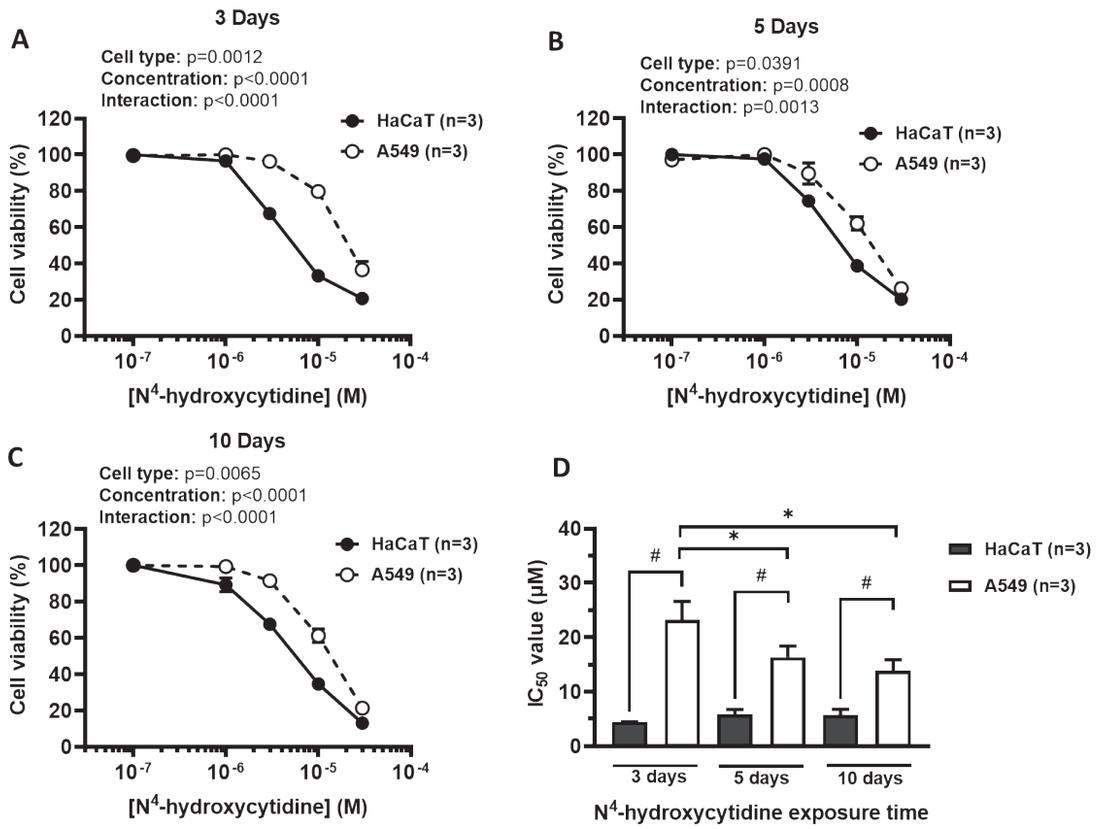

Figure 2 Differential cytotoxicity of HaCaT and A549 cells in response to NHC exposure over three different durations; 3 days (A), 5 days (B) and 10 days (C). IC_{50} of NHC against both cells after 3, 5 and 10 days of exposure (D). *significant differences among the different exposure durations, analyzed by one-way ANOVA with Tukey's multiple comparisons test. # significant differences between tested cells at each exposure duration, analyzed by unpaired t test. Data are presented as mean ± SD, n indicated number of replicates, p values shown in A-C were analyzed by two-way ANOVA.

Table 1 IC_{50} values of NHC on HaCaT and A549 cells

Cell lines	Exposure time to NHC (days)	N	IC_{50} value (µM) (Mean ± SD)	*P*-value
HaCaT (Human keratinocyte cells)				
	3	3	4.40 ± 0.09	0.128
	5	3	5.82 ± 0.91	
	10	3	5.41 ± 0.88	
A549 (Human adenocarcinoma alveolar basal epithelial cells)				
	3	3	23.21 ± 3.42	0.0107*
	5	3	16.35 ± 2.04**	
	10	3	13.83 ± 2.05**	

* significant difference among different exposure durations within the same cell type (one-way ANOVA)
** significant difference compared to day 3 exposure (one-way ANOVA with Turkey's multiple comparison test)
Data were mean ± SD; N = number of replicates.

Effect of NHC exposure on DNA damage

To assess the genotoxic activity of NHC, comet assay was performed in HaCaT and A549 cells. Comet assay is an economical and simple method using single-cell electrophoresis to detect DNA breaks in eukaryotic cells.[23] Figure 3 shows representative results of comet characteristics due to DNA damage in untreated cells (Fig. 3A) vs positive control H_2O_2 treatment (Fig. 3B). The analysis of DNA damage parameters included quantification of DNA tail length and %DNA in tail (Fig. 3C-D). Changes in DNA damage parameters after NHC exposure were quantified as shown in Fig. 4. DMSO 0.3% v/v (control solvent, CS) did not alter DNA tail length and % DNA in tail compared to control medium (CM) indicating changes in DNA damage parameters were induced by NHC. There were consistent increases in tail length and % DNA in tail of HaCaT cells exposed to NHC compared to CM in time-dependent (3-10 days), and concentration-dependent (1 and 10μM) manners (Fig. 4A-B). A549 cells exposed to NHC also showed consistent increases in DNA tail length and % DNA in tail in concentration-dependent (1 and 10 μM) manner, but not in time-dependent (3 and 10 days) manner (Fig. 4C-D).

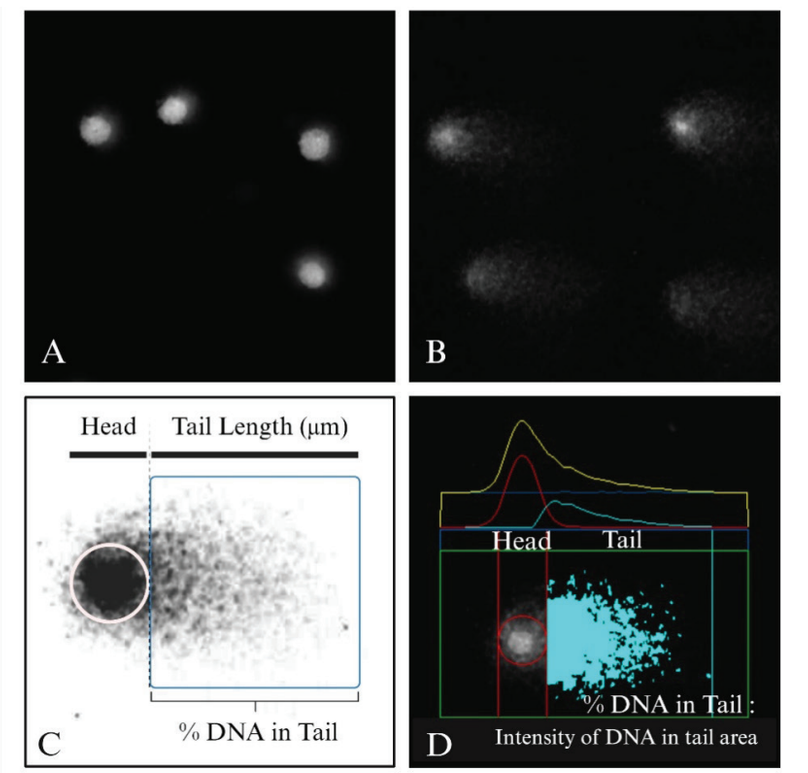

Figure 3 Representative images of alkaline comet assay in HaCaT cells. Untreated cells displayed intact round nuclei (A). Exposure to 20 mM H_2O_2 for 5 minutes caused DNA breaks, and DNA fragment migration from nucleoid body (comet-shape characteristics, (B)). Comet parameters for quantifying DNA damage were demonstrated in (C). Original color was adjusted to white background for better representation (C). Comet imager software captured fluorescence image, and individually identified cell head area, tail length and intensity of tail area (D). Tail length and % DNA in tail, were used to quantify DNA damage.

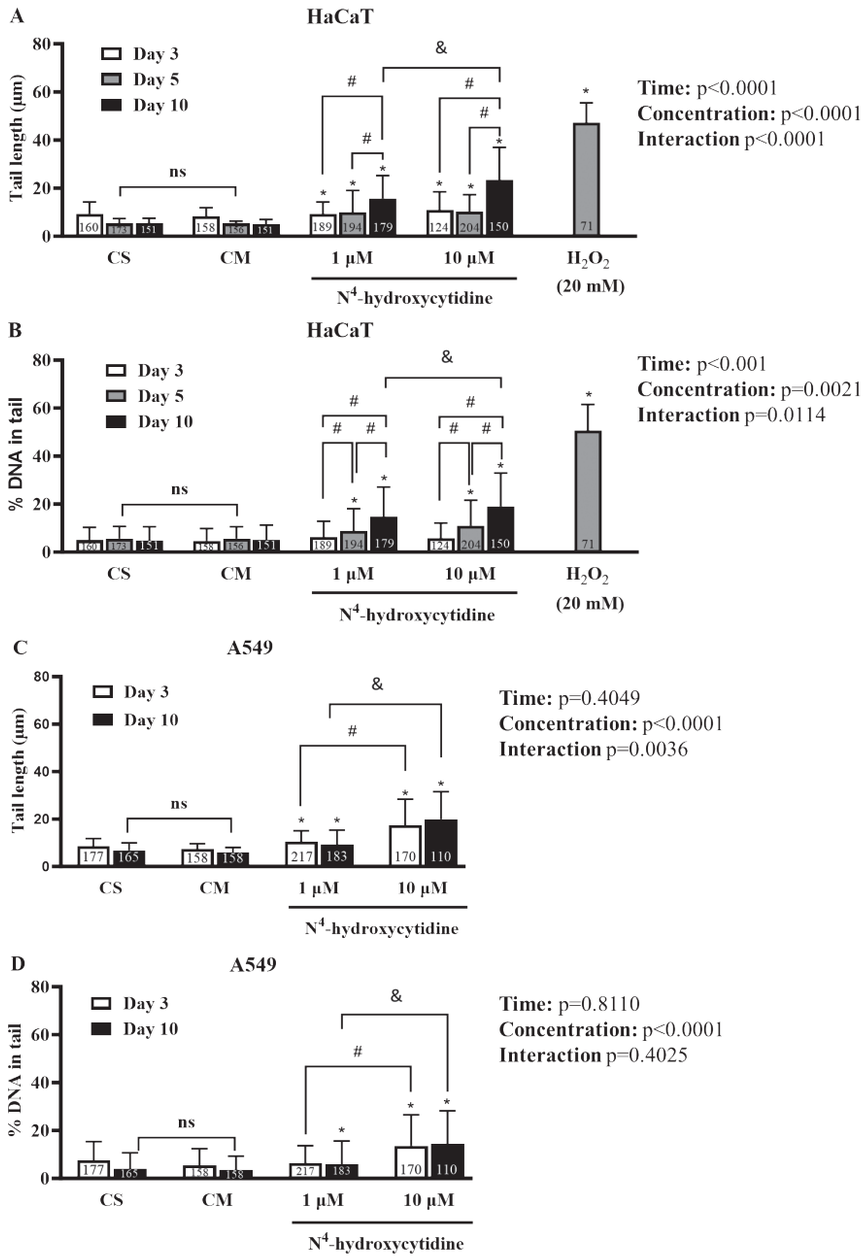

Figure 4 Comet assay analysis of DNA damage in response to N^4-hydroxycytidine exposure in HaCaT (A-B) and A549 (C-D) cells. DNA breaks were quantified in DNA tail length and % DNA in tail. A positive control was demonstrated by exposing day-5 cultured cells with hydrogen peroxide (A-B). P-values in (A-D) were analyzed by two-way ANOVA. *significant differences compared to CM at similar exposure time, analyzed by using unpaired t-test; #significant differences among different NHC concentrations (0 in CM, 1, and 10 µM) at similar exposure time, analyzed by using one-way ANOVA with Tukey's multiple comparison tests; &significant differences when comparing groups with different exposure durations and different concentration treatments, analyzed by using two-way ANOVA with Sidak's multiple comparison test. Data are presented as mean ± SD. The numbers on each bar indicates the number of cells in duplicate experiments. CM: control media; CS: control solvent (DMSO 0.3% v/v); ns: not significant.

Differential DNA damage after NHC exposure of HaCaT vs A549 cells

At 1μM NHC exposure for 3 days, DNA tail length in HaCaT was greater than A549 cells (9.28 ± 4.97 vs 10.57 ± 4.52μm, respectively, p = 0.0064), but %DNA in tail was similar in the two groups (6.31 ± 6.58 *vs* 6.38 ± 7.32μm, respectively, p>0.05). At longer duration of 1μM NHC exposure for 10 days, both tail length and %DNA in tail of HaCaT were greater compared to A549 cells (15.74 ± 9.48 *vs* 9.21 ± 6.13μm, p<0.0001, and 14.79 ± 12.35 *vs* 5.87 ± 9.76, p<0.001, respectively). Conversely, at 10 μM NHC exposure for 3 days, DNA tail length and %DNA damage were greater in A549 compared to HaCaT cells (17.45 ± 10.95 vs 10.92 ± 7.58, p<0.0001, and 13.61 ± 12.97 *vs* 5.74 ± 6.35, p<0.0001, respectively). At longer exposure of 10μM NHC exposure for 10 days, DNA tail length and %DNA damage of HaCaT were greater than A549 cells (2.47 ± 13.50 vs 19.91 ± 11.59, p = 0.0265, and 19.09 ± 13.97 vs 14.53 ± 13.69, p = 0.0086, respectively). The comparison analysis of comet assay between the two cell types was summarized in Table 2.

Table 2 Comparison of comet assay parameters between HaCaT and A549 cells after NHC exposure

Exposure conditions		Cell lines	N	Tail length (μm)		%DNA in tail	
Conc.	Time (days)			Mean ± SD	*P*-value	Mean ± SD	*P*-value
1 μM NHC	3	HaCaT	189	9.28 ± 4.97	0.0064*	6.31 ± 6.58	ns
	3	A549	217	10.57 ± 4.52		6.38 ± 7.32	
	10	HaCaT	179	15.74 ± 9.48	<0.0001*	14.79 ± 12.35	<0.0001*
	10	A549	183	9.21 ± 6.13		5.87 ± 9.76	
10 μM NHC	3	HaCaT	124	10.92 ± 7.58	<0.0001*	5.74 ± 6.35	<0.0001*
	3	A549	170	17.45 ± 10.95		13.61 ± 12.97	
	10	HaCaT	150	23.47 ± 13.50	0.0265*	19.09 ± 13.97	0.0086*
	10	A549	110	19.91 ± 11.59		14.53 ± 13.69	

* significant differences between the two cell types at similar concentration and exposure duration (unpaired t-test). Conc: concentration; N: the number of cells; NHC: N4-hydroxycytidine; ns: not significant.

Discussion

NHC induces cytotoxicity in vitro

European Medicines Agency reported *in vitro* data showing that NHC exerted antiviral activity to SARS-CoV-2 in various cell types including HUH-7, Calu-3, and A549-ACE2 with EC_{50} values in μM range. NHC antiviral activity is not due to cellular toxicity since its 50% cytotoxic concentration values (CC_{50}) were above the effective concentration values (EC_{50}).[24] Molnupiravir is rapidly hydrolyzed by carboxylesterases to NHC prior to reaching the systemic circulation.[25] NHC does not bind to plasma proteins.[25] Painter and coworkers reported that NHC was detected rapidly in the systemic circulation. The time to maximum plasma concentration (T_{max}) of NHC is ranged between 1-2 hours after a single oral dose of 800-1,600 mg molnupiravir. A single oral dose of 800, 1,200, and 1,600 mg results in maximum plasma concentration (C_{max}) of NHC at 3,640 ng/mL (14.04 μM), 4,500 ng/mL (17.36 μM), and 6,350 ng/mL (24.50 μM), respectively.[26] The NHC plasma molarity was calculated by using molecular weight of NHC at 259.22 Daltons.[27] Therapeutic dose of 800 mg molnupiravir every 12 hours results in NHC plasma concentration at steady state of 2,970 ng/mL (11.46 μM).[25] The human plasma concentrations of NHC detected in 1-10 μM range are relevant to the NHC IC_{50} values obtained from HaCaT cells *in vitro*. NHC-induced cytotoxic activity was more potent in HaCaT cells compared to human A549 cells. Less responsive NHC-induced cytotoxicity in A549 cells could be due to drug resistance characteristics of cancer cells. This finding is relevant to the work of Wallace and colleagues showing similar threshold of cytotoxicity by using SRB assay in the liver can-

cer cell HepG2 after 48 hours of NHC exposure.[28] Moreover, NHC-induced cytotoxicity in HaCaT human keratinocytes could be partly accountable for the NHC-related skin adverse events reported in patients with molnupiravir treatment.[29,30] Although a phase 2a clinical trial reported that low adverse events were associated with molnupiravir treatment (200-800 mg twice daily for 5 days),[31] this trial did not include participants with risk factors. Law and coworkers reported liver enzyme elevation after molnupiravir treatment in humans, and proposed caution when used in patients with impaired hepatic function.[32] Further assessment of NHC-induced cytotoxicity in other normal cell lines associated with vital organs is needed.

NHC induces DNA damage *in vitro*

Comet assay is a gel electrophoresis technique for detection of single cell DNA breaks.[33] As previously published data has reported NHC-induced mutagenesis in mammalian cells,[9] the current study aimed to determine whether NHC exposure caused phenotypic damage to DNA *in vitro*. The rapidly growing HaCaT and A549 cells were chosen for this study. Increases in the tail length and %DNA in tail were common parameters used for quantification of DNA breaks.[19] Our data suggested that DNA damage occurred in both cell types after exposure to 1-10 μM NHC for 3-10 days. NHC-induced DNA damage in HaCaT cells was concentration-dependent and time-dependent, whereas the effect in A549 was concentration-dependent, but not time-dependent. The resistance of NHC-induced DNA damage after a period of time was observed in A549 cells. This could be partly due to development of acquired resistance found in A549 cells.[34] Related mechanisms of this resistance include: overexpression of drug target genes[35] and multi-drug resistant genes[36], generation of tumor microenvironment,[34,35] and epithelial to mesenchymal transition.[34] Wallence group did not observe changes in mitochondrial gene expression in HepG2 cancer cells after 48 hours of NHC exposure. On the other hand, Miranda and coworkers showed that NHC caused an increase in genome-wide mutation frequencies in a concentration-dependent manner in mouse lymphoma L5178Y cells, and human lymphoblastoid TK6 cells.[37] Bian and colleagues suggested the antiviral drug prophylaxis after high-risk exposure for vulnerable cancer patients to prevent severe COVID-19.[38] Taken together, molnupiravir might not be a suitable candidate in this case, due to the possibility of drug resistance. Moreover, our findings were consistent with the data from Kobayahi et al., which revealed that NHC induced oxidative damage to isolated DNA from calf thymus was associated with the generation of NHC-induced hydroxylamine and Copper(I)-hydroperoxo complex generation.[39] Additional study of NHC-induced DNA damage and mutations in other normal cell lines associated with vital organs is suggested.

In conclusion, even though the safety profile of molnupiravir is still controversial, the study findings on cytotoxic and genotoxic activity of its active xenobiotic NHC, provide valuable information for the evaluation of the benefit/risk profile for molnupiravir. With the limitations of our *in vitro* study, we suggest that molnupiravir should be used with caution and supervision, especially in patients with impaired xenobiotic clearance. Furthermore, patients with cancer may be less responsive to molnupiravir treatment. In order to fulfill the safety profile of molnupiravir, the assessment of NHC-induced cytotoxicity and genotoxicity should be further performed in other normal cell lines associated with vital organs.

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Compliance with Ethics Requirements Experimental procedures and protocols were reviewed and approved by Institutional Biosafety Committee (IBC), Thammasat University (Approval number: 085/2565; Project code: 084/2565).

Conflict of interest All authors report no conflict of interest relevant to this article.

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Author Contributions

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Performed data analysis: Chamod, Sangsiri, Rimdusit

Wrote or contributed to the writing of the manuscript: Chamod, Poomirat, Prajuabjinda, Sangsiri

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