Title:

Comparative analysis of cytotoxicity, immunomodulatory properties and oxidative stress caused by three antimicrobials, Chlorhexidine, K18 and K21

Article type:

Short communication

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Abstract

We had created a novel quaternary ammonium silane, K21 and shown the antiviral effects of this compound on several different herpesviruses like HSV-1, HHV-6A and HHV-7 in *in vitro* cell culture infection models. K21 was able to inhibit viral infection by unknown mechanisms. Cytotoxic intracellular environment induced by the drug and resulting increased oxidative stress might be a factor that influences viral infection. These factors together with immunomodulatory properties of the drug influence the potential use of the compound for *in vivo* experiments. Here, we analyzed the extent of cytotoxicity of this compound and potential oxidative stress caused within the cell. We compared these properties of K21 to a previously published methacryloxy version of the compound called K18 and to a widely used antiseptic, chlorhexidine. Our results show comparatively less cytotoxicity and oxidative stress induced by K21 in comparison to chlorhexidine.

1. Introduction

Quaternary ammonium compounds (QACs) like K21 have both surfactant properties and broad-spectrum antimicrobial activity [1]. However the exact molecular mechanism behind the antimicrobial properties is not fully understood. One potential mechanism could be by induction of pro-inflammatory cytokines in phagocytic cells like macrophages that induce innate immune response to prevent pathogenic growth. Reactive oxygen species (ROS) is an important pro-inflammatory mediator and is induced by pro-inflammatory cytokines [2]. However, excess amounts of ROS can cause cellular toxicity and tissue damage [3]. Another widely used broad range

antimicrobials, chlorhexidine (CHX) has been shown to induce cytotoxicity and genotoxicity in macrophages through increased production of reactive oxygen species (ROS) [4] within host cell. As K21 was initially developed to be used in dental hygiene products, we choose to compare its effects to CHX as the later is one of the most widely used antiseptic compounds in dental products.

Here, we report less degree of cytotoxicity of K18 and K21 in comparison to CHX. Both K18 and K21 were comparable to CHX in terms of their immunomodulatory properties and were also effective in producing lesser amounts of ROS within macrophages suggesting potentially better antimicrobials than CHX in terms of host cell damage.

2. Material and methods

2.1. Drug

The compound K18 and K21 was obtained from KHG fiteBac technologies, USA. Chlorhexidine was purchased from Sigma (Cat. No. 282227).

2.2. Cell line

RAW 264.7 (ATCC TIB-71), a murine macrophage cell line was used for this study. RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 5% CO_2.

2.3. Flow cytometry

Cell viability and cytotoxic effects of K18, K21 and CHX was measured by Annexin V and Propidium Iodide (PI) staining using Dead Cell Apoptosis Kit (Life Technologies, Germany). Detailed protocol for this is described before [5].

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIZOL (SIGMA) and following manufacturers protocol. For quantitative Real time PCR (qRT-PCR), PerfeCTa qPCR SuperMix (Quanta Biosciences) was used and PCR amplifications were done on a StepOnePlus real time PCR platform (Applied Biosciences) using manufacturers protocol and SYBR Green chemistry. Amplified data were analyzed using StepOne Software v2.1. Primer details are – TNFα: TTGACCTCAGCGCTGAGTTG and CCTGTAGCCCACGTCGTAGC, IL-6: GGAAATCGTGGAAATGAG and GCTTAGGCATAACGCACT, IL-4: AACGAGGTCACAGGAGAAGG and TCTGCAGCTCCATGAGAACA, IL-10: AATATGCGAAGCACCTTGGAAGCC and GGGCATCACTTCTACCAGGT.

2.5. DCF assay for cellular ROS activity study

Total cellular ROS activity was measured using OxiSelect Intracellular ROS Assay Kit (Cell Biolabs Inc., USA) according to the manufacturer's protocol and an ELISA plate reader (TECAN Infinite M200) with an excitation filter of 480 nm and emission filter of 530 nm. In parallel, cells were pre-treated with 1000U of SOD for 6 h before drug treatment and were processed for DCF assay.

3. Results

3.1. Cytotoxic evaluation of K18, K21 and CHX

We have previously shown the CC50 value for K21 to be 9.45 μM in human foreskin fibroblasts [5]. Based on these data, we analyzed RAW 264.7 murine macrophages for cell viability, apoptosis, or necrosis using flow cytometry. We selected 4 different concentrations of K21 between 0.51 – 10.35 μM to evaluate cytotoxicity during

prolonged culture for 2 days. As expected 10.35 μM K21 showed ~15% increase in cell death in comparison to solvent control, with an increase in both apoptotic and necrotic cells (Fig. 1). Lower concentrations of K21 showed moderate effects on cell viability. K18 at similar concentrations showed comparable levels of cytotoxic effect. We then tested 4 different concentrations of chlorhexidine selected from previous publications [4] and found corroborating results. While 0.0001% CHX did not have any major effect on cell death, 0.002% CHX caused up to 90% cell death (Fig. 1). Bases on these results, we selected both cytotoxic and non-cytotoxic doses of CHX, K18 and K21 for further studies.

3.2. Evaluation of immunomodulatory properties

Upon exposure to pathogens, macrophages produce pro-inflammatory cytokines like tumor necrosis factor alpha (TNFα), Interleukin (IL)-1, IL-6, IL-8, and IL-12, which are essential to the functions of macrophages as they help in both innate and adaptive immune response. However, a tight regulation of amounts of inflammatory cytokines produced by macrophages is necessary as excess cytokines can induce cellular damage. TNFα is one of the first pro-inflammatory cytokines to be released in response to pathogenic infections. However excess of TNFα induces ROS-mediated apoptosis [6]. Antimicrobial compounds have been shown to enhance pro-inflammatory response of various cell types by inducing cytokine production [7, 8]. We first tested murine macrophages for their ability to induce TNFα and IL-6 mRNA expression upon exposure to K18, K21 and CHX. Bacterial lipopolysaccharides (LPS) stimulation was used as a positive control (Fig. 2A, 2B). Non-cytotoxic doses of all the three compounds tested showed no effect on TNFα as well as IL-6 expression (Fig. 2A, 2B). Out of different

doses of CHX tested, cytotoxic concentrations of 0.002% CHX was the only dose that was able to induce mild amount of TNFα and IL-6 mRNA expression. Similarly, 1.03 μM K18 and K21 also showed mild upregulation of TNFα and IL-6 mRNA expression (Fig. 2A, 2B).

Anti-inflammatory cytokines like IL-4, IL-10 inhibits inflammatory responses exerted by pro-inflammatory cytokines. For example, IL-4, IL-10, and IL-13 has been shown to decrease the production of TNFα-induced IL-8 in whole blood assay [9]. We tested IL-4 and IL-10 mRNA expression in murine macrophages upon exposure to all the three antimicrobial compounds. LPS served as a negative control in our assay as it decreased the basal levels of IL-4 and IL-10 in murine macrophages (Fig. 2C, 2D). None of the non-cytotoxic doses of CHX, K18 and K21 could induce IL-4 mRNA expression. However, 1.03 μM K18 and K21 caused mild upregulation in IL-4 mRNA expression. Only cytotoxic dose of 0.002% CHX was able to induce strong IL-4 mRNA expression (Fig. 2C, 2D).

3.3. Evaluation of oxidative stress

Pro-inflammatory cytokines like TNFα induces cellular ROS level leading to apoptosis. On the other hand, mitochondria-derived ROS act as signaling molecules to trigger pro-inflammatory cytokine production [10]. CHX has been previously shown to induce superoxide anions in macrophages [4]. Hence we tested potential ability of K18 and K21 to produce ROS in macrophages and compared the same to CHX. In contrast to previously published results, we found up to 3-fold increase in cellular ROS level upon treatment with 0.0001% CHX (Fig. 3). Whereas non-cytotoxic doses of 0.51 μM K18 and

K21 did not have any significant effect on cellular ROS levels. Pre-treatment of the cells with ROS scavenger superoxide dismutase (SOD) prevented the increase in ROS levels.

4. Discussion

Both K18 and K21 were primarily developed for their use in dental products. We have previously determined the CC_{50} values for K21 [5] and have shown effective anti-herpesviral properties of the compound. Both K18 and K21 are broad range antimicrobials *[11-15]*. Antimicrobial compounds are known to exert differential amounts of cytotoxic effects on various cell types [16]. Hence, we compared the potential cytotoxicity caused by K18 and K21 to a very commonly used oral antiseptic and antimicrobial compound, chlorhexidine. Chlorhexidine has been shown to cause both cytotoxic and genotoxic effects in macrophages [4, 16]. Our results show comparatively lesser cytotoxicity of K18 and K21 in comparison to CHX by flow cytometry where viable, apoptotic, or necrotic cells were counted by PI and Annexin V staining. We preferred using flow cytometry than MTT assay as the later has several limitations and cannot compare the metabolic state of the cells in differentially growing cell types in particular when cells are grown for a prolonged time with the test compounds.

Antimicrobial compounds exert their effect on pathogenic infections through modulation of host immune response and production of cytokines [17]. Anti-inflammatory properties of antibiotics have been shown to be advantageous in treating various diseases [18]. In our study, we found both K18 and K21 to be comparable to chlorhexidine in terms of their pro-inflammatory and anti-inflammatory properties. At non-cytotoxic doses, all the three compounds were found to be mildly anti-inflammatory. Genotoxic effects of chlorhexidine are well studied. Chlorhexidine is known to induce ROS-mediated DNA

damage. Hence, we compared the intracellular ROS levels in murine macrophages after prolonged drug treatment of two days. Our results showed no effect of K18 and K21 on intracellular ROS level whereas chlorhexidine induced up to 3 fold increase in ROS. These results suggest that both K18 and K21 are effective drugs when used at non-cytotoxic doses and have lesser cytotoxicity to commonly used antimicrobials like chlorhexidine.

Funding

Not applicable.

Conflict of Interests

None declared.

Author contributions

BKP, designed the study, NG and BKP, performed the experiments, wrote the manuscript; KK, developed and manufactured K21. All the authors read and approved the manuscript.

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Figure Legends

Figure 1. Cytotoxic effects of K18, K21 and chlorhexidine (CHX) on murine macrophages were studied using flow cytometry. Equal numbers of cells were seeded in 6-well plates and were treated with 4 different concentrations of each drug. In parallel cells were also grown either in absence of any compound /solvent or in presence of highest concentrations of solvent control. Cells were harvested on day 2 and were stained with Annexin V and PI. Subsequently cells were analyzed by flow cytometry. Data represents one set of experiments from the three biological replicates. Cells in left lower quadrant represent healthy cells. Cells in right lower quadrant represents early apoptotic cells. Cells in right upper quadrant represent late apoptotic cells whereas cells in left upper quadrant represent necrotic cells.

Figure 1

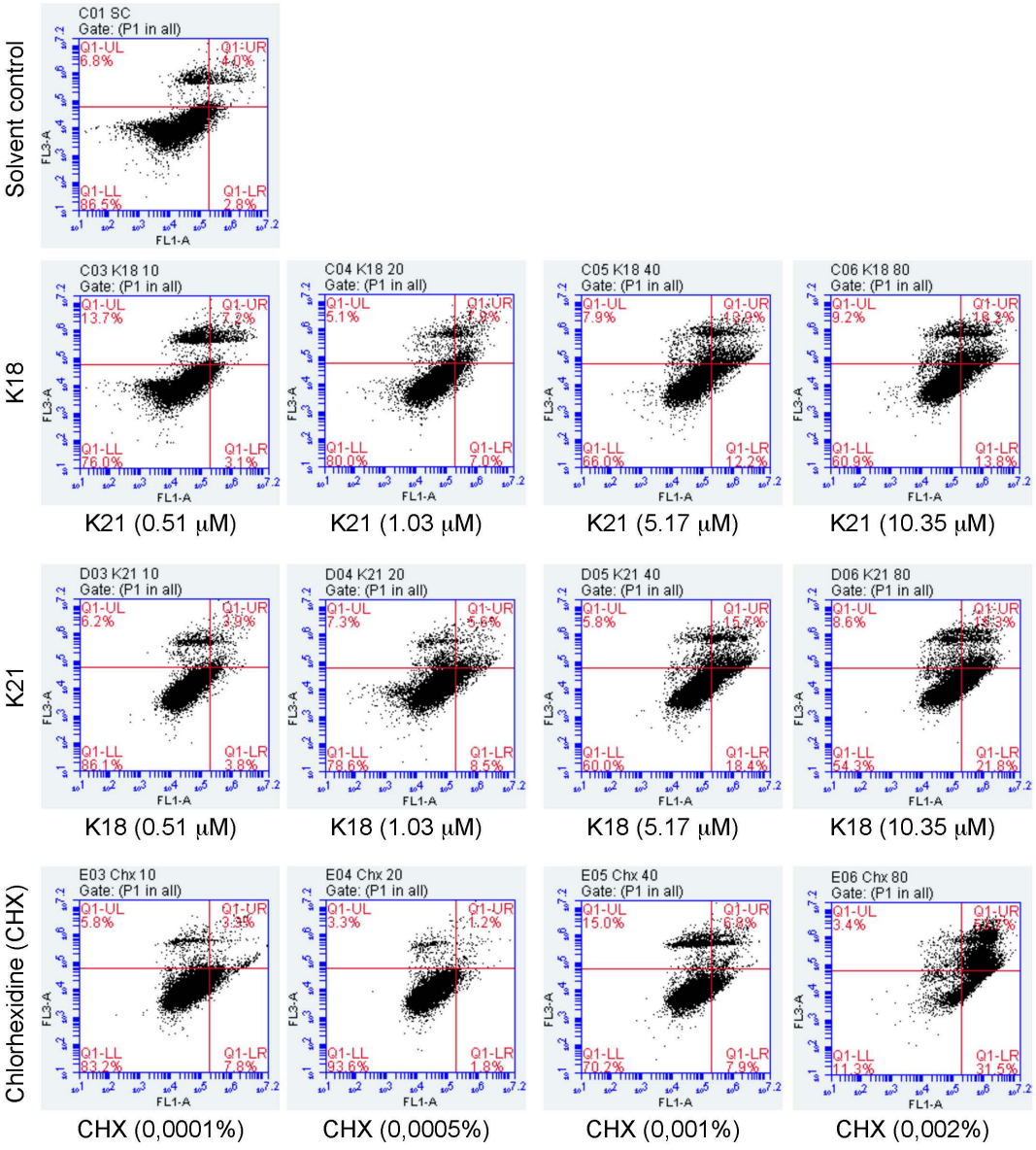

Solvent control
K18
K21
Chlorhexidine (CHX)
C01 SC
Gate: (P1 in all)
Q1-UL 6.8%
Q1-UR 4.0%
Q1-LL 86.5%
Q1-LR 2.8%
C03 K18 10
Gate: (P1 in all)
Q1-UL 13.7%
Q1-UR 7.2%
Q1-LL 76.0%
Q1-LR 3.1%
C04 K18 20
Gate: (P1 in all)
Q1-UL 5.1%
Q1-UR 7.5%
Q1-LL 80.0%
Q1-LR 7.0%
C05 K18 40
Gate: (P1 in all)
Q1-UL 7.9%
Q1-UR 12.9%
Q1-LL 66.0%
Q1-LR 12.2%
C06 K18 80
Gate: (P1 in all)
Q1-UL 9.2%
Q1-UR 18.3%
Q1-LL 60.9%
Q1-LR 13.8%
K21 (0.51 µM)
K21 (1.03 µM)
K21 (5.17 µM)
K21 (10.35 µM)
D03 K21 10
Gate: (P1 in all)
Q1-UL 6.2%
Q1-UR 3.9%
Q1-LL 86.1%
Q1-LR 3.8%
D04 K21 20
Gate: (P1 in all)
Q1-UL 7.3%
Q1-UR 5.6%
Q1-LL 78.6%
Q1-LR 8.5%
D05 K21 40
Gate: (P1 in all)
Q1-UL 5.8%
Q1-UR 15.7%
Q1-LL 60.0%
Q1-LR 18.4%
D06 K21 80
Gate: (P1 in all)
Q1-UL 8.6%
Q1-UR 15.3%
Q1-LL 54.3%
Q1-LR 21.8%
K18 (0.51 µM)
K18 (1.03 µM)
K18 (5.17 µM)
K18 (10.35 µM)
E03 Chx 10
Gate: (P1 in all)
Q1-UL 5.8%
Q1-UR 3.3%
Q1-LL 83.2%
Q1-LR 7.8%
E04 Chx 20
Gate: (P1 in all)
Q1-UL 3.3%
Q1-UR 1.2%
Q1-LL 93.6%
Q1-LR 1.8%
E05 Chx 40
Gate: (P1 in all)
Q1-UL 15.0%
Q1-UR 6.8%
Q1-LL 70.2%
Q1-LR 7.9%
E06 Chx 80
Gate: (P1 in all)
Q1-UL 3.4%
Q1-UR 50.7%
Q1-LL 11.3%
Q1-LR 31.5%
CHX (0,0001%)
CHX (0,0005%)
CHX (0,001%)
CHX (0,002%)
FL1-A
FL3-A

Figure 2. Immunomodulatory properties of K18, K21 and chlorhexidine. **(A)** TNFα mRNA expression was analyzed by qRT-PCR in presence of two different concentrations of each of the three compound. RAW 264.7 cells were seeded in 6-well plates and were treated either with K18, K21 or chlorhexidine (CHX) for 24 h. LPS treated cells were used as control. Cells treated with solvent control were used for normalization. Total RNA were extracted and used for qRT-PCR. **(B)** Same set of samples were used for quantitative analysis of IL-6 mRNA expression. **(C, D)** Same set of samples were used for quantitative analysis of IL-4 mRNA (C) and IL-10 (D) expression.

Figure 2

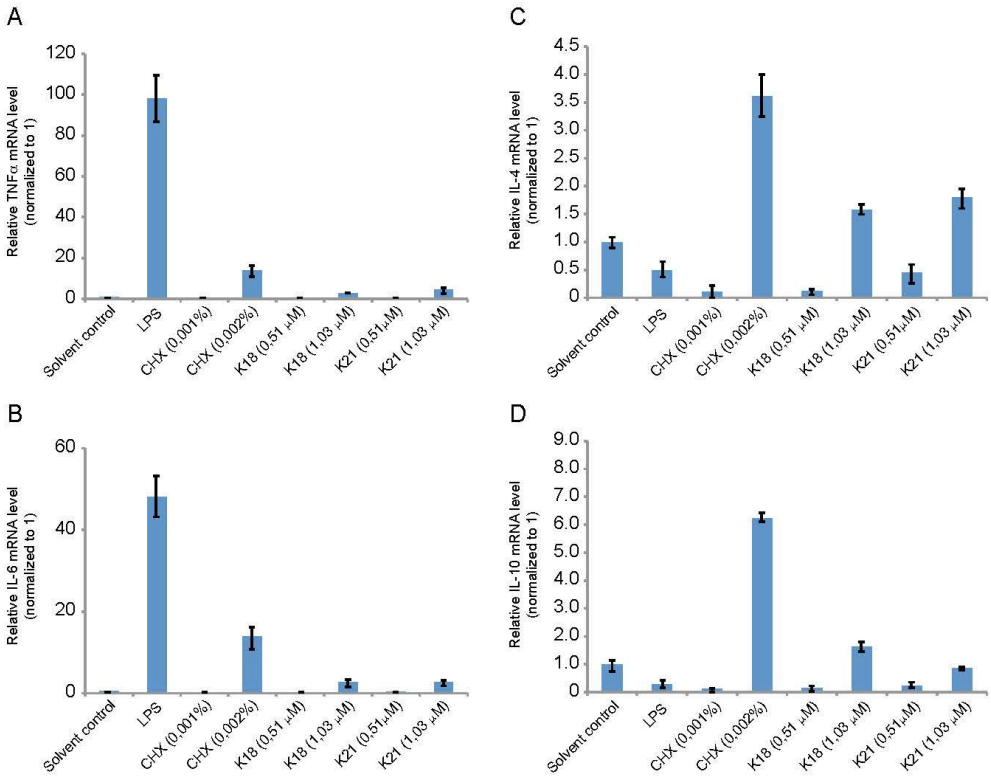
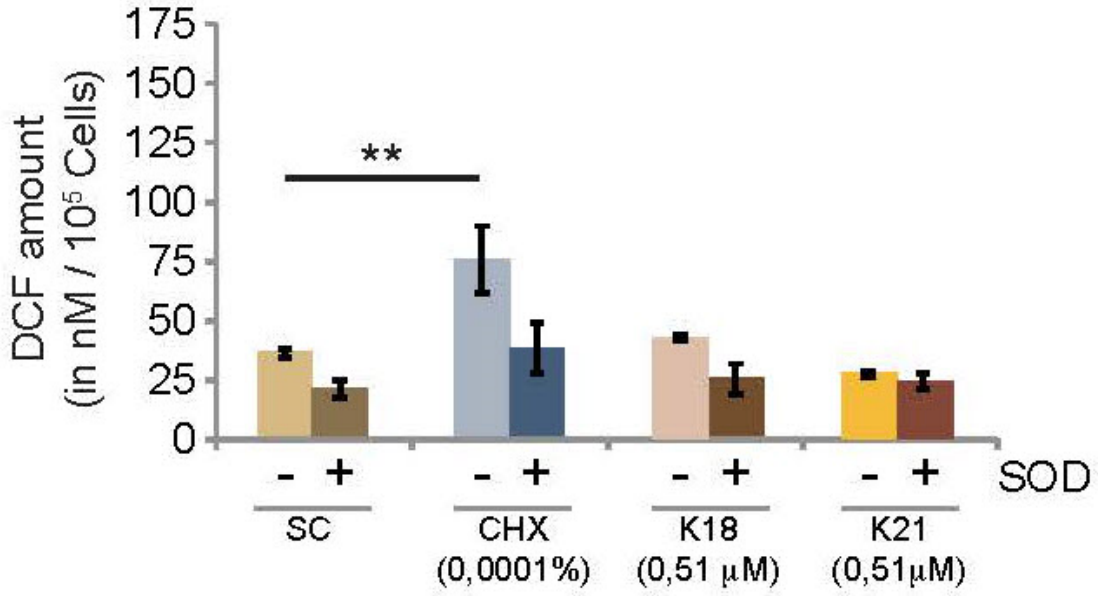

A
Relative TNFα mRNA level (normalized to 1)
120
100
80
60
40
20
0
B
Relative IL-6 mRNA level (normalized to 1)
60
40
20
0
C
Relative IL-4 mRNA level (normalized to 1)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
D
Relative IL-10 mRNA level (normalized to 1)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
Solvent control
LPS
CHX (0.001%)
CHX (0.002%)
K18 (0.51 μM)
K18 (1.03 μM)
K21 (0.51μM)
K21 (1.03 μM)

Figure 3. Intracellular ROS level in host cells changes upon drug treatment. RAW 264.7 cells were seeded in 6-well plates and were treated either with K18, K21 or chlorhexidine (CHX) for 24 h. In parallel, cells pre-treated with 1000U of SOD were treated with same amounts of K18, K21 or chlorhexidine for 24 h. At the end of the treatment time, cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) was added for 1 h at 37°C. Total cellular ROS content was measured using an Elisa reader. Data represent the mean ± SEM of three independent infection experiments performed at the same day. Statistical analysis was based on the Student t-test, and p values between different sample groups are mentioned above the respective line bars.

Figure 3


DCF amount
(in nM / 10⁵ Cells)
175
150
125
100
75
50
25
0
**
- +
- +
- +
- +
SOD
SC
CHX
(0,0001%)
K18
(0,51 µM)
K21
(0,51µM)