Vitamin K analogs influence the growth and virulence potential of enterohemorrhagic *Escherichia coli*

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) causes serious food-borne disease worldwide. It produces the very potent shiga toxin (Stx2). The Stx2-encoding genes are located on a prophage, and production of the toxin is linked to synthesis of Stx phages. There is, currently, no good treatment for EHEC infections, as antibiotics may trigger lytic cycle activation of the phages and increased Stx production.

This study addresses how four analogs of Vitamin K; phylloquinone (K1), menaquinone (K2), menadione (K3) and menadione sodium bisulfite (MSB) influence growth, Stx2-converting phage synthesis and Stx2 production by the EHEC O157:H7 strain EDL933. Menadione and MSB conferred a concentration-dependent negative effect on bacterial growth while phylloquinone or menaquinone had little and no effect on bacterial growth, respectively. All four vitamin K analogs affected Stx2-phage production negatively in uninduced cultures and in cultures induced with either hydrogen peroxide (H_2O_2), ciprofloxacin or mitomycin C. Menadione and MSB reduced Stx2 production in cultures induced with either H_2O_2 or ciprofloxacin. MSB also had a negative effect on Stx2 production in two other EHEC isolates tested. Phylloquinone and menaquinone had, on the other hand, variable and concentration-dependent effects on Stx2 production. MSB, which conferred the strongest inhibitory effect on both Stx2-phage and Stx2 production, improved growth of EHEC in the presence of H_2O_2 and Ciprofloxacin which could be explained by the reduced uptake of ciprofloxacin into the bacterial cell. Together, the data suggest that vitamin K analogs have a growth and potential virulence reducing effect on EHEC which could be of therapeutic interest.

Importance

Enterohemorrhagic *E. coli* (EHEC) can cause serious illness and deaths in humans by producing toxins that can severely damage our intestines and kidneys. There is currently no optimal treatment for EHEC infections, as antibiotics can worsen disease development. Consequently, the need for new treatment options is urgent. Environmental factors in our intestines can affect the virulence of EHEC and help our bodies fight EHEC infections. The ruminant intestine, the main reservoir for EHEC, contains high levels of vitamin K but the levels are variable in humans. This study shows that vitamin K analogs can inhibit growth of EHEC and/or production of its main virulence factor, the Shiga toxin. They may also inhibit the spreading of the Shiga toxin encoding bacteriophage. Our findings indicate that vitamin K analogs have the potential to suppress development of serious disease caused by EHEC.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a zoonotic pathogen responsible for food- and water-borne outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS), a disease with severe complications and 2 - 5% fatality (1). The World Health Organization (WHO) estimates that 10% of patients with EHEC infection develop HUS. EHEC infections affect young children most severely, and are difficult to treat, as administration of antibiotics may worsen the disease (1). The Shiga toxin (Stx) is considered the main virulence factor of EHEC. Stx binds to the globotriaosylceramide (Gb3) receptor, a glycolipid particularly abundant on kidney cells, that is also present on endothelial cells and in the brain (2, 3). It causes cell damage by inhibiting protein synthesis in its target cells, which is the main cause of the development of HUS and neurological symptoms during an EHEC infection (4).

62 There are two antigenically distinct main types of Stx. Stx1, produced by *Shigella dysenteriae*
63 and some Stx producing *E. coli* (STEC) strains, and Stx2, which is produced by mainly by
64 STEC/EHEC (5). Epidemiological data indicate that STEC strains that produce Stx2 is more
65 strongly associated with severe human disease than those that produce only Stx1 (6-9). Stx2 is
66 encoded by a chromosomally integrated (lysogenic) Stx-converting prophage (Stx phage) (10,
67 11). DNA damage, including that induced by antibiotics or reactive oxygen species (ROS), will
68 trigger the bacterial SOS response. This induces the lysogenic Stx2 phage to enter the lytic
69 (proliferative) cycle, leading to synthesis and release of phage particles and Stx2 (12, 13).
70 Single STEC cells in a population can start production of Stx phages even in the absence of an
71 external trigger. This phenomenon is called “spontaneous prophage induction” and occurs at
72 different frequencies in different STEC strains (14).

73 Ruminants are considered the major reservoir of EHEC (reviewed in (15)). Adult cattle, with a
74 mature rumen and ruminal microbiota, are usually unaffected by EHEC colonization (16).
75 Neonatal calves (<28 days old) may, however, develop symptoms from exposure to EHEC,
76 such as enterocolitis (17).

77 It is unknown why EHEC infections manifest differently among infected individuals. It has
78 been reported that various small molecules in the gastrointestinal tract influence the
79 pathogenicity of EHEC; fucose, cleaved from mucins, inhibits EHEC adhesion to the human
80 epithelial cells (18), succinate enhance EHEC adhesion to human epithelial cells (19), vitamin
81 B_{12} enhances Stx production (20), vitamin A deficiency exacerbates damage to the intestine and
82 increases EHECs survival in mice (21), vitamin D strengthens tight junctions and,
83 consequently, the intestinal barrier function (22), vitamin B7 (biotin) influences the target site
84 for colonization in the human intestine (23), and manganese blocks intracellular trafficking of
85 Stx and protects against shiga toxicosis (24).

Vitamin K occurs in various forms and at various levels in the human intestine. The amount of vitamin K present in the intestine is influenced by the diet, the composition of the gut microbiota and the age of the infected individual (25-27). Vitamin K exists in three main forms, phylloquinone (vitamin K_1), menaquinone (vitamin K_2) and menadione (vitamin K_3) (Fig.1). Phylloquinone is found in all organisms that perform photosynthesis, as it acts as an electron acceptor in photosystem 1 (28). It is therefore available to humans through the diet; fruits and leafy green vegetables are particularly rich in phylloquinone (29-32). Vitamin K is particularly abundant in the intestine of ruminants, mostly since they acquire nutrients from plant-based food, especially grass, which has a high content of phylloquinone, but also because the ruminal microbiota produces large amounts of menaquinone (33). Menaquinone is bacterially produced and can therefore be present in fermented foods such as cheese and yogurt (26, 34). Menaquinone is also present in meats and organ meats as phylloquinone is converted to menaquinone in tissues (35). The human intestinal microbiota also contains bacterial species that produce menaquinones (25, 36). Menadione, is the simplest form of vitamin K and an intermediate in the biosynthesis of menaquinone in bacteria (37). It is also an intermediate molecule in the metabolic conversion of phylloquinone to menaquinone in the metabolism of vertebrates (37). Furthermore, menadione is the main vitamin K analog found in enterocytes (38-40) . Different forms of menadione, such as menadione and menadione sodium bisulfite (MSB), can also be produced synthetically.

The levels of different types of vitamin K that are present in the intestine, enterocytes and in the blood varies between individuals and during different times of life (25). Vitamin K deficiency is quite uncommon in adult humans, but subclinical deficiency can easily be induced by limiting phylloquinone intake and by treatment with antibiotics (41, 42). Although healthy children rarely suffer from vitamin K deficiency, defined with respect to blood clotting, the blood levels of vitamin K in children are much lower than in adults (43, 44). It has also been

reported that menadione inhibit growth and exotoxin production by *Staphylococcus aureus*, *Bacillus anthracis*, *Streptococcus pyogenes* and *Streptococcus agalactiae* at a concentration of 10 - 200 µg/mL (45). This was, however, not observed for menaquinone and phylloquinone (45). Furthermore, it has previously been shown that menadione exhibits an anti-bacterial activity against the gastric pathogen *Helicobacter pylori* (46).

Treatment recommendations for EHEC infections are still mainly of supportive character, despite the serious nature of the disease. As antibiotic treatment remains a controversial issue, there is a dire need for alternative treatment procedures that can restrict the production of Stx and decrease the risk of developing HUS. In this work, we have studied how different vitamin K analogs influence the growth and virulence potential of EHEC. The rationale for studying the role of vitamin K in the complex interplay between EHEC and their Stx phages, lies in the fact that vitamin K is particularly abundant in the intestine of ruminants, the main natural habitat for EHEC, and that it is a relevant bio-molecule in the human intestine. Additionally, menadione has been shown to have an anti-bacterial and/or anti-virulence effect on several other pathogenic bacteria. Our study addresses how the four vitamin K analogs: phylloquinone, menaquinone, menadione and menadione sodium bisulfite (MSB), influence growth, Stx2 production, Stx2-phage release, and bacterial survival during induction of the phage lytic cycle, using the EHEC O157:H7 strain EDL933, which carries the Stx2-converting bacteriophage BP933W, as a model organism. The effect of MSB was also analyzed for the Norwegian EHEC O103:H7 outbreak strain NIPH-11060424 and the O157:H7 NVH-E7 strain.

RESULTS

The effect of vitamin K analogs on growth of EHEC

As a first step to explore the effect of different vitamin K analogs on EHEC, we cultured the EHEC strain EDL933 in the presence and absence of different concentrations of phylloquinone, menaquinone, menadione bisulfite (MSB) or menadione. As shown in Fig. 2 A and B and Table S1, phylloquinone and menaquinone had no discernible effect on bacterial growth, as *measured by CFU/mL* (Colony Forming Units/mL), at all concentrations tested, except for the highest concentration of phylloquinone (724 µM) that reduced the maximum rate of growth (V_{max}) by 25% compared to the negative control (culture without phylloquinone). The presence of menadione did not influence V_{max} significantly at any of the concentrations tested. However, the presence of 36 µM of menadione caused a significant reduction of CFU/mL at both 2 and 6 hrs of growth (Fig. 2C, Table S1). MSB reduced V_{max} significantly at all concentrations from 36 µM and above (Fig. 2D, Table S1). Similar results were obtained with strains EDL933, NIPH-11060424 and O157:H7 NVH-E7 when growth was assessed by measuring OD_{600} (Fig. S1A-D, FigS2 and Table S1).

Effect of vitamin K analogs on Stx production

In order to investigate how different vitamin K analogs influence production of Stx2, we grew EDL933 in the presence or absence of phylloquinone, menaquinone, MSB or menadione. After 3 hrs of growth, BP933W was induced to enter the lytic cycle, with concomitant Stx2 expression, with either H_2O_2, ciprofloxacin or MMC. The total level of Stx2 released in bacterial cultures 6, 8 and 20 hrs post-induction was assessed by LC-MS/MS, and the effect of vitamin K was determined through comparison with cultures induced with the same agents but without vitamin K. In un-induced cultures (i.e. no added inducing compound) the level of Stx2 was below the detection level (i.e., below 10 ng/mL). Without added vitamin K analogs, ciprofloxacin-induced cultures showed a higher level of released Stx2 compared to MMC and H_2O_2-induced cultures (Fig. 3A-C).

159 Of the vitamin K analogs tested, MSB demonstrated the strongest reducing effect on Stx2
160 production. In the presence of MSB, Stx2 production was significantly reduced in samples
161 collected 6, 8 and 20 hrs post-induction with H_2O_2 (between 81 - 86% reduction), 6 and 8 hrs
162 post-induction with ciprofloxacin (56 and 60%, respectively) and 8 hrs postinduction with
163 MMC (37%) (Fig. 3A-C, Table S3). Menadione treated samples from 20 hrs post-induction
164 with H_2O_2 also showed a 61% reduction in Stx2 production (Fig. 3A, Table S3).

165 No significant inhibitory effect was seen for the other combinations of inducing agents and
166 Vitamin K analogs tested although cultures containing menaquinone or phylloquinone showed
167 a dose-dependent effect on Stx2 production; during induction with H_2O_2 and ciprofloxacin. The
168 highest concentration (181 µM) of phylloquinone showed significantly inhibitory effect (47-
169 55%) on Stx2 production in H_2O_2 and ciprofloxacin-induced samples compared to lower
170 concentrations (Fig. 3A and B). Such effect was not observed in samples induced with MMC
171 (Fig. 3C). In contrast to phylloquinone, the lower concentration of menaquinone showed a trend
172 towards stronger inhibitory effect on Stx2 production compared to the higher concentration in
173 samples induced with ciprofloxacin and MMC (Fig. 3B and C).

174 The effect of MSB on Stx release was also tested for EHEC O103:H25 NIPH-11060424 and
175 EHEC O157:H7 NVH-E7 using a VTEC-RPLA kit. Similar to what was observed for strain
176 EDL933, these strains showed approximately 2- and 6-fold reduced Stx production during
177 treatment with MSB (Fig S3A).

178

179 **The effect of vitamin K on production of the Stx2-converting phage BP933W**

180 As Stx2 production is linked to induction of the lytic cycle and release of Stx converting phages
181 we wanted to test how different vitamin K analogs influence phage production under un-

induced conditions and after induction with either H_2O_2, ciprofloxacin or MMC in EDL933. By using a plaque assay for phage enumeration, we found that MMC acted as the most efficient inducer of BP933W production, while H_2O_2 and ciprofloxacin demonstrated similar, but lower, induction capabilities (Fig. S4). All vitamin K analogs tested reduced the BP933W titer in uninduced cultures and the strongest reducing effect was observed in cultures containing menadione or MSB (88.4% ± 5.6 and 85.0% ± 13.9, respectively) (Fig. 4A). In H_2O_2-induced cultures, the presence of phylloquinone or menaquinone resulted in variable and much weaker reducing effects on the BP933W titer (33.4% ± 26.9 and 39.0% ± 30.8, respectively) compared to H_2O_2-induced cultures containing menadione or MSB (97.6% ± 1.6 and 97.2% ± 1.7, respectively) (Fig. 4B). MSB also exhibited a strong inhibitory effect on BP933W production in ciprofloxacin-induced cultures (94.2% ± 2.5) while the other vitamin K analogs showed similar inhibitory effects (from 60% to 74% inhibition) (Fig. 4C). Of the vitamin K analogs tested, menadione and MSB showed the strongest inhibitory effects on BP933W titers in cultures induced with MMC (83.8% ± 11.4 and 82.4% ± 11.1, respectively) (Fig. 4D).

The reduced BP933W titers observed in cultures containing vitamin K analogs could either be due to reduced bacterial growth, reduced synthesis of phages or to a direct effect of these compounds on released phages, affecting their ability to infect the recipient *E. coli* strain. To determine the effect of vitamin K analogs on the infectivity of BP933W, phage filtrates were incubated with high concentrations of phylloquinone, menaquinone, menadione or MSB before they were used in the plaque assay. The results showed that both menaquinone and phylloquinone reduced the infectivity of BP933W i.e., reduced plaque-formation. MSB did, on the other hand, cause an increased infectivity of the Stx converting phage while no positive or negative effect on phage infectivity was observed for menadione (Fig. 5).

Effect of vitamin K on *stx2* and *recA* transcription

207 Since vitamin K analogs inhibited production of BP933W we wanted to explore if they affected
208 induction of the SOS-response and thereby *stx2* transcription. EDL933 were grown in the
209 presence or absence of vitamin K analogs and induced with either H_2O_2, ciprofloxacin or MMC.
210 Quantitative real-time PCR was used to examine the effect of the different vitamin K analogs
211 on *recA* (indicative on SOS-response activation) and *stx2* transcription two hrs post-induction.
212 The results from the qRT-PCR analyses are shown in Fig. 6.

213 Both MSB and menadione had a reducing effect on *recA* transcription when H_2O_2 was used as
214 the phage inducing agent. At the examined time point, the different vitamin K analogs had little
215 or no effect on *recA* transcription during ciprofloxacin or MMC treatments. MSB had a
216 reducing effect on *stx2* transcription, regardless of the inducing agent, while menadione only
217 showed a reducing effect on *stx2* transcription during induction with H_2O_2. Phylloquinone and
218 menaquinone did not confer any noticeable effect on *stx2* or *recA* expression at the time point
219 tested, regardless of phage inducing agent used (Fig. 6). MSB also conferred an inhibitory effect
220 on both *stx2* and *recA* transcription in EHEC strains NIPH-11060424 and NVH-E7 (Fig. S3B).

221

222 **The presence of menadione or MSB prevent antibiotic-induced morphological changes**.
223 Previous reports have shown that exposure to ciprofloxacin and mitomycin induces
224 morphological and biochemical changes in *E. coli* cells (47, 48). Similar to these reports, we
225 observed that EHEC cells demonstrated elongated (filamentous) morphology when grown in
226 the presence of ciprofloxacin or mitomycin. Ciprofloxacin-induced samples containing
227 menadione or MSB did, on the other hand, contain fewer filamentous cells or much shorter
228 filaments (Fig. 7). MMC treated cells showed even more elongated appearance compared to
229 ciprofloxacin treated cells and also here, the presence of menadione or MSB reduced the level
230 of cell elongation. Cells induced with H_2O_2 did not show a markedly different morphology and

there was no obvious difference between H_2O_2-induced samples with or without vitamin K (data not shown).

Menadione and MSB influences the survival of EHEC in the presence of phage-inducing agents.

As some vitamin K analogs had a negative effect on production of BP933W it seemed likely that these compounds also prevent phage-mediated lysis of the bacterial cells (i.e. bacterial death). A decline in bacterial growth, as measured by CFU/mL, was observed in all cultures immediately after addition of H_2O_2. The decline in bacterial growth was, however, much less pronounced in cultures containing menadione or MSB (Fig. 8A). As the exposure of EHEC to ciprofloxacin or MMC made EHEC grow into unseptated filaments, it was not relevant to measure bacterial growth in cultures containing these antibiotics by counting CFU/mL, since one filament containing multiple bacterial genomes will count as one colony. As measurement of optical density provided similar results as measurement of CFU/mL (Fig. 2 and Fig. S1B-C), the effect of vitamin K analogs on bacterial survival was determined my measurement of OD_{600} in cultures containing ciprofloxacin or MMC. Addition of 1 x MIC of ciprofloxacin or 0.5 µg/mL of MMC did not confer an immediate negative effect on bacterial growth (Fig. 8B and C). The presence of MSB slowed down growth after induction with ciprofloxacin t (Table. S5). After 5 to 6 hrs of growth, there was a strong decline in OD_{600} in both ciprofloxacin and MMC-induced cultures, indicating cell lysis (Fig. 8B and C). In MMC-induced cultures, MSB seemed to have a slight positive effect on bacterial survival (Fig. 8B). This was not seen for the other types of vitamin K tested. MSB also conferred a positive effect on bacterial survival when strain NIPH-11060424 and NVH-E7 was treated with 0.5 µg/mL MMC in the presence or absence of 72 µM MSB (Fig. S5).

255

256 **MSB reduces uptake of ciprofloxacin into the bacterial cells**

257 To test whether the vitamin K analogs decreased phage and/or Stx2 production by reducing the
258 uptake of ciprofloxacin into the bacterial cell, we cultured strain EDL933 in the presence or
259 absence of different types of vitamin K and induced the cultures with 1 x MIC (0.06 µg/mL) of
260 ciprofloxacin. The presence of MSB in the growth media caused an approximately 6-times
261 reduction in the intracellular level of ciprofloxacin. Both menaquinone and menadione reduced
262 uptake of ciprofloxacin (48.9% ± 9.6 and 40.5% ± 11.4, respectively). No significant effect on
263 ciprofloxacin uptake was observed for phylloquinone ($p = 0.42$) (Fig. 9).

264

265 **Discussion**

266 In this work, we have studied the effect of four vitamin K analogs on the growth, Stx2
267 production, production and infectivity of the Stx2-phage, and survival of the EHEC O157:H7
268 strain EDL933 during induction of the phage lytic cycle. The study was done as part of the
269 search for novel treatment regimens for EHEC infections. Our strategy was to study the vitamin
270 K analogs as previous data have shown that these biomolecules could potentially limit growth
271 and virulence of other pathogenic bacteria. Firstly, we showed that two of vitamin K analogs
272 tested, menadione and MSB, inhibited growth of strain EDL933, while the presence of
273 phylloquinone or menaquinone did not seem to seem to have a pronounced effect on growth
274 under the tested conditions. Menadione generates reactive oxygen species, such as superoxide
275 anions (O_2^-), which are toxic for the bacteria, and this could explain the negative effect of these
276 compounds on bacterial growth (49).

277 The effects of the various vitamin K analogs on production of the Stx2-converting phage and
278 Stx2 were tested under un-induced conditions and by using H_2O_2, ciprofloxacin, or MMC as
279 phage-inducing agents. H_2O_2 represents a natural inducing agent in the host as it is produced
280 by neutrophils and other cells in infected humans and by protists that predate on bacterial cells
281 (50, 51). Ciprofloxacin is clinically relevant as it is used to treat different types of *E. coli*
282 infections, but for fear that antibiotic treatment will exacerbate the symptoms, it is not used to
283 cure EHEC infections (52, 53). MMC is an efficient inducer of the SOS-response in *E. coli* and
284 is frequently used in research to induce phages to enter lytic cycle, but it is normally not used
285 for treating human infections due to its toxicity and mutagenicity (54-58). The presence of MSB
286 reduced the levels of released Stx2 in H_2O_2-induced cultures, and the same outcome was
287 observed for MSB in ciprofloxacin-induced cultures. A similar reducing effect of MSB on Stx2
288 production was also observed in two other EHEC strains tested, which suggests that this effect
289 could be a general response among EHEC strains. Results from the plaque assay showed that
290 the presence of all four types of vitamin K reduced plaque formation independent of which
291 inducing agent that was used to trigger activation of lytic cycle. MSB exhibited the strongest
292 reducing effect on plaque production. The reduction in plaque formation was not due to that
293 MSB treatment reduced the ability of BP933W to infect the recipient strain, as the plaque count
294 increased when phage filtrates were treated with MSB. The mechanism for the positive effect
295 of MSB on the infection rate of BP933W is unknown. However, as phage production results in
296 lysis and death of the bacterial cell, the reduced production of phages in cultures containing
297 menadione or MSB could explain why menadione and MSB had a positive effect on bacterial
298 growth/survival in the presence of H_2O_2 and that MSB had a slight positive effect on bacterial
299 survival in cultures containing MMC.

300 The reduced Stx2-phage synthesis and Stx2 production led us to the hypothesis that vitamin K
301 analogs could inhibit the uptake of molecules into the bacterial cell. Indeed, by LC MS/MS

measurements, we could show that the intracellular level of ciprofloxacin taken up into the bacterial cell was reduced when strain EDL933 was grown in the presence of MSB, menadione and menaquinone. In *E. coli*, exposure to redox cycling drugs such as menadione, leads to activation of the OxyR protein, a global transcriptional regulator important in oxidative stress resistance (reviewed in (59)). This leads to increased expression of the homologous MarA, SoxS, and Rob proteins that are involved in regulation of the adaptive response of *E. coli* to chemical stresses, oxidative stressors, and antibiotic compounds (60-62). Their upregulation is associated with altered expression of genes involved in the efflux of antibiotics (*acrAB* and *tolC*), decrease in outer-membrane permeability (*ompF*) and superoxide resistance (*fpr* and *sodA*) (63-68). The reduced uptake of ciprofloxacin suggests that the *mar/sox/rob* regulon could be involved in the increased tolerance against H_2O_2 and the reduced uptake of ciprofloxacin in MSB-treated EHEC strains observed in the present study. Furthermore, the increased tolerance against H_2O_2 and reduced uptake of antibiotics could inhibit activation of SOS response and induction of the lytic cycle of the Stx2-phage, followed by reduced production of Stx2.

A previous study has shown that nitric oxide (NO) exhibits an inhibitory effect on production of Stx converting phages, *stx* expression and MMC-induced killing of strain EDL933 [48]. This effect resembles the menadione- and MSB-mediated resistance to the growth inhibitory/killing effect of H_2O_2 and MMC observed in our study. Like menadione, NO also activates the SoxRS response system in *E. coli*, which confers protection against subsequent exposure to harmful compounds such as H_2O_2 and antibiotics, which induce the lytic cycle of Stx phages [48]. However, further studies are required to elucidate the mechanisms behind the anti-bacterial/anti-virulence effects of menadione, MSB and vitamin K on EHEC. Non-targeted proteomic or transcriptomic methods could be employed to get a global view on the biological processes behind their effects on EHEC. Further studies should also include strains of different serotypes and Stx profiles.

Earlier studies have suggested that Stx converting phages exhibit a diverse host-range and are able to infect commensal *E. coli* strains (14, 69). Gamage *et al*., showed that commensal non-O157 *E. coli* strains were susceptible to both lytic and lysogenic infections by Stx2 converting phages from an EHEC O157:H7 strain (69). Based on their findings, they suggested that commensal *E. coli* strains can amplify Stx production if they are susceptible to infection by Stx phages. Similarly, the Stx2 phage (ϕ734) from the Norwegian outbreak strain NIPH-11060424 was shown to lysogenize commensal *E. coli* strains from healthy children below 5 years of age (14). When commensal ϕ734 lysogens were induced to enter the lytic cycle by H_2O_2, most of the commensal strains produced more ϕ734 phages than the donor NIPH-11060424 strain. Notably, five of the commensal strains spontaneously (non-induced) produced more ϕ734 phages than the NIPH-11060424 strain did under either H_2O_2-or MMC induced conditions (14). Altogether, the reports by Gamage and Iversen suggest that phages that are released by EHEC and subsequently infect commensal strains, can potentially increase the pathogenic potential of EHEC during infection. With this in mind it is tempting to speculate that if vitamin K analogs, from the diet and from the metabolism of the host intestinal microbiota, inhibit production and dissemination of infective Stx phages, they may also restrict development of severe disease. A similar effect could potentially be achieved by using MSB or menadione therapeutically. It is also tempting to speculate that the high concentrations of vitamin K in the ruminant intestine could contribute to persistence and long-term carriage of EHEC by ruminants, by preventing phage induction with concomitant lysis of the EHEC cells.

Menadione has been shown to produce carbon monoxide (CO) endogenously *in vivo* in rat brain microsomes and also *in vivo* (70). CO has primarily a reputation as a toxic gas when inhaled in large quantities. It does, however, have important anti-inflammatory, cytoprotective and vasodilatory properties *in vivo*, that are beneficial to health and have many therapeutic applications. CO also has antimicrobial properties and CO-releasing molecules (CORMs) are

pointed out to be potential “antimicrobial agents” in a post-antibiotic era (71-74). It could be that the inhibitory effect of menadione and MSB we see on the growth of EHEC could, at least partly, be due to CO production.

Together, our results suggest that MSB, menadione, phylloquinone and menaquinone could function as supportive agents to prevent severe outcomes from EHEC infections by reducing the virulence of the infecting EHEC strain. In theory, by targeting virulence factors, the resilience of EHEC within the human host could be impaired allowing the host immune system to combat the infection. To evaluate the potential of vitamin K analogs as therapeutic agents we need an increased understanding of the effects of these compounds on the interaction between EHEC and the human host. By studying the effect of vitamin K analogs on the interaction between EHEC and cultured cells we could gain an increased knowledge on how these compounds the initial infection process, colonization and pathogenesis. However, the intestinal environment is complex and cannot be adequately simulated *in vitro*. For example, the host immune response and the normal microbiota are factors that are not considered in *in vitro* models and are probably of utmost importance for the outcome of EHEC infections. The use of *in vivo* models is, therefore, necessary to further evaluate if vitamin K analogs could be used to treat EHEC infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions

EHEC O157:H7 strain EDL933 [47] was used to study the effect of vitamin K on the growth and virulence potential of EHEC. EDL933 carries both *stx1* and *stx2* genes, but it has previously been shown that *stx1* is poorly expressed in this strain and not upregulated under MMC

375 treatment [48]. The *E. coli* laboratory strain DH5α [49] was used as a recipient strain in the
376 plaque assay. The Norwegian outbreak strain NIPH-11060424 of serotype O103:H25 [50] and
377 NVH-E7, a non-sorbitol fermenting O157:H7 strain belonging to MLST type 11 were also used
378 to test the effect of MSB on growth and Stx2 production. NIPH-11060424 is Stx1 negative and
379 Stx2 positive, while NVH-E7 is both Stx1a and Stx2 positive. The *stx2*-negative EHEC
380 O157:H7 strain NVH-E961 was used as a Stx2 negative matrix in LC MS/MS samples.
381 EDL933 and DH5α were grown under agitation (200 rpm) in Luria Bertani (LB) broth at 37 ºC.
382 Strains NIPH-11060424 and NVH-E7 were grown in Brain Heart Infusion (BHI) broth (Oxford
383 Limited, Basingstoke, UK) under agitation (225 rpm) at 37 ºC. Ciprofloxacin (AppliChem,
384 Darmstadt, Germany) at 0.03 µg/mL (1/2 MIC for EHEC EDL933 [51]), MMC (Sigma
385 Aldrich) at 0.5 µg/mL (75) or H_2O_2 (NAF, Oslo, Norway) at 3 mM were used to induce the
386 phage lytic cycle when the cultures had reached an optical density at 600 nm (OD_{600}) of 0.5, i.e.
387 when the cultures had reached the exponential growth phase. After addition of inducing agents,
388 the cultures were incubated in the dark.

389 To determine the dose-response effect of menadione sodium bisulfite (MSB) (Sigma Aldrich,
390 St. Louis, MO), menaquinone (Supelco, Bellefonte, PA), phylloquinone (Supelco, Bellefonte,
391 PA) and menadione (Sigma Aldrich, St. Louis, MO), the EHEC strains were grown overnight
392 at 37 ºC under agitation. 20 µL of the overnight culture was inoculated into an Erlenmeyer flask
393 containing 20 mL of LB broth, when appropriate, a defined concentration of menadione, MSB
394 or either of the two types of vitamin K tested. MSB was solved in water, while phylloquinone,
395 menaquinone and crystalline menadione are lipid soluble and therefore solved in DMSO. The
396 final concentration of DMSO in the cultures was 0.05%. The OD_{600} was monitored every 30
397 min for 10 hrs, and samples were taken every 2 hrs for 10 hrs for enumeration of CFU/mL

398 **Sampling for Stx measurements**

399 EDL933 was grown in the presence or absence of the different forms of vitamin K and induced
400 as described above. The induced cultures were incubated at 37 °C in dark with shaking at 200
401 rpm, and samples were collected at 6, 8 and 20 hrs post induction. The samples collected after
402 6 and 8 hrs were kept at 0 °C on ice overnight, for minimal loss of Stx during storage. The
403 choice of storing the samples on ice was done after examining what temperatures were ideal
404 for storing Stx overnight with minimal loss of toxin. The temperatures tested were -80 °C, -20
405 °C, 0 °C and 4 °C. Storage at 0 °C (in the dark) on ice showed the best yield of Stx2 toxin.

406 **Protein reduction, alkylation and digestion**

407 Protein reduction, alkylation and digestion was done using a modified version of the method
408 described in Silva et. al, 2014 [54]. After harvesting EDL933 cells at 20 hrs post induction, 100
409 µL of the bacterial cultures were added to a 1.5 mL centrifuge tube and diluted with 20 µL of
410 milliQ® water. A volume of 100 µL of the Stx negative matrix strain was spiked with 20 µL of
411 milliQ® water with the following concentrations: 0, 10, 28, 52.5, 70 and 140 ng/mL of the
412 peptide standard YNEDDTFTVK (Biomatik, Cambridge, Canada) to create a calibration curve.
413 The EDL933 samples and the calibration standards were thereafter treated identically
414 throughout the preparation for LC/MS analysis. Disulfide bond reduction was performed by
415 adding 2 µL of 100 mM dithiothreitol, (DTT, Sigma Aldrich) solved in 25 mM ammonium
416 bicarbonate (buffer A) to the samples and incubating them for 1 h. at 37 °C. To ensure alkylation
417 of the free sulfhydryl groups on cysteine residues in the toxin, the samples were cooled to room
418 temperature and 8 µL of 100 mM iodoacetamide (IAA, Sigma Aldrich) solved in buffer A was
419 added. The samples were then incubated in darkness at room temperature for 1 h. Subsequently,
420 4 µL of 100 mM DTT, solved in buffer A, was added to quench excess iodoacetamide, followed
421 by addition of 10 µL of Sequencing Grade Modified Trypsin (100 µg/mL, Promega). The
422 samples were incubated at 37 °C for 2 hrs (76). All samples were then transferred to 0.3 mL PP

Short Thread Micro-vials (VWR) and capped. Samples that were expected to have Stx2 levels above the highest point in the calibration curve, was diluted 1/5 in a Stx2 free matrix. A MS-standard and a Spiked Matrix blind were also created to ensure the accuracy of the analyses.

LC/MS analysis

The quantification of Stx2 was done by LC-MS/MS. The analysis was performed using an Agilent 1290 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent G6490 MS/MS (Agilent Technologies, Santa Clara, CA, USA) containing an Agilent jet stream electrospray ion source. Separation was done using a 2.1 x 50 mm Agilent Zorbax SB-C18 column (1.8 µm). The chromatographic method was 5.5 min. The gradient started at 98% mobile phase A, that within two minutes was decreased to 60% A. Mobile phase B was increased to 100% in 0.2 min, held for 1.8 min, and then returned to 98% mobile phase A, which was held for 1.5 min. The flow was held constant at 0.6 mL/min. Mobile phase A consisted of 0.5% acetic acid in water, and mobile phase B of 0.5% acetic acid in 90% acetonitrile. The column compartment and autosampler were held at 25 °C and 4 °C, respectively. Stx2 was detected using multiple reaction monitoring (MRM), with mass transitions set at 616.3 m/z → 135.9 m/z for quantification, and 616.3 m/z → 277.9 m/z as qualifier transition.

Semi-quantification of Stx2 levels using VTEC RPLA kit

The VTEC RPLA toxin detection kit (Oxford Limited, Basingstoke, UK) was used to determine Stx2 production in culture supernatants of strains NIPH-11060424 and NVH-E7. The assay was performed according to the manufacturer's instructions. The cultures were induced by MMC as described above and the samples were harvested 4 hrs after induction. The amount of toxin in each test-well was reduced 2-fold at each dilution. The reciprocal of the highest dilution causing latex agglutination was considered as the Stx-titer.

447 **Plaque assay**

448 The plaque assay used for quantification of infectious phage particles was modified from a
449 method previously described by O'Brien et al, 1984 (10). Briefly, cultures of EDL933, grown
450 to an OD_{600} of 0.3 - 0.6, were induced with either ciprofloxacin, H_2O_2 or mitomycin and
451 incubated overnight under dark conditions. The cultures were centrifuged (3,900 x g for 10
452 min) and filtered using 0.22 µm pore filters Minisart® syringe filters (Sartorius, Göttingen,
453 Germany). To eliminate the bias of cell lysis by colicins (77), tryptic digestion of the phage
454 filtrates were performed using 0.1 mg/mL trypsin-EDTA (Gibco™, Fischer Scientific,
455 Loughborough, England) for 1 h. at 37 °C with shaking (200 rpm). A volume of 100 µL of the
456 phage filtrate was mixed with 900 µL of a culture of the *E. coli* strain DH5α (OD_{600} 0.3 - 0.6)
457 and the mixture was added to 3 mL of liquid soft agar (0.7% agar, 55 °C) supplemented with
458 10 mM $CaCl_2$ and overlaid on LB agar plates. The plates were incubated at 37 °C overnight
459 and the phage titers were determined by visual plaque recognition and counting the following
460 day.

461 **Vitamin K's effect on the infectivity of BP933W**

462 A phage stock was made by inoculating 150 mL of LB broth in an Erlenmeyer flask with 1.5
463 mL of overnight culture of EDL933. After growth to OD_{600} 0.3 - 0.6, the cultures were induced
464 with 0.5 µg/mL mitomycin C, covered with aluminum foil to deprive the cultures of light, and
465 incubated at 37 °C with shaking at 200 rpm for 24 hrs. The cultures were then centrifuged (10
466 min, 4,000 x g, 4 °C) and sterile filtered with 0.22 µm Minisart® syringe filters. The different
467 vitamin K variants and solvents were added to 20 mL of the phage filtrates. DMSO was added
468 to a final concentration of 0.05%, the same concentration as in the cultures with vitamin added.
469 The vitamin K concentrations were 724 µM for phylloquinone and menaquinone, 36 µM for
470 MSB, and 72 µM for menadione. The samples were incubated for 2 hrs at 37 °C, in the dark

under shaking at 200 rpm. The vitamin K treated phage stocks were treated with trypsin as described above and tested with a plaque assay.

qPCR

Quantitative real-time PCR (qRT-PCR) was used to measure the expression level of *stx2* and *recA*. EDL933 was cultured in the presence or absence of different forms of vitamin K and induced as described above. Two hrs after induction, the cultures were mixed with ice cold methanol and kept at -80 ºC before isolation of RNA. Total RNA was extracted using the Purelink RNA mini kit (Life technologies, Carlsbad, USA) and the DNA was removed using the Turbo DNA-free kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity (A_{260}) and purity ($A_{260/280}$) of the RNA were measured in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and an Agilent 2100 bioanalyzer was used to assess the quality of the RNA. Only mRNA samples with a purity of 1.90 – 2.10 $A_{260/280}$ and with integrity over RIN 9 were used for cDNA synthesis. Next, cDNA was synthesized from 500 ng of RNA using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. Five microliters of a 1:100 dilution of the cDNA preparations was used as templates for qPCR amplification in a total volume of 25 µL containing 12.5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Carlsbad, USA) and primers at a concentration of 400 nM. The primers used for qPCR are listed in the supplemental material (Table 1). The qPCR amplification was performed using a StepOne system (Applied Biosystems, Carlsbad, USA). The thermal cycling conditions were 2 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 15 sec at 95 °C and 30 sec at 60 °C. The fluorescence was recorded during each extension phase, and a melting curve analysis was carried out after each run to verify the amplification of specific transcripts. Each assay was performed in three biological replicates and three technical

495 replicates. Samples containing no cDNA template functioned as negative controls. The slope
496 of the standard curve and PCR efficiency for each primer pair were obtained by amplifying
497 serial dilutions of genomic DNA of EDL933 containing the target sequence. The mRNA level
498 for each gene was determined relative to the reference gene *gapA* (glyceraldehyde-3-phosphate
499 dehydrogenase) and the results were analyzed using the Pfaffl method (78).

500 **Microscopy**

501 The EDL933 strain was grown in the presence of absence of different forms of vitamin K and
502 induced as described above. The samples were incubated for 20 hrs post induction, and the
503 samples were immediately prepared for microscopy analysis. The microscopy was done with
504 an Olympus BX51 microscope, and the pictures were taken with an Olympus UC-90 color
505 camera (Olympus, Tokio, Japan) and treated with the cellSens software (Olympus, Tokio,
506 Japan). Slightly different shades were observed between quadrants in all images. The reason
507 for the different shades is due to a mismatch in the graphic board requirements between the
508 camera and the laboratory computer connected to the camera. The computer connected to the
509 camera has a too low capacity (windows VGA 19201080 x 32 Bit (96DPI)) compared to what
510 the camera requires (3840 x 2160 pixel @ 30 Hz). This should, however, not influence the
511 results presented.

512

513 **Survival assay**

514 EHEC was grown overnight in LB broth, and the next day 20 µL was inoculated into 20 mL
515 LB in Erlenmeyer flasks. Vitamin K variants were used in the following concentrations:
516 phylloquinone and menaquinone (72 µM), MSB (36 µM) and menadione (7 µM). The OD_{600}
517 was determined every hr and H_2O_2 (3 mM) or Ciprofloxacin (0.06 µg/mL i.e. 1 x the minimum

518 inhibitory concentration) and 0.5µg/mL MMC was added when the cultures had reached an
519 OD_{600} of 0.5 ± 0.05. The OD_{600} was measured every hr after induction for eight hrs, and samples
520 were taken for enumeration of CFU/mL every second hr.

521 **LC-MS/MS quantification of intracellular ciprofloxacin**

522 Cytoplasmic extracts were prepared from EDL933 grown in LB with or without vitamin K
523 analogs (phylloquinone and menaquinone 72 µM, MSB 36 µM and menadione 7 µM) at 37 °C.
524 The cultures were induced with 0.06 µg/mL of ciprofloxacin when they reached an OD_{600} of
525 0.5. After 20 min of growth, 6 mL of the cultures were harvested and pelleted by centrifugation
526 at 18,000 x g for 30 s and washed three times in PBS (pH 7.4). Prior to the last wash, a 10 µL
527 portion of the samples was harvested, diluted and plated on LB agar for enumeration. The
528 samples were pelleted and vacuum dried (Savant Spd 121P speed vac concentrator, Thermo
529 Scientific, Waltham, Massachusetts, USA) for 5 - 10 min at 35 °C. The pellets were solubilized
530 in a solution of 200 µL water and 10 µL chloroform, and centrifuged for 8 min at 18,000 x g.
531 The supernatants were transferred to 0.3 mL PP Short Thread Micro-vials (VWR) and capped.
532 Aliquots of 5 µL were analyzed for the concentration of ciprofloxacin with LC-MS/MS as
533 described previously (79). The instrumentation used was an Agilent 1200 SL HPLC system
534 equipped with an Agilent G6490 triple quadrupole mass spectrometer with an electrospray ion
535 source. An Agilent Zorbax Rx C18 column, 150 x 3.0 (ID) mm with 3.5 µm particles was used
536 for separation. Calibration standards were prepared in a filtered cell extract matrix of EDL933
537 without ciprofloxacin added and ciprofloxacin at concentrations of 0, 0.5, 1, 5, 7.5, 10 ng/mL.
538 The calibration curve was forced through zero and was linear with correlation coefficient above
539 0.99. The values from the MS analysis were normalized according to the number of CFU/mL
540 counted, and relative percentages of uptake of ciprofloxacin was calculated.

541

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Author Contributions

MA is the corresponding author and primary contact during manuscript submission, review and publication process. The work was done under her supervision as the principal investigator. She significantly contributed to the study design, drafting, revisions and interpretation of data. AK is the major player in the conception, design, conduct, revision, analysis and interpretation. TL, ILW, HR, TLL and YW contributed to the design and conduction of different sections of the work as well as to editing the manuscript. All authors have approved the final version of the manuscript before submission.

Conflict of Interest Statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figure 1: The chemical structure of phylloquinone (Vitamin K_1), menaquinone (Vitamin K_2) and menadione (Vitamin K_3) and menadione sodium bisulfite (Vitamin K3).

564

565 **Figure 2.** The effect of four different vitamin K variants on the growth of EDL933 as measured
566 by the increase in CFU/mL. **(A)** phylloquinone, **(B)** menaquinone, **(C)** menadione, **(D)** MSB.
567 The solvents used for solubilization of the different types of vitamin K were used as negative
568 controls. DMSO was used at a concentration of 0.05%. Results are given as means of three
569 independent experiments, with bars showing ± standard deviation (SD).

570

571 **Figure 3.** Stx2 production by EDL933 in the presence or absence of vitamin K analogs. Stx2
572 production under **(A)** H_2O_2-induced and **(B)** ciprofloxacin and **(C)** MMC -induced conditions,
573 in the presence or absence of menadione, MSB or vitamin K, was measured by LC-MS/MS.
574 The error bars represent the standard deviation (SD) of three independent experiments. An
575 asterisk indicates statistically significant difference ($p < 0.05$) in Stx2 levels compared to
576 negative control-cultures with the same inducing agent and same solvent as used for
577 solubilization of the vitamin K variants i.e., DMSO or water. * = $P < 0.05$, ** = $P < 0.01$, ***=
578 $P < 0.001$ (Student's *t*-test).

579

580 **Figure 4.** The effect of vitamin K analogs on production of BP933W. The level of BP933W
581 produced by EDL933 was investigated under **(A)** uninduced, **(B)** H_2O_2-induced, **(C)**
582 ciprofloxacin-induced and **(D)** MMC-induced conditions, in the presence or absence of
583 different types of vitamin K analogs. The concentrations of vitamin K analogs used were 36
584 µM for MSB, 72 µM for phylloquinone, 72 µM for menaquinone and 7 µM for menadione and
585 36 µM for MSB. The error bars represent the SD of three independent experiments. * = $P <$
586 0.05, ** = $P < 0.01$, ***= $P < 0.001$ (Student's *t*-test).

587

588 **Figure 5.** The effect of the four vitamin K analogs on the infectivity of BP933W on *E. coli*
589 DH5 α. The concentrations of vitamin K analogs used were 362 µM for MSB, 724 µM for
590 phylloquinone, 724 µM for menaquinone and 72 µM for menadione, and the phage filtrate was
591 treated with the analogs for 2 hrs. The error bars represent the SD of three independent
592 experiments. * = $P < 0.05$, ** = $P < 0.01$ (Student's *t*-test).

593

594 **Figure 6.** Relative fold change in transcript levels of *stx2* and *recA* in vitamin K treated EHEC
595 cultures compared to untreated cultures. The concentrations of vitamin K used were 72 µM for
596 phylloquinone and menaquinone, 36 µM for MSB and 7 µM for menadione. Data represent
597 mean of three individual experiments. The error bars represent the standard deviation (SD) of
598 three independent experiments.

599

600 Figure 7. Microscopy analysis of EHEC cultured with four vitamin K variants and their
601 respective controls induced with ciprofloxacin and mitomycin C. Slightly different shades were
602 observed between quadrants in all images. The reason for this is a mismatch in the graphic
603 board requirements between the camera and the laboratory computer. This artifact do not
604 influence aim of this figure which is to show differences in cell morphology.

605

606 **Figure 8.** The effect of vitamin K analogs on the growth and survival of strain EDL933 in the
607 presence of H_2O_2, ciprofloxacin and MMC. **(A)** H_2O_2 (measured by the increase in CFU/mL)
608 H_2O_2. **(B)** ciprofloxacin (measured by the increase in OD_{600}) or **(C)** MMC (measured by the

609 increase in OD_{600}). Phylloquinone and menaquinone were used at concentrations of 72 µM,
610 menadione at a concentration of 7 µM and MSB at a concentration of 36 µM. The results are
611 given as means of three independent experiments, with bars showing ± standard deviation (SD).

612

613 **Figure 9.** Influence of vitamin K on the intracellular concentration of ciprofloxacin. The
614 concentrations of vitamin K used were 72 µM for phylloquinone and menaquinone, 36 µM for
615 MSB and 7 µM for menadione. The error bars represent the standard deviation (SD) of three
616 independent experiments. * = P < 0.05, ** = P < 0.01 (Student's *t*-test).

617

618 **Table 1.** Primers used in this study

Primer sequences

Gene	Forward (5' to 3')	Reverse (5' to 3')	Slope[a]	% Eff[b]
stx2	GAACGTTCCGGAATGCAAA	CCATTAACGCCAGATATGATGA	-3.40	98.00
recA	TTGACCTGGGCGTAAAAGAG	CGGTTTCCGGGTTATCTTTC	-3.10	90.00
gapA	AGGTCTGATGACCACCGTTC	AACGGTCAGGTCAACTACGG	-3.30	99.70

619 [a]The slope was calculated from the regression line of the standard curve
620 [b]The efficiency was calculated using the slope of the regression line of the standard curve

621

622

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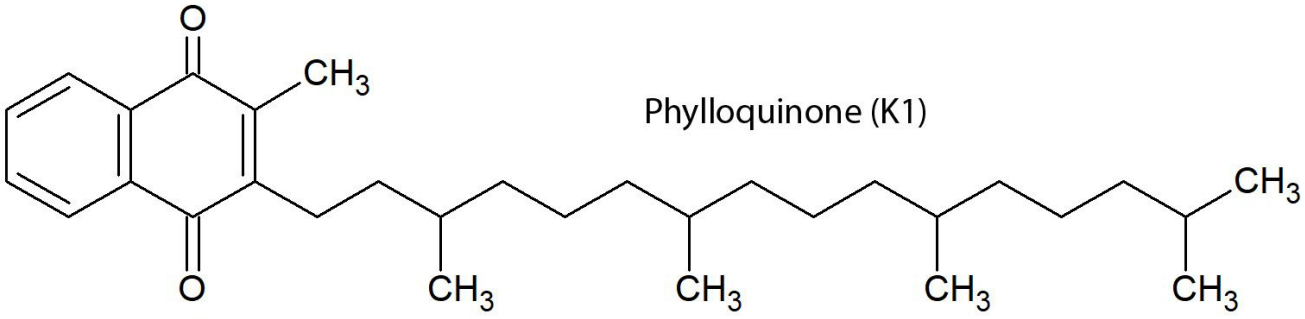
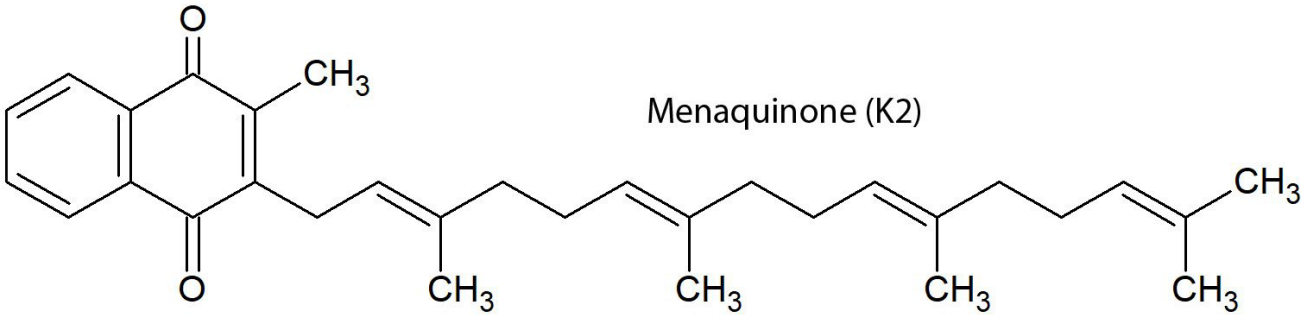
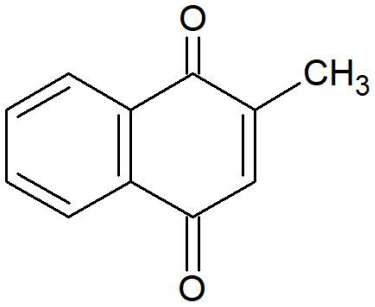


Phylloquinone (K1)



Menaquinone (K2)

Menadione (K3)



Menadione sodium bisulfite (K3, MSB)