ASSESSING CYTOKININ INHIBITION OF FROG VIRUS 3 REPLICATION AND CHANGES TO NUCELAR MORPHOLOGY

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ABSTRACT

Assessing Cytokinin Inhibition of frog virus 3 Replication and Changes to Nuclear Morphology

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Cytokinins (CKs) are adenine derivative molecules that are present in all kingdoms of life. CKs are known to have a role in cell growth and development in plants. However, the role of CKs in vertebrate systems is not well understood. Frog virus 3 (FV3) is a type species of the *Iridioviridae* family, genus Ranavirus. FV3 is a major contributor to the amphibian population decline in North America. In this study, we demonstrate that concurrent and pretreatment of 20 μM of either N^6-isopentyldenine (iP), N^6-ispoentyladenosine (iPR), N^6-furfurladenine/kinetin, and N^6-furfurladenosine/kinetin riboside (KR) decreased FV3 replication. To understand the mechanism of inhibition, we assessed morphological changes in host cell nuclei to assess the effect of CKs on infected nuclei. Our results show that infection with FV3 and 20μM treatment of iP or iPR reduced nuclei size. These results are the first to reveal insight into the potential mechanism in which FV3 replication is inhibited by iP and iPR.

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"An intelligent heart acquires knowledge, and the ear of the wise seeks knowledge."

Proverbs 18:15

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LIST OF ABBREVIATIONS

AHK	histidine kinase
AHP	histidine-containing phosphotransferase
ARRA	type-A RRs
ARRB	type-B RRs
BA	N^6-benzyladenine
BAR	N^6-benzyladenosine
BHSD	beta-hydroxysteroid dehydrogenase
CARD	caspase activation and recruitment domain
CHASE	cyclase/histidine kinase associated sensory extracellular
CK	cytokinin
CKX	cytokinin oxidase
CRE1	cytokinin response 1
CRF	cytokinin response factor
CTL	catalase
***c*Z**	*cis*-zeatin
DE	delayed-early
DHZ	dihydrozeatin
DMAPP	dimethylallyl diphosphate
eIF-2alpha	eukaryotic initiation factor
EPC	*Epithelioma cyprini papulosum*
FV3	frog virus 3
GP	glutathione peroxidase
GSH-PX	glutathione peroxidase

HO-1	heme oxygenase 1
HPt	histidine phosphotransfer protein
IE	immediate early
iP	N^6-isopentenyladenine
iPMP	isopentenyladenosine-5'-monophosphate
iPR	N6-isopentenyladenosine
iPRP	N^6-isopentynylphosphate
IPT	isopentenyltransferease
KIN	N6-furfuryladenine/Kinetin
KR	N6-furfuyladenosine/Kinetin Riboside
LOG	LONELY GUY
MCP	major capsid protein
MEP	methylerythritol phosphate
MOI	multiplicity of infection
***m*T**	*meta*-topolin
MVA	mevalonate
NCLDV	nucleocytoplasmic large DNA viruses
***o*T**	*ortho*-topolin
PIN1	*PIN-FORMED 1*
***p*T**	*para*-topolin
ROS	reactive oxidative species
SOD	superoxide dismutase
T-SOD	system super oxide dismutase
tRNA-IPT	tRNA-specific adenylate *IPT*
tZ	*trans*-zeatin

***t*ZR**	*trans*-zeatin riboside
VTAP	virion-associated transcriptional trans-activators
WOL1	wooden leg 1

1

1. GENERAL INTRODUCTION

1.1 Cytokinins

The discovery of cytokinins (CKs) in the 1950s in plant systems revealed their roles in plant development and growth[1,2]. Originally, cytokinins were classified as phytohormones that at low concentrations were effective in regulating seed and root germination, nutritional signaling, cell growth and development, defense response, and leaf senescence[3–5]. CKs' role in plants have been well documented and studied, including their signaling pathways and biosynthesis[6,7]. Kinetin, one of the first CKs to be discovered, was extracted from autoclaved sperm of herring[8]. Kinetin was named for its ability to promote cell division and was isolated and through series of experiments was shown to promote the growth of tobacco stems[8,9]. Although natural occurring CKs were once thought to be exclusive to plants, several studies have documented the presence of naturally occurring CKs in all kingdoms of life[10,11]. In bacteria and fungi, CKs were shown to have similar biosynthesis pathways to that of plants[12]. Additionally, CKs have been detected in mammalian tissues and fish cell lines[13,14]. Since the existence of CKs outside of plants was discovered, their role in other kingdoms of life include functions relating to cell growth and development and a host defense[15–17].

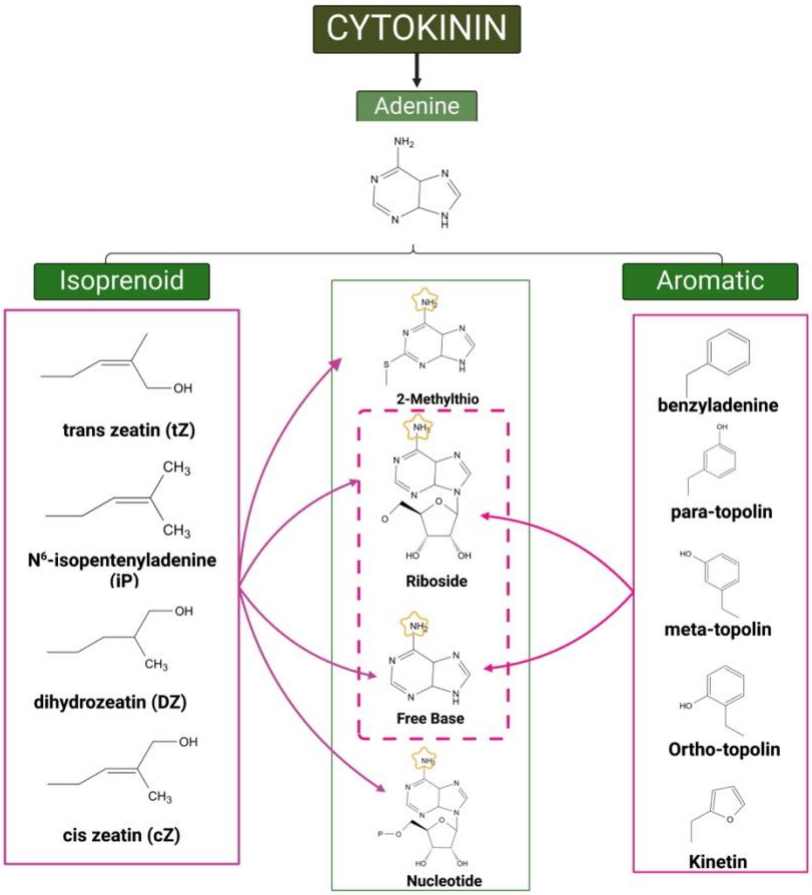
1.2 Structural Forms of Cytokinins

Cytokinins can be naturally occurring or synthetic. Both types of CKs are biologically similar despite being structurally different[18]. Naturally occurring CKs are N^6-substituted adenine derivatives that can be characterized as either an isoprenoid or aromatic CK[3,19]. The presence of an isoprenoid or aromatic side chain is the determining factor to differentiate between the two types of CKs. Structurally, CKs can exist as a free base, a riboside, nucleotide, or as 2-methylthio (2MeS)-forms based on the attachment of the defining group [2,11]. In plants, the free base form of CKs is the most active. Ribosides are considered the

transport signaling CKs and nucleotides are the inactive yet the most stable precursor form [3,20]. In a study done in detecting CKs in mammalian tissues, ribosides, nucleotides, and 2MeS-type CKs were detected, yet no free bases were detected[14]. The authors suggest that the precursor forms are rapidly metabolized into the active free base form. These structural derivatives of CKs are precursors for the biosynthesis and regulation of CK activity. Isoprenoid CKs are more abundant and active in plants systems than aromatic CKs and this same pattern can be found in all kingdoms of life[21]. Aromatic CKs are only found in a few life forms[22].The first isoprenoid CK to be isolated was zeatin found in immature maize endosperm[23,24]. Isoprenoid CKs can be characterized further based on a side chain attached at the N^6 position. These side chains include N^6-isopentenyladenine (iP), *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), and dihydrozeatin (DZ)[5,25] (**Figure 1**).

Compared to isoprenoid CKs, there are fewer studies focused on aromatic CKs. What is known about aromatic CKs began with the first isolation and identification of kinetin from the sperm of herring[8]. At the time of its discovery, kinetin was thought to be a synthetic product of DNA rearrangement, until it was later demonstrated that it can be synthesized *in vivo* as a product of DNA damage[26]. After its discovery, scientists began to synthesize other aromatic CKs like N^6-benzyladenine (BA)[27,28]. BA has been studied in plant research and its findings has contributed to a vast amount of knowledge on the function of CKs in plants[29]. This sparked a thorough search for the understanding of CKs, more specifically, aromatic CKs and their roles in plants[30,31]. It was first thought that aromatic CKs were not natural to plant systems until Strand et al in 1996 performed an HPLC screening for aromatic CKs in the tissues of poplar leaves and *Solanum* teratoma shoot culture[32]. From the screening, endogenous aromatic CKs like *meta*-topolin and *ortho*-topolin were found. The name topolin was derived from the Czech name for poplar.

Similar to isoprenoid CKs, aromatic CKs consist of an adenine base but its differences lie in the presence of an aromatic ring. They can be further characterized based on a side chain (an aromatic ring) attached at the N^6 position. These side chains include kinetin, BA and its hydroxylated conjugates- *meta*-topolin (*m*T), *para*-topolin (*p*T), and *ortho*-topolin (*o*T) (**Figure 1**). These CKs can be further characterized based on conjugates attached to the compounds. Some of these conjugates include ribosides, ribonucleotides, *N*-glucosides, *O*-glucosides, *O*-xylosides, lupinic acid or discadenine. Although aromatic CKs can be found in plant systems, its occurrence is rare compared to isoprenoid CKs.



58

59 **Figure 1: Cytokinins can be characterized as either an isoprenoid or an aromatic.**

60 Cytokinins are adenine-based derivatives that can be divided into two classes based on a group

61 attached at the N^6-position[21]. Isoprenoid CKs have an isoprenoid side chain attached where

62 aromatic CKs have an aromatic ring attached. Isoprenoid CKs can exist as a free base,

63 nucleotide, riboside, and 2-Methylthio-form[11]. Most research on aromatic CKs include the free

64 base and riboside form of these CKs[22]. The addition of the side chain gives it its final structure

65 and function[33]. Isoprenoid side chains include *trans*-zeatin (*t*Z), N^6-isopentyladenine (iP), *cis*-

66 zeatin (*c*Z), and dihydrozeatin (DZ). Aromatic CKs include benzyladenine (BA), *para*-topolin,

67 *meta*-topolin, *ortho*-topolin, and kinetin[22,34].

11.3 Biosynthesis of Cytokinins

1.3.1 Isoprenoid Cytokinins

The synthesis of CKs was first examined in *Dictyostelium discoideum*[35]. Since then, there are now two accepted pathways for the biosynthesis of isoprenoid CKs. There is the methylerythritol phosphate (MEP) or *de novo* pathway, and the mevalonate (MVA) or tRNA-degradation pathway[34,36]. Both the *de novo* and the tRNA degradation pathways synthesize CKs in plants and produces CKs from the isopentenylation of free adenine nucleotides[19,37]. The tRNA degradation pathway synthesizes CKs in all eukaryotes and some bacteria and produces these CKs from the degradation of tRNA[3,36]. The *de novo* pathway synthesizes iP, DH, and *t*Z type CKs; *c*Z, 2-MeSi and iP type CKs are synthesized in the MVA pathway[11]. During the early stages of CK biosynthesis research, experts thought that the degradation of tRNA was responsible for the formation of CKs in plants. The slow turnover rate of tRNA in comparison to the large amount of CK produced in the plant, put the theory of CKs being produced in plants via tRNA degradation into question[24]. However, this pathway produces CKs although at a much lower concentration compared to the tRNA degradation pathway. In the *de novo* pathway, the synthesis of CKs begins with the *N*-prenylation of adenine 5'-phosphates (AMP, ADP, or ATP) with dimethylallyl diphosphate (DMAPP) (**Figure 2**)[38]. IPTs in higher plants are known to prefer binding to AMP and ATP over ADP[3,39]. This reaction synthesizes an active CK, isopentenyladenosine-5'-monophosphate (iPMP)[35]. The side chain formed during this reaction can be further hydroxylated by cytochrome P450 monooxygenase[40]. From the production of iP-type CKs other types of CKs like *t*Z can be synthesized. There are two ways in which *t*Z can be formed, the iPMP-dependent way and the iPMP-independent way[41]. In the iPMP-dependent route, iP nucleotides are converted into *t*Z nucleotides by cytochrome P450 monooxygenase CYP735A1 and CYP735A2 (**Figure 2**)[19,42]. This pathway is the widely accepted pathway. In the iPMP-independent pathway, *t*Z

93 riboside 5'phosphates are produced directly by *IPT*. This pathway, however, is still not well
94 understood. In plants, free base forms of CKs are most active although their riboside forms
95 are frequently most abundant[43]. The LONELY GUY (LOG) enzymes are responsible for the
96 conversion from the riboside to the free base form of the CKs[44]. CK degradation is mediated
97 by cytokinin oxidases which is encoded by the cytokinin oxidase (CKX) gene family[45].
98 The tRNA degradation pathway, which occurs in the cytosol of eukaryotes and
99 bacteria, first begins with the transfer of an isoprenoid group from DMAPP to an adenine
100 molecule at position 37 of the tRNA molecule[14,46]. This step is catalyzed by the tRNA-
101 specific adenylate *IPT* (*tRNA-IPT*) to produce N^6-isopentynyladenosine phosphate (iPRP)[9,47].
102 The tRNA IPT proteins contains two domains: a large core and a small insertion domain. The
103 tRNA anticodon which has a structure of a stem loop binds the two domains together. When
104 the isoprenylation at position A37 occurs, it allows DMAPP to enter into the channel of the
105 core domain. At this point, iPRP can be converted to N^6-isopentynladenosine (iPR) and
106 further converted iP. Prenylated-tRNA has a *cis*-hydroxyl group and its degradation generates
107 cZ and 2-MeS-type CKs **(Figure 1)**[4].

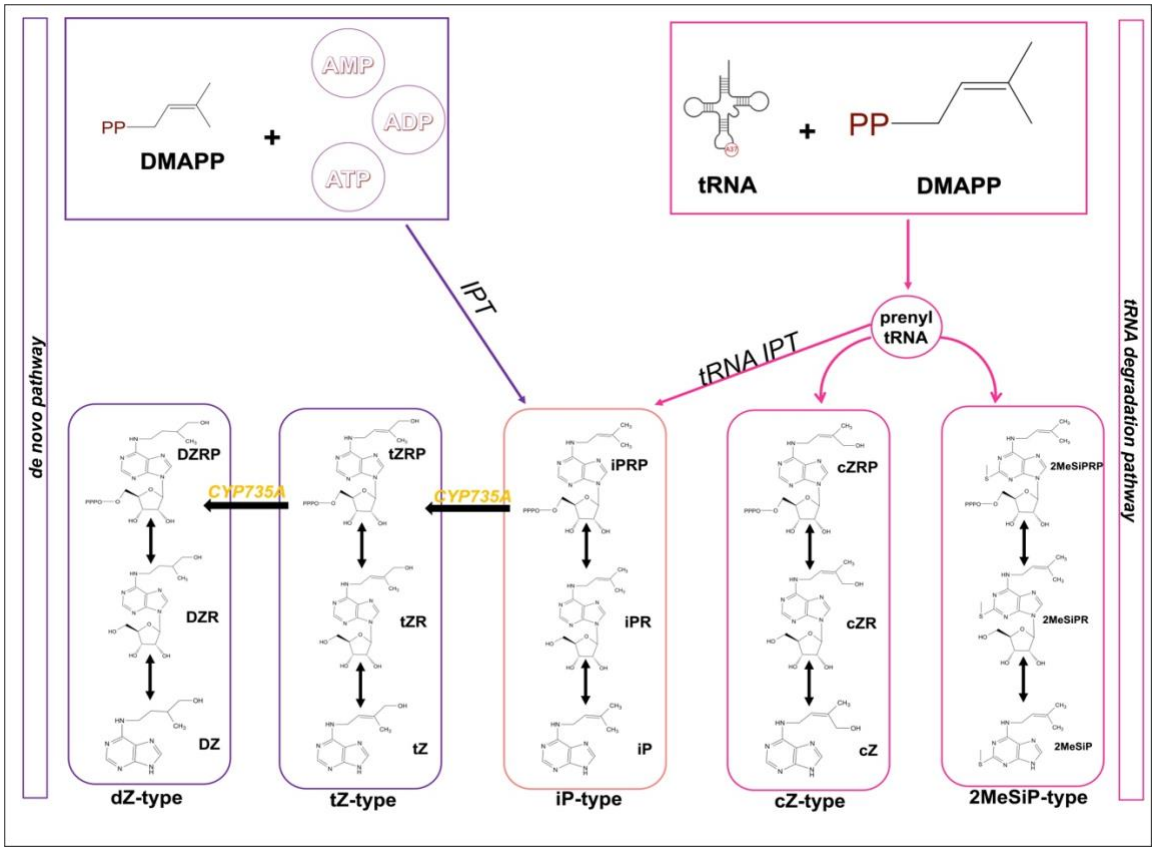


Figure 2: Isoprenoid cytokinins can be formed via the *de novo* pathway or the tRNA degradation pathway. Isoprenoid CKs can be synthesized via two pathways. The *de novo* pathway produces DZ-type, *t*Z-type, and iP-type CKs[19]. DMAPP binds to ADP, AMP, or ATP and produces iPMP. The reaction is regulated by the enzyme *IPT.* iPMP then goes on to synthesize *t*Z-type and DZ-type CKs via the enzyme, CYP735A[2,19]. The iPMP can be interconverted to the riboside form, iPR, and then further synthesized to produce the free base form, iP. In the tRNA degradation pathway, DMAPP binds to tRNA at the A37 position to create prenyl-tRNA[48]. This then is degraded further to produce iP-type, *c*Z-type, and 2MeSiP-type CKs. *Adapted from Aoki et. al. (2020)*[11].

1.3.2 Aromatic Cytokinins

The synthesis pathway of naturally occurring aromatic CKs is unknown. However, researchers have been able to synthetically synthesize aromatic CKs and determine the optimal conditions for maximum product. Kinetin was first synthesized from the reaction of furfuryl chloride and adenine condensation under alkaline conditions using sodium bicarbonate [1,8]. Shortly after this, other aromatic CKs were synthesized. BA was first synthesized using 6-methylthiopurine reacting with benzylamine at a temperature of 140ºC for 16 hours. Miller et al were able to achieve a 40-60% yield of product[8]. The production of aromatic CKs was time consuming under extreme conditions yet yielded an unsatisfactory amount of product[8]. Through trial and error, improvements were made to protocols which allowed for a higher yield of product under less extreme conditions[29]. For example, kinetin was initially prepared by heating 6-methylmercaptopurie and freshly redistilled furfurylamine at 115-120ºC for nine hours[33,49]. Currently, kinetin is produced by the nucleophilic substitution of 6-chloropurine by furfurylamine under alkaline conditions[29,34]. The reaction at first took 3 hours producing 80% of product[50]. Now, the reaction takes several minutes and can produce roughly 98% of product[29,34].

After the initial synthesis of BA, derivatives of BA were produced introducing functional groups in the meta-, ortho- and para positions on the benzyl ring. Testing of the BA derivatives showed that the position of the functional group significantly influences the biological properties of the derivates[31].

Although the synthesis pathway of aromatic CKs is still unknown, in mammalian systems, kinetin is produced during DNA damage. Hydroxyl radical oxidation at the carbon 5' of DNA produces the aldehyde, furfural. After the production of furfural, it then reacts with adenine, and after intramolecular rearrangement yields kinetin **(Figure 3)**[51,52]**.** Kinetin has earned the name "free radical sink" for being a part of the salvage pathway of hydroxyl

143 radicals[53]. Its riboside is synthesized via the enzyme, adenosine phosphoribosyl transferase

144 (APRT)[54].

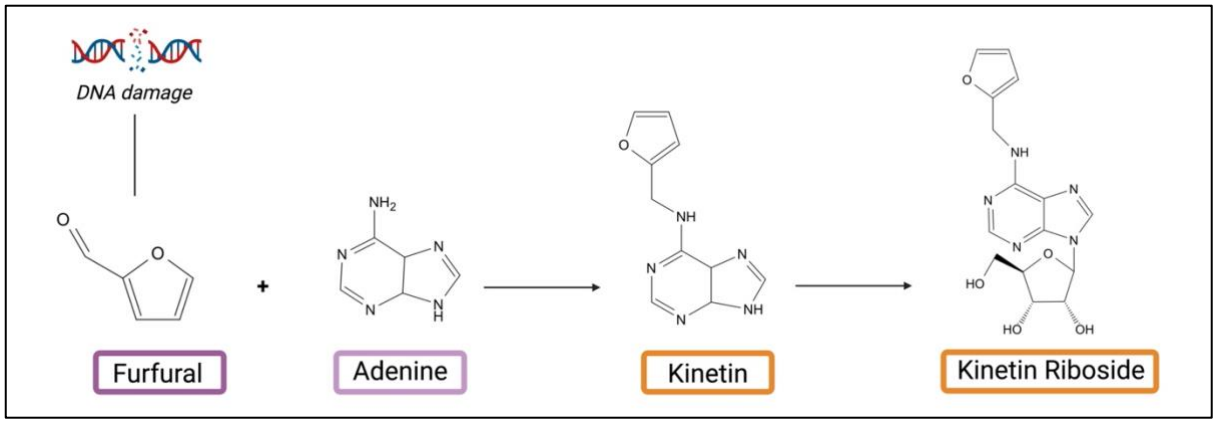


Figure 3: Kinetin and kinetin riboside are formed during DNA damage.: When DNA damage occurs, hydroxyl radical oxidation of the carbon 5- DNA produces the aldehyde, furfural[51,52]. These furfural groups bind with adenine to produce kinetin. Kinetin then is converted to kinetin riboside via the enzyme, APRT[55]. Kinetin is deemed as a "free radical sink" for its ability to salvage free radicals produced during DNA damage[56].

1.4 Cytokinins in Plants

Free bases are the most active form of CKs in plants[3,6]. However, there is a larger amount of inactive CKs in plants (ribosides and nucleotides) suggesting that the concentration of active CKs is strictly regulated to prevent excessive signaling from occurring[57]. The coordination of enzymes involved in the biosynthesis, modification, and degradation of CKs is important in this regard[19]. Much of what is known about the CK signaling pathway was studied in the model plant *Arabidopsis (Arabidopsis thaliana)*[2,27] with iP and *tZ* being the most prominent CKs in this species[58].

1.4.1 Signaling and Gene Regulation

In plants, CKs are known for their involvement in plant growth and development[59]. In early studies to identify factors involved in plant cell growth and development, kinetin and later on zeatin were found to be regulators of plant growth [23,49,60]. In addition to regulating plant growth and development, CKs regulate plant defense systems in response to stressors and they help build immunity[61,62]. In plants, a histidine-to-aspartate (His-to-Asp) phosphorelay system act for CK signaling. This is a two-component signaling system with histidine kinase (AHK) functioning as a sensor, a response regulator, and a histidine-containing phosphotransferase (AHP) domain[63]. The His-to-Asp signaling pathway was thought to be restricted to prokaryotic systems, but it has been seen in eukaryotic species[63,64]. In bacteria, the signaling pathway is said to be more complex and is a multi-step His-to-Asp pathway[65,66]. In lower life eukaryotes like fungi, the phosphoryl group transfer from HK to the response regulator occurs in a multi-step phosphorelay that also involves the intermediate histidine phosphotransfer protein[64,67].

There are three CK receptors vital to this pathway: *Arabidopsis* histidine kinase 2, 3, and 4 (AHK2, AHK3, and AHK4)[68]. AHK4 is the only one of the three that contains intrinsic phosphatase activity at a constant level[69]. AHK4 is often referred to as wooden leg 1

(WOL1) and cytokinin response 1 (CRE1) as it was the first CK receptor to be indentified[45,70]. Additionally, its binding ability to CKs is most active out of the three[71]. AHK proteins contain a cyclase/histidine kinase associated sensory extracellular (CHASE) domain to which the CKs bind[19,59]. They primarily localize to the ER membrane **(Figure 4)**. On binding to the CHASE domain, cytosolic histidine-kinase is activated and autophosphorylated[70]. Then, the phosphate group from the AHK is transferred to a conserved Asp in the receiver domain. The Asp then transfers the phosphoryl group it received to an AHP protein[63,72].

The downstream phosphorelay events are regulated by six AHPs and 23 response regulators (ARRs)[70]. Out of the six AHPS, five are involved in the phosphorelay signaling and contain a conserved amino acid that allows these proteins to act as a histidine phosphotransfer protein (HPt) **(Figure 4)**[68]. They are considered histidine phosphotransfer proteins based on their ability to transfer phosphate groups to the response regulators. The AHPs main role involves being an intermediate for the phosphorelay signaling between the CK receptors on the response regulators[73]. When CKs bind to the AHPs, they travel from the cytosol and begin to accumulate in the nucleus[42]. They are phosphorylated by the CK receptors, which then phosphorylates the response regulators[74]. The two types of response regulators that are involved in the CK signaling pathways are: type-A RRs (ARRA) and type-B RRs (ARRB)[75]. These ARRs are activated once the Asp residue is phosphorylated by the AHPs.

Type-A ARRs are made up of ten family members that contain a single receiver domain with a conserved Asp residue[76]. The ARRAs act opposite to ARRBs in that they act as negative-feedback regulators of CK signaling **(Figure 4)**. Like ARRBs, they do contain a receiver domain, but the characteristic that separates the two types of ARRs lies in the fact that ARRAS do not have an output domain for transcriptional regulation[42]. They are also

stabilized by CKs in a phosphorylation-dependent manner. ARRAs are rapidly transcriptionally upregulated during CK signaling. They contain short C-terminal DOMAIN[77].

Type-B RRs contain 11 family members. ARRBs are positive regulators of CK signaling[68]. Type-B RRs are activated when the Asp residue in their receive domain are phosphorylated. This step is important for the transcriptional response to CKs[78]. Type-B RRs have a receiver domain and a C-terminal extension that includes a DNA-binding domain. The preferred DNA-binding motifs for type-B RRs are found upstream of many CK-regulated genes[73]. Studies show that type-B RRs in plants will bind to their target sites in a CK-dependent manner. It is assumed that the binding happens in response to the phosphorylation of the receiver domain. As mentioned before, type-B RRs control the expression of transcription factors in a CK-dependent manner **(Figure 4)**. These transcription factors are involved in chloroplast development and shoot apical meristem (SAM) function[58,71].

CK signaling is also encoded by CRFs[79]. These are responsible for encoding transcription factors that are vital to the signaling pathway **(Figure 4)**[79,80]. They are similar to the type-A RRs as they also are upregulated in response to CKs, as they are dependent on the activation of type-B RRs. CRFs localize to the nucleus from the cytosol in response to CK signaling as well[81]. Studies show that while mutated CRF genes can reduce the induction of CK genes that influence the induction of type-B RRs, the mutations do not reduce responses to CKs[80].

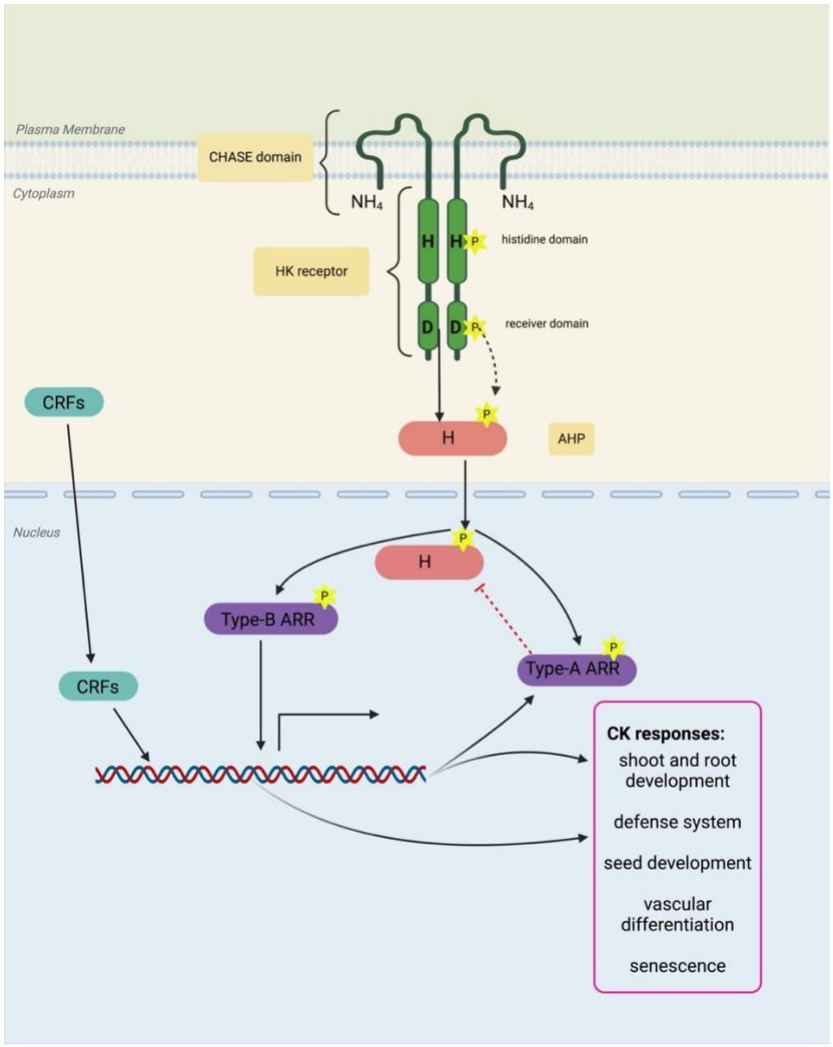


Figure 4: Cytokinin signalling in plants leads to development of plant systems. CK signaling begins with the activation of the CHASE domain where the CKs bind[12]. The binding causes autophosphorylation of the HK receptor. The phosphate group is then transferred to the AHP which acts as a phosphate group transport[58]. Once in the nucleus, the phosphate group is transferred to the receptor responses[42]. Type-B ARR phosphorylation leads to the up regulation of CK genes that results in the different CK responses. Type-A ARR is transcribed by Type-B ARR[78]. It also causes negative feedback of CK responses. Another group that is involved in the transcription of CK genes are the CK response factors (CRFs) which encode transcription factors involved in plant growth and development[80].

1.4.2 CK Roles

Cytokinins are involved in almost all aspects of plant growth and development[82,83]. As an extension of their role in development, CKs regulate stress in plants[9]. CKs respond to various abiotic stressors such as extreme temperatures, drought, and levels of salt[84]. These disruptions alter cell processes which leads to secondary stress in the cell such as ROS production, osmotic stress, and protein denaturation[85]. The stress response includes the sensing of the CKs followed by transmission of signals from regulators to effectors causing a protective function. Increase of ROS production is indicative of stress in not only plant systems, but other systems, including animals[86,87]. Specific ROS that are produced under a stressful state in plant cells include superoxide, hydroxyl radicals, and hydrogen peroxide. CK signaling activated from abiotic stress can either lead to a higher resistance to the abiotic stress leading to the survival of the plant, or it is ineffective in combating or resisting the stress (excessive ROS accumulation can cause this) which triggers environmental-triggered cell death processes[84,88].

Cytokinins were first discovered based on their role in cell division[49]. In plants, this has been extensively studied. The cell cycle in plants is similar to other eukaryotes[89]. Essentially, the end results during mitotic cell division includes replicated DNA that has been integrated into nuclei of new daughter cells. In plants and other eukaryotes, the phases are also similar: a synthesis phase, mitosis phase, G1 phase and G2 phase. The phases are regulated by cyclin-dependent kinases[90]. The cell cycle in plants involve three cyclin D gene homologues- CycD1, CycD2, and CycD3[90,91]. These cyclins are similar to mammalian D-type cyclins which are active during middle-to-late G1 phase. CKs are shown to have both negative and positive roles during cell division that overall work for the success of this process[89]. For example, while endogenous CKs are involved in ensuring the success of cell division to be completed, exogenous application of CKs can halt cell division[90]. Studies have

attributed this to the oscillation levels of CKs being a vital factor during various parts of the cell cycle. CKs activate mitosis and cell division at low concentrations and is inhibitory at higher concentrations[90]. Recently, the Yang group looked at the molecular mechanism in which CKs activate cell division in Arabidopsis[92]. In this study, they were able to identify the different genes involved in this process. CKs stimulate cell proliferation through promotion of the gene Myb-domain protein 3R4 (MYB3R4) to be transported from the cytoplasm into the nucleus where it activates mitotic gene expression.

11.5 Cytokinins in Bacteria and Fungi

1.5.1 Detection

Outside of plants, CKs have been detected in many taxa including bacteria and fungi[12]. CK studies have focused on the phytopathogenic and plant symbiotic bacteria. In bacteria, CKs are produced via the *de novo* pathway and the tRNA degradation pathway. However, like plants, the slow turnover of tRNA in bacteria contributes to very little CK production, and so the *de novo* pathway is the main contributor to CK production[93]. One study developed an immunological method that rapidly identified CKs in bacteria[94]. From this method, the authors identified trans-zeatin and trans-zeatin riboside in *Agrobacterium*[94]. Other bacterial species examined included *Pseudomonas syringae* which contained trans-zeatin, dihydrozeatin, and 1-mehtyl-trans-zeatin riboside; and *Corynbacterium fascians* which produced cis-zeatin and isopentenyladenine and isopentenyladenosine[94].

Many fungi species produce CKs via the *de novo* or the tRNA degradation pathway[95]. In one study over 30 species of fungi were screened for CKs; the authors cultured the fungi in CK-deficient medium[96]. The results showed that the species *R. roseolus* produced zeatin[96]. CKs in fungi species are involved in many different processes including spore germination, virulence, and nutrient uptake[95]. Many studies observed the different area of fungi development where CKs play a role. For example, one study demonstrated CKs' abilities to

tolerate oxidative stress in the fungi species, *Magnaporthe oryzar*[97]. Regarding virulence activity of fungi, one study investigated this in the fungi *C. purpurea*[98]. The study conducted by Hinsh et. al. deleted the CK synthesis genes preventing the production of cZ-type CKs and expressing a CXX gene.[46] This resulted in the inhibition of virulence activity. Another virulence study done by Morrison et al in 2015 found that in *U. myadis*-infected tissues, CKs accumulated in the infected tissues in the plant host[99]. When they deleted the fungal tRNA-IPT gene, it resulted in the decrease of CK production and virulence.

1.5.2 Signaling and Function

In cyanobacteria, CK signaling, and synthesis differs. These bacteria produce CKs during the light period[100]. In fact, CKs are ineffective without the presence of light in these bacteria. During the light phase, several genes responsible for encoding HK receptors and RRs are transcribed. Their domain arrangement is similar to that seen in *X. campestri*[12]*s.* A specific gene, *all2875,* encodes a CHASE domain-containing HK, and binds to iP and trans zeatin, although it has a lower binding affinity for iP[12]. Overall cyanobacteria regulate CK biosynthesis in the presence of light.

To date, there is no evidence of the CHASE domain being present in CK-producing fungi and fungal phytopathogens[12]. Studies have shown that fungi can sense CKs, and researchers suggest that despite the lack of CHASE domain CKs they are able to sense through their HKs[101,102]. Fungal HKs are called hybrid histidine kinase (HHKs) because the HK/HATPase region is fused to the C-terminal receiver domain. In fungi, the histidine phosphor-transfer shuttle proteins are referred to as HPT, where in plants they are referred to as AHP[95]. CK signaling in fungi plays a role in fungal virulence, development, host nutrient allocation, and host immunity regulation[95].

1.6 Cytokinins in Vertebrate Systems

1.6.1 Detection

Initially, it was thought that CKs were exclusively found in plants. CKs have begun to be detected in other kingdoms, including vertebrates. In 2018, Seegobin et. al. were able to detect numerous CKs in canine tissue from different sources[14]. Some of the notable CKs detected include, iPR, iPRP, cis-zeatin-9-riboside, and 2-methylthiso-N^6-isopentynyladenine[14]. Another study showed that CKs are present in fish cells particularly, iPR, iPNT, cZNT, and 2MeSZ. In 2010, Beres et al developed a new reversed-phase high performative liquid chromatography with mass spectrometry detection to quantify CKs found in human K-562 leukemia cells treated with iPR[103]. This study identified CK nucleotides[103]. Nucleotides are said to function as triggers for apoptosis in cancer cells when they are treated with iPR[104]. More recently, CKs levels were assessed in skeletal muscles under normal conditions and under stress to determine if CKs play a role in muscle secretome and to determine if they are responsive to stress[15]. Tobin et al found that CKs, specifically iP-type CKs, were detected intracellularly and extracellularly[15].

The presence of CKs in vertebrate systems has sparked interest in understanding more about the metabolism of CKs[62]. There is a good understanding of the biosynthesis of CKs in vertebrate systems and many studies have been done on their biological function in these systems[13,14,20].

1.6.2 Signaling and Function

In vertebrate systems, the full mechanism behind CK signaling is not well understood. A receptor, A_{2A} has been identified, however, it has only been shown to bind to zeatin and kinetin in human models[105,106]. The function of CKs in vertebrate systems has focused on CKs' abilities as an antioxidant, anti-viral, anti-cancer and anti-ageing agent[62]. The molecular mechanism behind these functions is not well understood yet. Studies have investigated how

CKs are modulators of the immune system to help fight diseases and illnesses[106]. The A_{2A} receptor is a target of interest in neurodegenerative diseases in addition to various other types of diseases[105]. Because of its presence in many immune systems, they have found that its expression is upregulated during inflammatory responses[62].

Lee et. al. investigated different CKs as a neuroprotectant against Huntington Disease[105]. To test this, the authors focused on the A_{2A} receptor. In their study, the authors found that *trans*-zeatin riboside (*t*ZR) and KR were able to inhibit serum-starved apoptosis in Huntington disease-mutant transfected PC12 cells[105]. This was due to the activation of the A_{2A} receptor acting on an A_{2A} antagonist and a protein kinase A (PKA) inhibitor[105]. When *t*ZR and KR acted on this receptor, the downstream effect led to the decrease of Huntington disease mutant aggregations that are known to inhibit proteasome activity[107]. When PKA is activated it in turn increased proteasome activity. In addition to testing *t*ZR and KR, they also tested their free base forms and found that they were ineffective in activating the A_{2A} receptor[105].

In the study by Lappas et. al., *t*ZR is shown to activate the A_{2A} receptor leading to the increase of cAMP production, an anti-inflammatory enzyme[106]. Simultaneously, this *t*ZR mediated activation of A_{2A} has led to the inhibition of pro-inflammatory cytokines, TH1 and TH2 by CD4+ and CD8+[106]. It also limits the expression of CD25, CD40, and CD69[106].

CKs have shown great potential at being antioxidant agents[62]. In 1996, Okada & Okajima demonstrated that kinetin mediated its antioxidant effect by controlling the level of ROS in the cell[108] through the furan ring in kinetin which acts as a scavenger of ROS[62,108].

(i) Antioxidant activity

In addition to anti-inflammatory responses, CKs act as an antioxidant in vertebrate systems. Othman et. al. assessed the antioxidant activity of kinetin in the presence of stressed cells[87]. At high concentrations (50–10000 nM), they found that kinetin exhibited cytotoxic

effects whereas at a low concentration of 1-100 nM, kinetin became a protective agent against oxidative stress in various mammalian cell lines[87]. A review study supported this activity for kinetin, but also looked into the mechanism behind the antioxidant activity. Kadlecova et. al. explained the mechanism could be the result of kinetin forming a super complex with copper that have superoxide dismuse-like activity[109]. Another proposed mechanism is kinetin protecting cells by inducing antioxidant enzymes like superoxide dismutase (SOD), catalase (CTL), and glutathione peroxidase (GP)[87,109].

Another study done by Jablonska-Trypuc et. al. investigated CKs effect on basic oxidative stress parameters like antioxidant enzyme activity, reduced glutathione, and thiol group content as well as lipid peroxidation[110]. In addition to observing oxidative stress parameters, the authors investigated BA and kinetin's potential to stimulate oxidative stress in fibroblasts or to prevent free radical synthesis. Their results showed that at concentrations of 10^{-5} and 10^{-6} M, kinetin significantly increased the proliferation of fibroblasts while BA decreased the total cell number[110]. This was expected as BA is known to inhibit the proliferation of cells by enhancing the expression of the CYCD3 gene which is responsible for encoding cyclins[28,111].

Oxidative stress is an issue associated with the post-thaw quality of samples in cryopreservation[112]. Kinetin was assessed for its antioxidant activity to preserve the structure and functional integrity of dog sperm during cryopreservation[113]. By supplementing the samples with 50 μM kinetin, the sperm count increased while maintaining plasma membranes, normal acrosomes, mitochondria, and chromatin[114]. Additionally, there was an increase in the expression levels of BCL-2, an anti-apoptotic protein, and protamine-related genes. There was a decrease in the expression of pro-apoptotic proteins and mitochondrial ROS genes[114]. A similar study done in 2016 by Hashem et al found that kinetin was able to preserve the quality of ram sperm during storage at refrigerator temperature[115]. They

concluded that kinetin improved the spermatozoa motility and viability and the function of the spermatozoa plasma membrane and the antioxidative markers of the ram sperm[115].

In a study done by Liu et al., kinetin was able to protect cultured astrocytes and mouse brain against D-galactose-induced oxidative damage and enhanced cell viability through attenuation of the antioxidant system super oxide dismutase (T-SOD), glutathione peroxidase (GSH-PX) and malonyl dialdehyde concentration in the cell membrane[116].

Kinetin has shown the ability to enhance the antioxidant system of mouse cells that were induced by aluminum chloride and D-galactose causing cognitive impairment and oxidative damage[95]. The study done by Wei et al in 2000 showed kinetin was able to do this by enhancing the activities of SOD, GSH-PX, and heme oxygenase 1 (HO-1). Kinetin was also able to scavenge free radicals and showed its ability to suppress hydroxyl radical formation in a dose-dependent manner[117].

(ii) Anti-viral activity

While many studies have looked at the therapeutical potential of CKs extensively in other areas, very few studies have assessed its potential as an antiviral agent. The inhibition of Marburg and Lassa virus by BAR had been demonstrated using mammalian bioassays[17]. The authors report that iPR had an antiviral efficiency (EC) of 1.0 ± 0.2 μM and a selectivity index of 5.7[17]. CKs are shown to inhibit TBEV replication[118]. The authors assessed anti-TBEV activity through plaque formation assay in porcine embryo kidney cells. The infected cells were treated with various CKs at a final concentration of 50 μM. Of the CKs that have been tested in this study, kinetin and iP were amongst the selected CKs that showed a decrease of 50% plaque formation compared to the control[118]. Through structure-activity relationship between CKs and tick-borne encephalitis virus (TBEV), Orlov et. al. found that selected CKs inhibit the reproduction of the virus[118]. They hypothesize that this inhibition could be due to the interaction of the TBEV non-structural protein 5 methyltransferase or

RNA-dependent RNA polymerase domains or somehow through viral entry being inhibited[118].

Recently, kinetin has been shown to inhibit *in vitro* replication of SARS-COV-2 in human hepatic and pulmonary cell lines[119]. In this study, kinetin was converted into its triphosphate nucleotide that in turn inhibits viral RNA synthesis to induce error-prone virus replication[119]. Kinetin was proposed to go into rapid clinical development as an anti-viral oral drug. This is based on previous and extensive studies done on kinetin and its ability to be used a therapeutic agent[119].

Several iP-type CKs have been shown to inhibit viral activity in frog virus 3 (FV3)[120]. Concurrent treatment of iP and iPR specifically caused a significant decrease in plaque formation. The study was the first of its kind to provide evidence of the antiviral activity of CKs against a DNA virus while also highlighting that investigation of their structure related to antiviral activity[120].

1.7 Ranavirus

1.7.1 Taxonomy

The *Iridioviridae* family are large, linear double stranded DNA (dsDNA) viruses[121]. Members of this family are approximately 120-350 nm in diameter and encodes between 97 to 211 open reading frames[122]. Their genomes range from approximately 100,000 to just over 200,000 base pairs. The *Iridioviridae* family is subdivided into two families (*Alphairidovirinae* and *Betairidovirinae*) which are further divided into six genera[123,124]. The families are divided based on host preferences (vertebrates vs invertebrates)[125]. Members of the Alphairidiovirinae include *Lymphocystivirus*, *Megalocytivirus* and *Ranavirus*[126]. This subfamily affects primarily ectothermic vertebrates[124,127]. *Lymphocystivirus* and *Megalocytivirus* infect solely fish species, whereas *Ranavirus* infects fish, amphibian, and reptiles[126,127]. *Ranavirus* and *Lymphocystivirus* both contain approximately 20% methylated

residues[128]. Betairidovirinae comprises of the other three genera- *Iridovirus, Chloriridovirus* and *Decapodiridoviru.* this subfamily infects mainly invertebrates like crustaceans and insects[121,124].

Aside from host-specific tropism, the subfamilies are divided for additional reasons. Initially, the sub-families were divided based on GC content of the genome, serology, virion morphology, particle size, and clinical signs of disease[124]. Iridovirids contain genomes that are circularly permuted and terminally redundant[129]. The terminal regions make up almost 50% of the genome resulting in the total length of the genome being longer than the length of the unique region[130]. Unlike, the *Alphairidovirinae* genera family, *Betairidovirinae* do not contain any methylated residues[131]. This is the result of the absence of a virus-encoded DNA methyltransferase gene in *Aplhairidovirinae* and its presence in *Betairidovirinae*. In Ranavirus and Lymphocystivirus, the GC content is about 27-29% while the other three contains about 48-55%. The codon usage is influenced by the percentage of GC content in the genome. The *Iridioviridae* family are phylogenetically linked to other families of viruses that are similarly categorized as nucleocytoplasmic large DNA viruses (NCLDV)[131]. Some of these viruses include *Poxviridae, Asfaviridae, Ascoviridae, Phycodnaviridae* and *Mimiviridae*[132].

1.7.2 Morphology

The genome and virion size differs within the family; however, the morphology of the virion particle is similar[133]. The structure for this family is an icosahedral capsid and can exist as an enveloped or non-enveloped/naked virion[133,134]. The non-enveloped virions are comprised of three main layers: an outer capsid layer, an internal lipid membrane, and an inner core containing the viral genome and associated virus-encoded proteins[135]. The outer capsid layer is made up of multiple copies of a major capsid protein (MCP). The MCP makes up 40% of the total virion protein content. The difference between the virions lies in the

presence or absence of a viral envelope which contains surface proteins on the external capsid[133]. Both virions infect host cells, however, it is shown that enveloped virions exhibit higher infectivity[123]. The virion core contains a single linear double stranded DNA molecule roughly 140-300 kbp[123,133].

1.8 Frog Virus 3

Frog virus 3 (FV3) is a type species of the Ranavirus genus[136]. It is currently the best characterized Iridovirid[133]. FV3 was first isolated from the kidney of the leopard frog *Rana pipiens*[137]. The genome of FV3 is 105,903 kb[138]. Due to it being a significant factor in amphibian population declines, FV3 is the most well studied species in the Ranavirus genus[139]. Its replication strategy serves as the model for other virus species in the Ranavirus genus[125,133]. Viral transmission of FV3 can occur via feeding, parental injection, or environmental exposure[140]. FV3 can grow in cultured fish, amphibian and reptilian cells in temperatures ranging from 4- 32ºC[123]. Ranaviruses are part of the NCLDV family where the virus replicates within the cytoplasm of the host cell, although some of the members, including Ranavirus, have some of the replication stages occurring in the nucleus[123].

1.8.1 Replication

The entry of the virions into the host cell first begins with either the non-enveloped or the enveloped virion into the host cell's cytoplasm **(Figure 5)**[134,141]. The non-enveloped virion interacts with the plasma membrane and uncoats[123]. The viral DNA core enters into the cytoplasm. In contrast, the enveloped virion enters the cell via receptor-mediated endocytosis, outer membrane is shed, and the non-enveloped virion enters the cytoplasm **(Figure 5)**[123]. The virion then travels to the nuclear membrane and the viral DNA enters the nucleus.

FV3 gene expression is divided into three temporal stages: immediate early (IE), delayed-early (DE), and late transcription[142,143]. There are 33IE genes, 22 DE genes, and 36 late genes identified[144]. IE and DE transcription take place in the nucleus, which is unique

compared to other NCLDV viruses where all transcription happens in the cytoplasm [123,125]. IE and DE transcription are catalyzed by the host cell's RNA polymerase II[145]. The viral DNA polymerase is then synthesized which leads to the replication of unit-sized copies of the viral genome within the nucleus[146]. IE transcription requires the presence of one or more virion-associated transcriptional trans-activators (VTAP)[147].

The product of first-stage DNA replication is the production of genome-size to twice genome-size copies of the viral genome[123]. The newly synthesized DNA then transports from the nucleus into the cytoplasm where it serves as a template for second-stage DNA synthesis **(Figure 5)**[144]. Once the newly synthesized DNA enters the cytoplasm, 20- 25% of cytosine residue from the CpG motifs become methylated by the virus-encoded DNA methyltransferase, DMTase[128]. Some researchers hypothesize that the methylation of the viral genome plays a role in inhibiting the activation of pro-inflammatory cytokines[123]. This is one way in which the virus inhibits the host's defense system. After methylation of the viral DNA occurs, a second round of DNA synthesis begins[144]. The viral DNA is then converted into large concatemeric DNA at the designated "assembly sites." These concatemeric DNA are up to ten times larger than the genome-sized units[123]. Late transcription occurs and is catalyzed by RNA polymerase II[144]. At the assembly sites, virions are packaged together and appear as electron-lucent areas that contain viral DNA and proteins **(Figure 5)**. These sites will often be close to the host cell's mitochondria[133]. Researchers hypothesize that the proximity of the sites to the mitochondria is to provide the energy needed for virion assembly[123].

The budding event is how enveloped virions get their viral envelope while non-enveloped virions are released from the host cell through lysis. In cultured cells, FV3 replication takes roughly 12-24 hours[125]. Early DNA synthesis takes place within 4 hours post-infection. Late transcription and proteins can be seen 8 hours post infection[131]. Several

503 factors like multiplicity of infection (MOI), temperature, and metabolic state of the host cell
504 can influence the kinetics of FV3 replication[123].

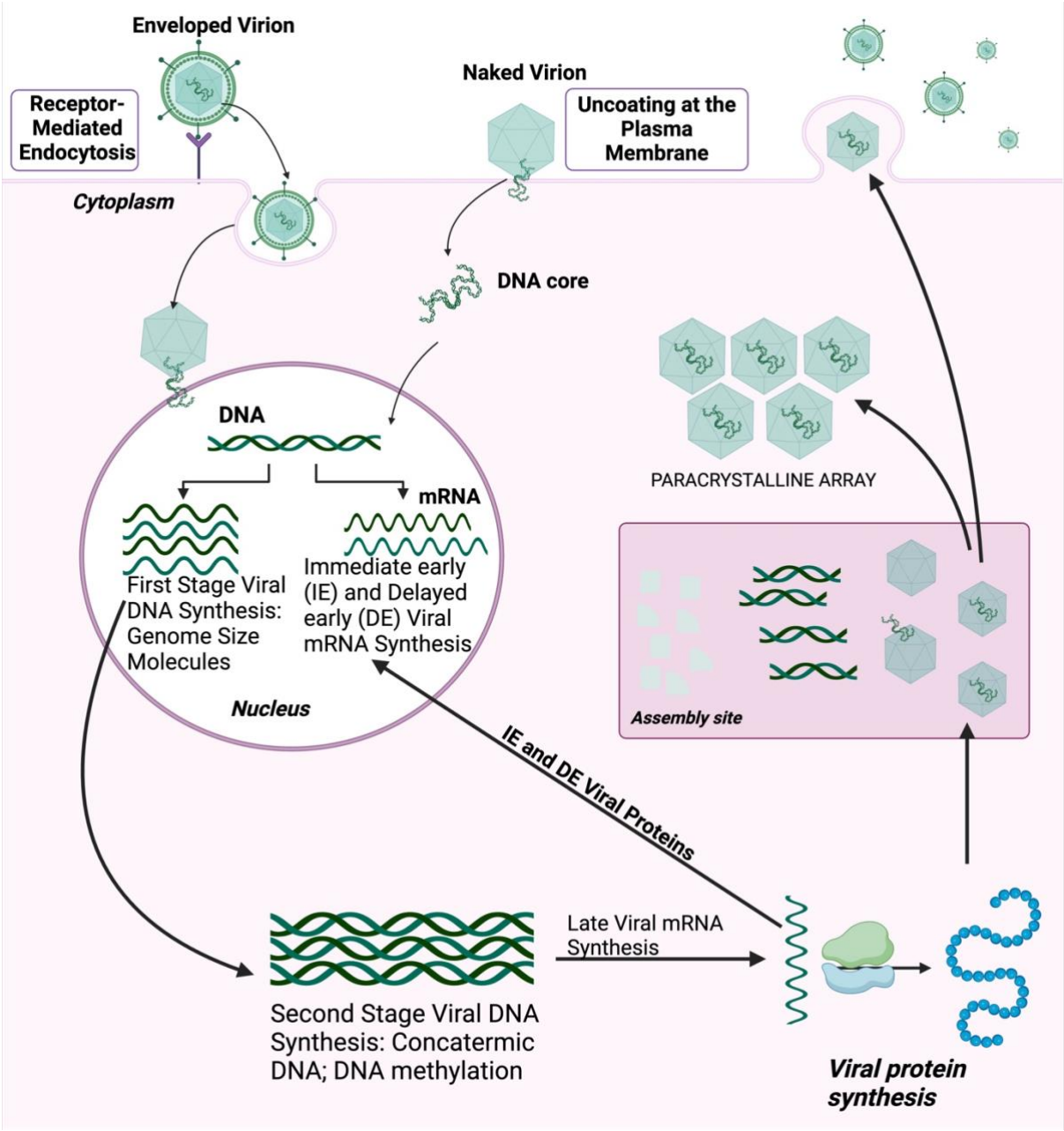


Figure 5: Frog virus 3 replication results in the formation of enveloped or non-enveloped virions.: FV3 replication begins with either an enveloped virion entering the cell via receptor-mediated endocytosis or as a non-enveloped virion entering the host cell via uncoating at the plasma membrane[123]. Once in the host cell's cytoplasm, the DNA core enters the nucleus where first stage viral DNA replication takes place to produce genome sized molecules; and immediate early and delayed early viral mRNA synthesis occurs[141]. The second stage of viral DNA synthesis then takes place in the cytoplasm to produce concatermic DNA which is

methylated[123]. Following this, late mRNA viral synthesis occurs followed by viral protein synthesis. At the assembly site, these proteins then come together to form new virions which either remain in the cell and is released once the cell has lysed, or it buds out of the cell which gives it its envelope[123].

1.9 Impact of Virus Infection on Host Cell

Upon infection of Ranavirus, the host DNA, RNA, and protein synthesis is shut off leading to swift cell death[148]. FV3 inhibits a variety of cellular processes including host mRNA and the phosphorylation and inactivation of the alpha subunit of eukaryotic initiation factor (eIF-2alpha)[149,150]. This enzyme is activated during cellular stress and is a key part of the immune response, and when activated inhibits viral replication[149].

1.9.1 Apoptosis

Ranavirus-induced apoptosis is dependent on caspase activation and can be prevented by caspase inhibition[148]. DNA fragmentation occurs 6–7-hours post FV3 infection while more prominent DNA fragmentation occurs at the 17-hour mark[148]. Researchers have suggested that virion proteins themselves are able to induce apoptosis. This was confirmed through a series of tests done when cells were infected with UV-inactivated FV3 virions, key viral proteins had been denatured[148]. Additionally, UV-inactivated viruses induced apoptosis suggesting that FV3 replication and gene expression is not required to induce apoptosis. Apoptosis can be triggered by either translational shut off or PRK activation[151]. By interacting with the transmembrane signaling protein, FV3 may be able to induce apoptosis. PKR activation results in protein synthesis being inhibited in infected cells which will trigger apoptosis[148]. Also, it is possible that at the point of contact or interaction with the host cell, virion-associated proteins indirectly or directly induce apoptosis[148].

1.10 Pathology

Biological consequences from Iridovirid infections vary depending on the specific virus and the host organism. Ranavirus outbreaks in amphibians are seen most often in larvae and recently metamorphosed animals. Ranaviruses, more specifically FV3, can cause systemic infections that target the host's kidney, liver, and GI tract[140]. The mortality rate for infected species is up to 100%[152]. The larvae are more susceptible to lethal effects than

adults. In fact, most adult hosts are asymptomatic and are carriers of the virus[131,140]. With the high mortality rate for infected larvae, there is a significant decline in amphibian population worldwide[125,127]. Some of the gross pathology seen in infected amphibian larvae include erratic swimming, edema of the body, and internal hemorrhages[152]. In adults, some of the pathology seen involves lethargy, skin ulcers, and edema of legs, feet, and internal soft tissues[152].

1.11 Diversity and Distribution

The genus Ranavirus is capable infecting captive and wild ectothermic animals[153]. The virus has impacted these animals on every continent except for Antarctica. Roughly 175 species across 52 families have been infected by Ranavirus[139]. Ranaviruses are classified as emerging pathogens as over the years the host range and geographical distribution range has grown. One cause of the range increasing is due to international trading of amphibians. Aside from Hawaii, Ranaviruses can be found across North America[139]. In Canada, there are at least nine species of amphibians that are infected with Ranavirus[153]. The two main species of Ranavirus found in North America are FV3 and ATV[131]. The latter is the most dominant species of Ranavirus found in North America[139].

1.12 Antiviral Immunity

There are currently limited treatments for Ranavirus infections. Allender et. al. proposed the use of guanine antiviral drugs[154]. Lei et al also reported antiviral activity of DNA aptamers[155]. Heat treatments is another possibility and is effective in inactivating pathogens of ectothermic vertebrates. Heat treatments, however, would vary dependent on host species. Most Ranaviruses do not replicate at temperatures above 32ºC, and so elevating body temperatures past this may be effective. More research is needed to fully assess heat treatments as an effective treatment for Ranaviruses. Any commercially available vaccines are targeted towards fish species within the aquaculture industry[156]. Limitations of a vaccine

lies in the intramuscular injection, which is not feasible in wild populations. Researchers are investigating oral antivirals for a more feasible delivery method[156].

Based on the study done by Seegobin et. al., it is evident that CKs inhibit FV3 activity when used as a concurrent treatment at 20 μM[157]. iP and iPR were able to inhibit FV3 activity in *Epithelioma cyprini papulosum* (EPC) cells at 48% and 52% respectively. In addition to concurrent treatment, the study used pre-treated host cells with the CKs for 12 hours prior to infection. Again, there was a significant decrease in plaque formation. To expand on this study, this project focuses on comparing concurrent treatment and pre-treatment of CKs while aiming to answer the question what is the minimum time EPC cells can be pre-treated with CKs while still seeing a significant decrease in plaque formation. In addition, aromatic CKs, kinetin and kinetin riboside's anti-FV3 activity were investigated to determine if the results seen with iP and iPR could be seen with other types of CKs. Finally, understanding the mechanism of how iP and iPR inhibit viral replication was the next step. To do this, morphological changes in the infected nuclei of EPC cells were examined. Gaining answers to these questions will further our understanding of CKs anti-viral activity.

2. Materials and Methods

2.1 Cell Culture and reagents

Epithelioma cyprini papulosum (EPC, American Type Culture Collection, ATCC, VA, USA) cells were grown and maintained in Lewitzbock's L-15 medium (L-15; ThermoFisher Scientific, Waltham, MA, USA). The medium was supplemented with 10% fetal bovine serum (FBS), 1.5% penicillin streptomycin, and 1.6 µg/mL amphotericin B (ThermoFisher Scientific, Waltham, MA, USA) at 25°C-27°C. The cells were grown in non-vented culture flasks (T-175 mL, Sarstedt AG & Co. KG, Numbrecht, Germany) Cells were subcultured every 5-7 days by treatment with TrypleE (ThermoFisher Scientific, Waltham, MA, USA) to detach cells adhered to the flasks.

2.2 Cytokinin preparation

Stock solutions of N^6-isopentyladenine (iP), N^6-isopentenyladenosine (iPR), N6-furfuryladenine (KIN), and N6-furfuyladenosine (KR) (OlChemIm Ltd., Olomouc, Czech Republic) were prepared by dissolving in 100% DMSO for a final concentration of 10 mM. The CKs were used in increasing concentrations (0.5-20 µM). iP and iPR were used as a pre-treatment at a final concentration of 20 µM for 30 minutes. Kinetin and KR were used as a pre-treatment at a final concentration of 20 µM for one hour.

2.3 Virus Isolation and Titering

EPC cells were grown to 80% confluency to form a monolayer in 75cm^2 flasks. Once the confluent monolayer was formed, the cells were inoculated with FV3 at a multiplicity of infection (MOI) of 0.1 PFU/cell. The infected cells were then incubated at 25°C-27°C for five days. After the 5 days, the cells were harvested once approximately 90% of the cells displayed cytopathic effects. A freeze/thaw cycle was then carried out three times to promote cell lysis followed by purification by centrifugation (4000 RPM for 5 minutes). The virus

stock was then aliquoted into 500 mL falcon (UltiDent Scientific, St Laurent, Quebec, Canada) tubes and stored at -80℃. Viral titering was determined by plaque assay.

2.4 Plaque Formation Assay

Serial dilutions at 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were prepared from the stock virus. The dilution EPC cells were seeded into 6 well plates at 2.4 x 10^6 using 10% L-15 media. Once a confluent monolayer was formed after 24 hours, the EPC cells were infected with the FV3 dilutions. The plates were then incubated at 25℃-27℃ for 24 hours. The cells were then washed twice with phosphate buffered saline (PBS, pH 7.2, ThermoFisher Scientific, Waltham, MA, USA) and were overlaid with 0.75% methylcellulose (Sigma-Aldrich, Oakville, ON, Canada) in L-15 supplemented with 2% FBS. The cells were then incubated for seventy-two hours at 25℃-27℃ to allow plaques to grow. The plaques were then counted on the third day using a tally counter viewed through a Nikon Ts2R-FL inverted microscope (Nikon Canada Incorporated Instruments Division, Mississauga, ON, Canada). Plaque formations were calculated, and statistical significance was assessed in GraphPad Prism 9 (GraphPad Software Incorporated, La Jolla, CA, USA) using a Kruskal-Wallis test followed by Dunn's post hoc analysis. A *p*-value of <0.05 was considered statistically significant.

2.4.1 Concurrent treatment of CKs

Confluent monolayers of EPC cells were prepared in six-well plates at a density of 2.4 x 10^6 per well. After 24 hours, the cells were infected with FV3 at an MOI of 0.1 and treated with 40 µM of iP, iPR, KIN, or KR. After 24 hours, the inocula was removed and the cells were washed twice with PBS. The cells were then overlaid with 0.75% methylcellulose and were left to incubate for 5 days at 25℃-27℃. After 5 days, a plaque assay was performed.

2.4.2 Pre-treatment of CKs

EPC cells were seeded at 2.4 x 10^6 in each well of a 6-well plate and were grown to 100% confluency to form a monolayer. After 24 hours, the media was removed, and the cells

were treated with 20 µM iP or iPR for 30 minutes or KIN or KR for one hour. After the allotted time had passed, the solution was aspirated from the cells and was infected with FV3 at an MOI of 0.1. The cells were left to incubate for 24 hours at which point, the inoculum was removed, and the cells were washed twice with 1x PBS. The cells were then overlaid with 0.75% methylcellulose and were left to incubate once again for 5 days. After the allotted time, the formed plaques were counted.

2.5 Assessing Nuclei Size During Viral Infection and CK treatment.

2.5.1 Concurrent Treatment with iP and iPR

EPC cells were seeded at a density of 2.4 x 10^6 into 6-well plates with coverslips. After 24 hours, the cells were either treated with iP or iPR, infected with FV3 at an MOI of 5, infected with FV3 at an MOI of 5 and treated with either iP or iPR, or had neither CKs nor FV3 on them. The treatments were left on the cells for either 6, 12, 18 or 24 hours.

2.5.2 Immunofluorescence Assay

Once the treatments were taken off the cells at the allotted time points, the cells were washed twice with 1x PBS (7.4) for 2-minute intervals and then fixed to coverslips with 3.7% paraformaldehyde (Sigma-Aldrich, Oakville, ON, Canada) for 10 minutes. The cells were then washed again twice with PBS for 2-minute intervals. The PBS was then removed and replaced with 1 mL of 0.1% Triton X-100 to wash the cells for 5 minutes. After the 5 minutes, the cells were then washed again with fresh PBS twice for 2-minute intervals. At that point, the PBS was removed and replaced with 2mL of blocking buffer for 2 hours at room temperature. After blocking, the solutions were removed, and the cells were washed with fresh washing buffer twice for 3-minute intervals. After removing the washing buffer, the coverslips were moved from the plates and placed on to microscopic slides. The washing buffer was then removed, and the coverslips were removed from the plate and mounted on the microscopic slides with VECTASHIELD mounting media with DAPI (Vector

Laboratories, Burlington, ON, Canada) and sealed with clear nail polish. The stained nuclei were then detected using a Leica DM 1RE2 fluorescent microscope.

2.5.3 Nuclei Area Measurement

The protocol for nuclei measurement was adapted from the Eidet et. al[158]. The 16-bit photomicrographs of the DAPI-stained nuclei were first converted to 8-bit images before being converted to binary photos using the "Make Binary" function in ImageJ. Touching nuclei were separated by the "Watershed" function and small fragments of nuclei were discarded based on the "Analyze Particle" function. Cells also touching the edges were excluded based on the "Exclude particles touching the edge" feature. The "Analyze Particle" feature provided morphological parameters including cell count and nuclear area. A one-way ANOVA test was carried out using Prism GraphPad. *P*-values below 0.05 were considered statistically significant.

3 Results

3.1 Exogenous application of iP and iPR do not reduce plaque formation when used as a 15-minute pre-treatment.

To assess iP and iPR's ability to inhibit FV3 activity EPC cells were pre-treated for 15-minutes with iP and iPR followed by an FV3-infection. A plaque assay was performed. iP (20 μM) showed no significant decrease in plaque formation compared to the control (p= 0.0010) **(Figure 6A**). Similarly, iPR (20 μM) did not significantly inhibit plaque formation (p= 0.0399) (**Figure 7B**). These data suggested that 15-minute pre-treatment was not enough time for iP and iPR to significantly inhibit FV3 activity. Length of time for pre-treatment was increased to determine the minimum time needed for significant inhibition of FV3 activity.

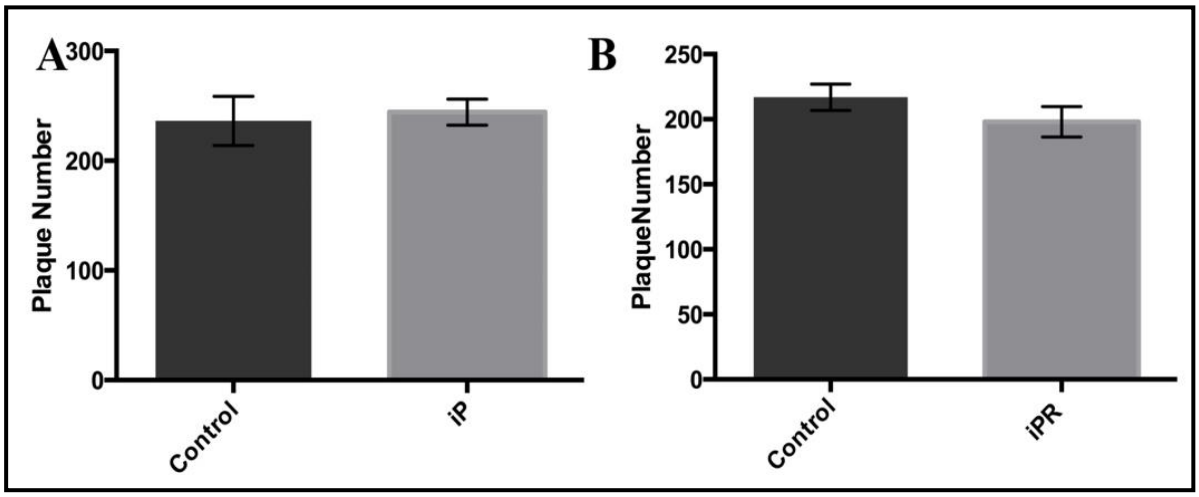
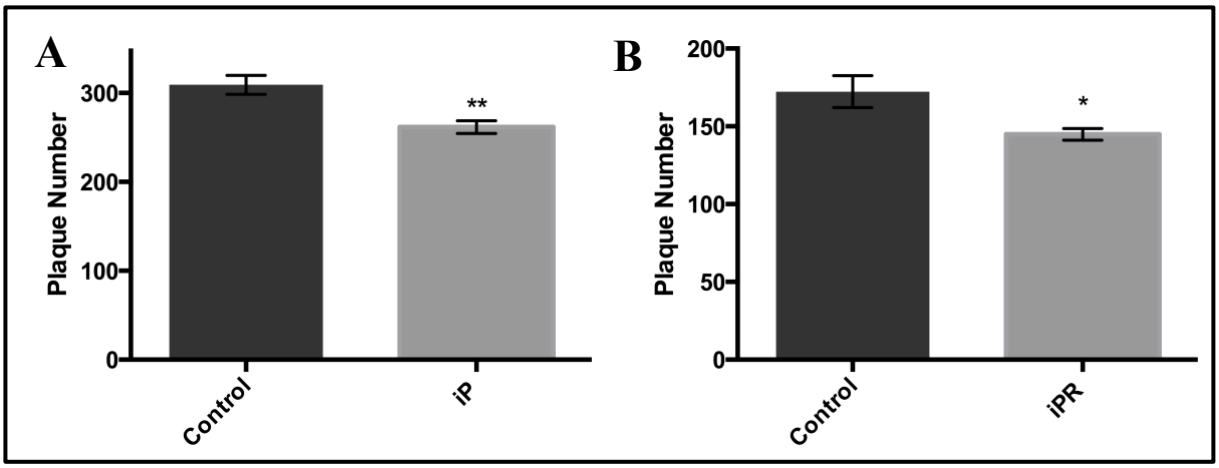


Figure 6: iP and iPR do not inhibit FV3 replication when used as a 15-minute pre-treatment. EPC cells were treated with CKs iP **(A)** and iPR **(B)** for 15 minutes prior to FV3 infection. Methylcellulose overlay (0.75%) was added 24 hours post infection and plaque formation assay was carried out 5 days post overlay. Data are presented as mean plaque formation ±SEM. Statistical analysis was assessed using nonparametric t-test analysis. ($n \geq 3$)

3.2 Exogenous application of iP and iPR reduce plaque formation when used as a pre-treatment.

To assess iP and iPR's ability to inhibit FV3 activity EPC cells were pre-treated for 30-minutes with iP and iPR followed by an FV3-infection. A plaque assay was performed. iP (20 μM) showed a 14% decrease in plaque formation compared to the control (p= 0.0010) **(Figure 7A**) while iPR (20 μM) showed a 16% decrease in plaque formation (p= 0.039) (**Figure 7B**). These data show that as a pre-treatment for 30 minutes of iP and iPR can significantly inhibit FV3 activity. Other CKs were explored to determine if FV3 inhibition was only specific to iP and iPR or a broader range could inhibit FV3 replication.



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694 **Figure 7: iP and iPR inhibits FV3 replication when used as a pre-treatment for 30**
695 **minutes.** EPC cells were treated with CKs iP **(A)** and iPR **(B)** for 30 minutes prior to FV3
696 infection. Methylcellulose overlay (0.75%) was added 24 hours post infection and after 5 days,
697 plaque numbers were counted. Data are presented as mean plaque formation ±SEM. Statistical
698 analysis was assessed using nonparametric t-test analysis. ($n \geq 3$; $*p \leq 0.05$ $**p \leq 0.01$)

3.2 Exogenous application of kinetin reduces plaque formation when used as a 1-hour pre-treatment.

Kinetin has shown the ability to inhibit viral replication in SARS-Cov-2[119]. Additionally, both kinetin and KR are known to have an antioxidant effect on vertebrate cells[62]. Because of this, kinetin and KR were two CKs appropriate for testing against FV3 activity. EPC cells were pre-treated with kinetin and KR at a concentration of 20 μM 1-hour prior to FV3 infection. Kinetin (20 μM) showed a 21% reduction in plaque formation compared to the control ($p = 0.0075$) (**Figure 8A**). However, KR (20 μM) did not significantly decrease plaque formation ($p = 0.0809$) (**Figure 8B**). iP, iPR and kinetin showed FV3 inhibition ability when used as a 30-minute pre-treatment. Since a short pre-treatment of CKs partially inhibited FV3 infection, the presence of both virus and CK's at the same time was assessed to determine if concurrent treatment was more effective at inhibiting FV3.

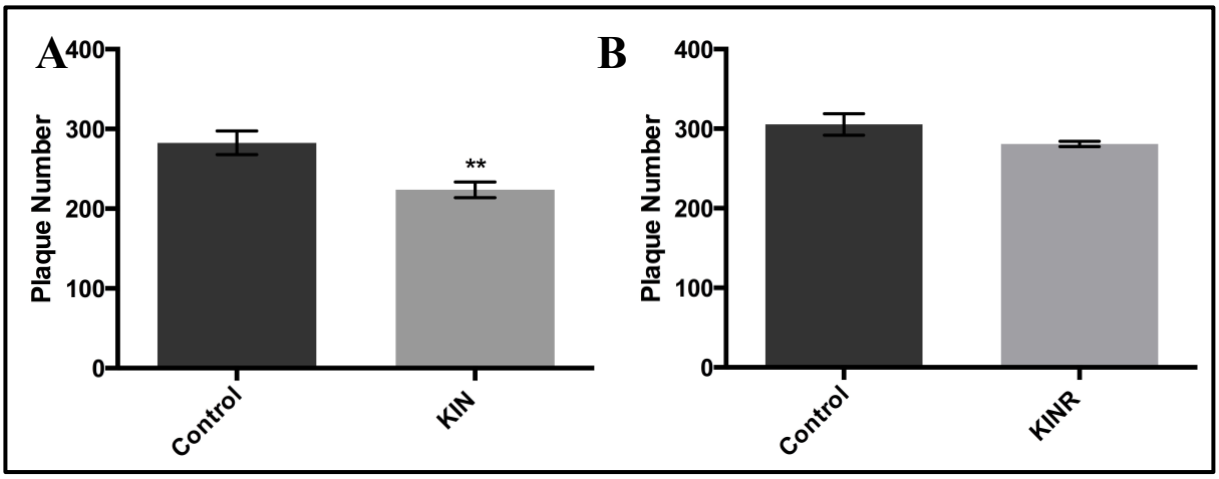
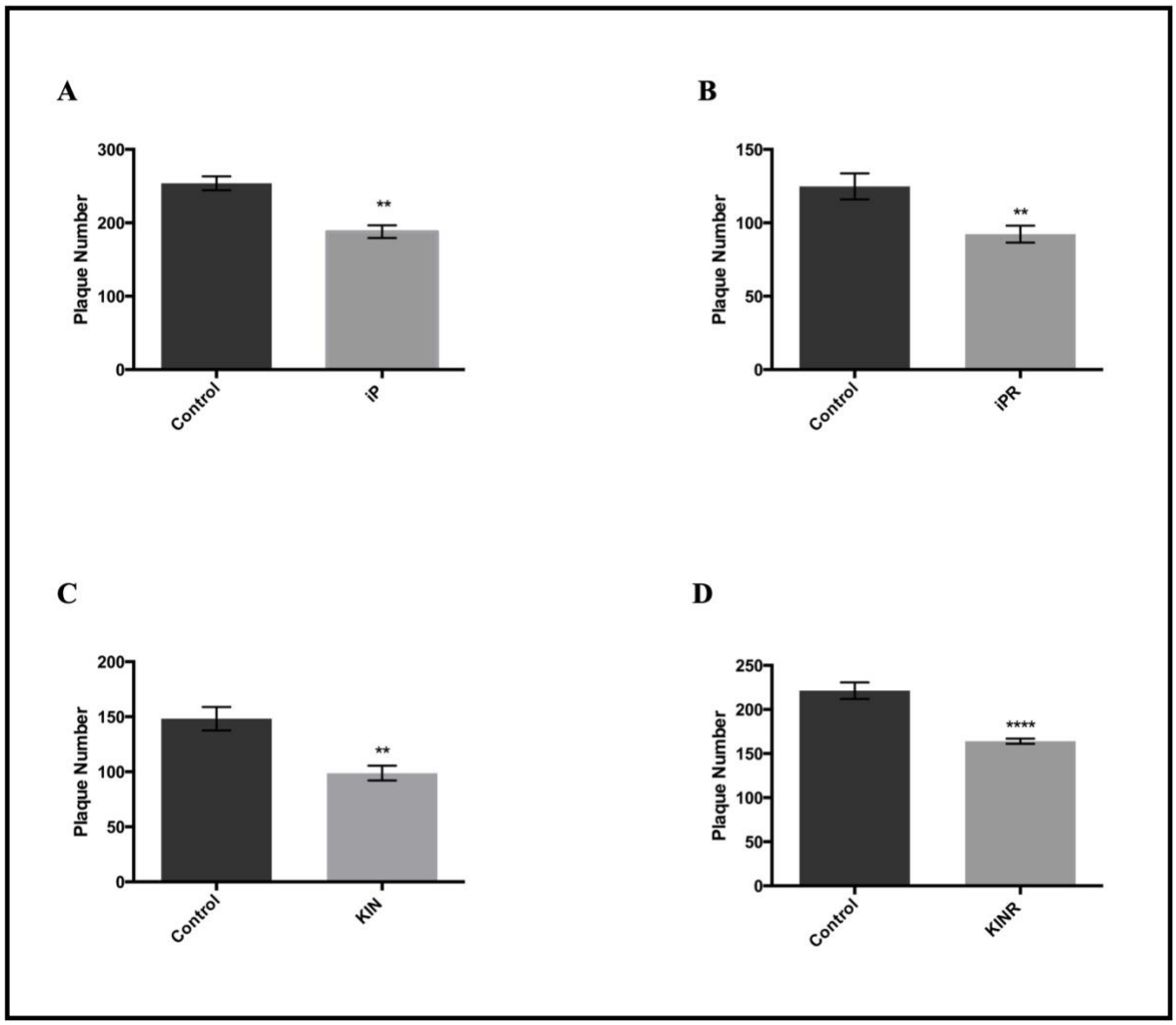


Figure 8: Exogenous application of kinetin and KR as a pre-treatment. EPC cells were treated with CKs KIN **(C)** and KINR **(D)** for one hour prior to FV3 infection at an MOI of 0.1. Methylcellulose overlay (0.75%) was added 24 hours post infection and after 5 days plaque formation numbers were counted. Data are presented as mean plaque formation ±SEM. Statistical analysis was assessed using nonparametric t-test analysis. ($n \geq 3$; $^{**}p \leq 0.01$)

3.3 Exogenous Application of iP, iPR, kinetin, and KR reduce plaque formation when used as a concurrent treatment.

To determine if concurrent treatment of kinetin and KR was more effective than pretreatment, EPC cells were infected with FV3 and 20μM of iP, iPR, kinetin or KR. Plaques were counted and compared with the control of FV3-infected cells. The results show that iP decreased plaque formation by 21% compared to the control (p =0.0050) (**Figure 9A**). iPR (20 μM) showed a 26% decrease in plaque formation (p =0.0040) (**Figure 9B**). These results were similar to Seegobin et al. Kinetin (20 μM) showed a 33% decrease in plaque formation (p =0.0060) (**Figure 9C**); and KR (20 μM) showed a 26% decrease in plaque formation (p <0.0001) (**Figure 9D**). These data (Figure 8&9) demonstrate that kinetin and KR can inhibit FV3 replication.



729

730 **Figure 9: Concurrent treatment of CKs show significant decrease in FV3 replication.**:

731 EPC cells were treated with CKs iP **(A),** iPR **(B)**, kinetin **(C)**, and KINR **(D)** and infected with

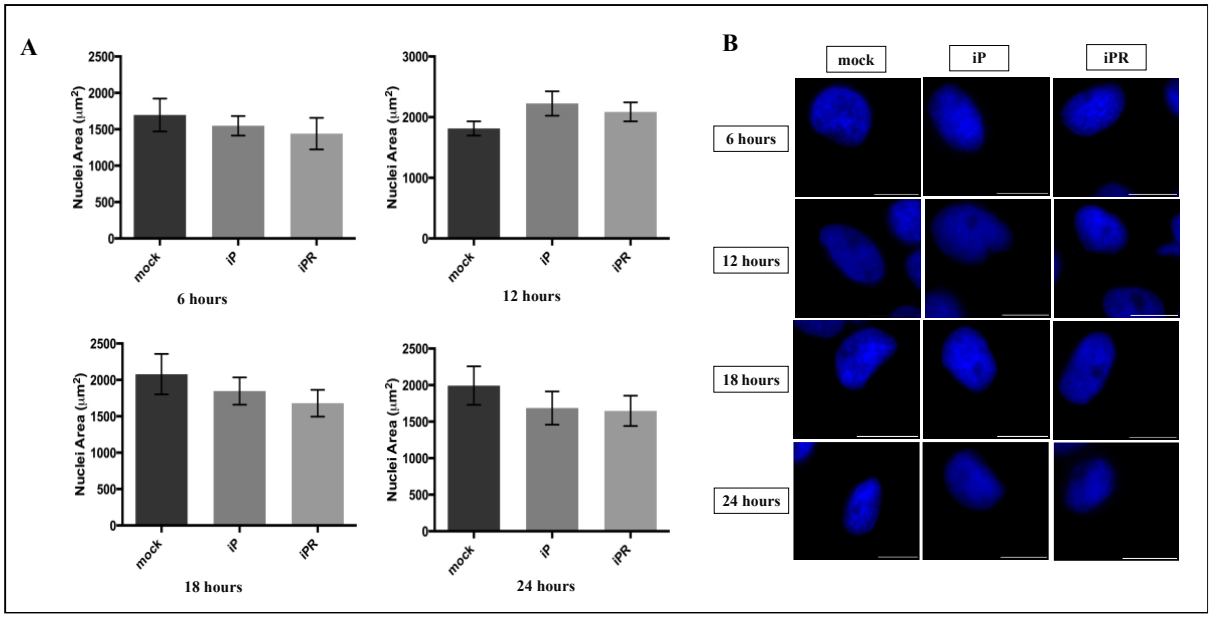
732 FV3 at an MOI of 0.1. Methylcellulose overlay was added 24 hours post infection and plaque

733 numbers were counted. Data are presented as mean plaque formation ±SEM. Statistical

734 analysis was assessed using nonparametric t-test analysis. ($n \geq 3$; $^{**}p \leq 0.01$ $^{****}p \leq 0.0001$)

3.4 Application of iP and iPR cause no morphological changes to nuclei.

The pre-treatment and concurrent treatment results concluded that iP and iPR inhibit FV3 replication, and so the next step was to understand the mechanism of this inhibition. To look for morphological changes caused by the presence of CK's during an FV3 infection, the nuclei of infected cells were analyzed. To begin, iP and iPR were assessed to determine if they affected nuclear size. EPC cells were treated with iP/iPR (20 μM) at various time points- 6, 12, 18 and 24 hours and the nuclei were stained with DAPI. My results show that at 6, 12, 18, and 24 hours, there was no significant change in nuclei area for both iP and iPR compared to a mock treatment used as the control **(Figure 10A)**. DAPI staining of nuclei indicated there is no apparent physical changes in nuclei size at all time points between control and iP/iPR treated cells **(Figure 10B)**.

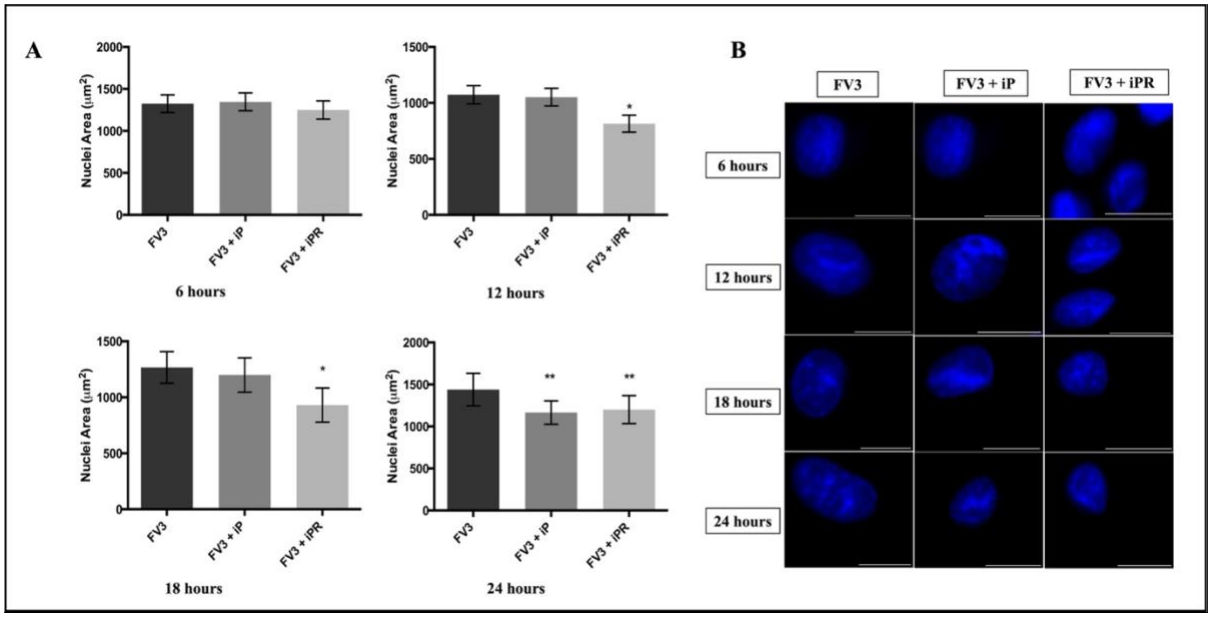


746

747 **Figure 10: iP and iPR-treated cells show no significant changes in nuclei area.** EPC cells
748 were treated with mock solution, 20 µM iP, or 20 µM iPR. The area of the nuclei was observed
749 at 6 hours 12 hours 18 hours and 24 hours (**A**). Data are presented as average of area of EPC
750 nuclei ±SEM. Statistical analysis was assessed using Kruskal-Wallis test followed by Dunn's
751 post hoc analysis. ($n \geq 3$) Scale bar = 50µm.

3.5 Application of iP and iPR decreases nuclei area when used as a concurrent treatment during viral infection.

There was no significant change in nuclei size observed with iP and iPR treatment. We next assessed whether iP and iPR along with an FV3 infection altered nuclei morphology. EPC cells were treated with iP/iPR (20 μM) and infected with FV3 at an MOI of 0.1. At 6, 12, 18 and 24 hours the nuclei were stained with DAPI. At 6 hours, there was no significant difference in nuclei area between the FV3 infected cells (control) versus the infected and iP/iPR treated cells (**Figure 11A**). At 12 and 18 hours, there was no significant difference between the control and the FV3/iP treated cells. However, there was a notable decrease in FV3/iPR treated cells (**Figure 11A**). At 24 hours, there was a significant decrease in nuclei area between FV3/iP (23%) and FV3/iPR (25%) cells versus the control (**Figure 11A**). These data suggest that concurrent treatment with iPR and iPR have an effect on the infected nuclei; iPR's effect is seen after 12 hours and iP's effect is seen at 24 hours.



765

766 **Figure 11: iP and iPR shows changes in infected nuclei area.** EPC cells were infected with
767 FV3 at an MOI of 5 and treated with 20 μM iP or iPR. The area of the nuclei was observed at
768 6 hours, 12 hours, 18 hours, and 24 hours. **(A)** Data are presented as average of area of EPC
769 nuclei ±SEM. Statistical analysis was assessed using Kruskal-Wallis test followed by Dunn's
770 post hoc analysis. ($n \geq 3$; $*p \leq 0.05$, $**p \leq 0.01$). Scale bar = 50μm.

4. Discussion

Based on a previous study, it is known that iP and iPR inhibit FV3 replication[120]. To expand on that study, the mode of application was investigated to determine efficiency. Specifically, pre-treatment of iP and iPR was assessed to compare inhibition of FV3 versus concurrent treatment of iP and iPR during FV3 infection. Additionally, alternative CK forms derived from a different cellular source (DNA vs tRNA) were assessed in this study as they are known to be effective in mitigating neurodegenerative disease pathways[53,105,159]. Thus, kinetin and its riboside, KR were tested to determine if they affect FV3 replication. To better understand the state of the cells during concurrent treatment with CKs, the effect of CKs on EPC cells morphology during FV3 infection was tested. Specifically, the nuclei size during concurrent treatment with iP or iPR was compared to the nuclei size during FV3-infection alone.

Pre-treatment of iP and iPR for 15 minutes was tested and the results show no significant decrease in plaque formation. However, when testing 30-minute pretreatment of iP and iPR, the results show that iP and iPR were able to inhibit FV3 replication. In a previous study, Seegobin et al showed that when iP and iPR were applied 12 hours prior to infection, FV3 plaque formation decreased by 48% and 60% respectively[157]. The inhibition of FV3 by exogenous application of iP and iPR demonstrates that CKs can have a prolonged effect on the cells. Although this study demonstrated the ability of exogenous application of CKs to affect FV3 replication, there have been no studies on the continuous effect of exogenous application of CKs on FV3 activity. The main question is, after pre-treating cells with CKs, what is the maximum amount of time that can pass before infecting the cells and still seeing a significant decrease in FV3 replication. To do this, a pretreatment for 30 minutes of EPC cells with CK's would be informative. The CKs would be removed after 30 minutes, and the cells would be incubated for varying lengths of time in the absence of CKs

and FV3. After the incubation period, the cells would be infected, and a plaque formation assay would show FV3 activity. A longer incubation time would have higher FV3 activity compared to shorter incubation time. These results would help to better understand CK metabolism in EPC cells and the amount of time CK metabolism takes.

One mechanism of how CKs could disrupt FV3 replication is through activating interferons (IFNs). IFNs are a group of immunomodulatory molecules that are produced by the immune system in response to the presence of pathogens during viral infections[160,161]. FV3 open reading frames (ORFs) are known to aid the virus in evading these IFNs allowing for successful replication[156]. For example, the FV3 ORF 53 does this by intercepting a viral homolog of the tumour necrosis factor-alpha signaling[162]. In addition, FV3 DNA methyltransferase prevents induction of IFN[156,162]. Cytokinins are known to stimulate pro and anti-inflammatory immune responses[62]. It is possible that treatment with iP and iPR could upregulate IFN production, leading to a decrease in FV3 activity. To test this, a study could be done to assess if there is an increase of IFNs 24 hours post FV3 infection in the presence or absence of iP and iPR. An increase in IFNs in the infected cells could indicate that the presence of exogenous CKs stimulates a greater production of IFNs. To quantify the production of IFNs, and ELISA could be performed. There are ELISA kits that detect IFNs[163]. Taken together, these results could demonstrate that iP and iPR utilize IFNs to decrease FV3 activity.

This study was the first to show that kinetin and KR can inhibit FV3 plaque formation (**Figure 8 & 9**) However, KR was unable to inhibit FV3 replication with a 1-hour pre-treatment (**Figure 8**). With an aromatic ring and riboside attached to KR, it is possible that one-hour is not enough time for the CK to enter the cell or be interconverted to an active form to cause a significant decrease in FV3 replication. In the study by Aoki et al, the authors examined pre-treatment of the aromatic CK, N^6-benzyladenosine (BAR) in HeLa cells at 6

and 12 hours. At these pre-treatment times, BAR was taken up into cells and converted to the free base form via the enzyme APRT[20]. It is possible that a longer pre-treatment time (more than 60 minutes) is needed for KR to be taken up by the EPC cells and converted to an active form to have an effect on FV3 activity.

Future work will determine if KR can inhibit FV3 infection with a 6- or 12-hour pre-treatment. In addition, completing a single-step growth curve of FV3 infected cells with kinetin or KR would identify at what stage of the viral replication cycle the inhibition is occurring. This could provide clues about potential function and could be compared to the published results of iPR and iPR to determine if CK's inhibit similar stages of viral replication.

Furthermore, studies could be done looking into CKs applied after infection. The cells would be infected with FV3. After 6, 12, 18, or 24 hours, the inocula would be removed and replaced with kinetin or KR solution. In the study done by Souza et al, the authors infected their Calu-3 cells with SARS-Cov-2 and then did a dual post-treatment of kinetin, one right after infection and then another 24 hours post infection[119]. They observed an EC50 of 0.31 ± 0.05 and the EC90 showed 2.8 ± 0.3[119]. This study also confirms what we are seeing in our study for kinetin and KR. From our study and this study, we see that kinetin and KR can be used as an anti-viral agent against both DNA and RNA viruses. The difference in the time of treatment of the CKs is a potential reason there is a higher inhibition of the SARS-Cov-2 compared to our study with inhibition of FV3[119]. This study only performed one treatment and that occurred at the same time of infection or 1-hour prior to infection, whereas in the Souza 2024 study, kinetin was applied as two post-infection treatments, once right after infection and again 24 hours later[119].

Another area to explore is whether kinetin and KR reduce reactive oxidative species (ROS). Viral infections are known to increase the induction of ROS which results in

apoptosis[164]. To determine if kinetin and KR affect ROS production, a ferric reduction antioxidant power (FRAP) assay could be performed. The FRAP assay shows the antioxidant potential of a sample which is visibly seen as a color change during the reduction of Fe^{3+} to Fe^{2+} [87,165]. The aim of the study would be to compare the ROS production in FV3 infected cells versus kinetin or KR/FV3-infected cells. If the FV3 infected cells alone have a higher ROS activity than the CK-treated infected cells, then this could mean that CKs are able to affect FV3 activity through a mechanism affecting ROS production. In a study conducted by Aoki et al, the authors observed an increase in mitochondrial activity when there was a deletion of adenylate isopentenyltransferase (*iptA)* during the vegetative growth state of *Dictyostelium discoideum*[166]. Disruption of IPT genes is known to alter CK levels in plants and non-plant organisms. Their results suggest a link between CKs activity and mitochondrial activity. In this case, a decrease in CK levels caused *D. discoideum* to go into a stress state resulting in increase in mitochondrial activity. In the case of viral infections, ROS production is related to mitochondrial function; a future study that could be conducted would be to compare ROS activity to mitochondrial morphology and activity between FV3-infected cells and CK-treated infected cells.

CKs like kinetin and zeatin are known to activate anti and pro-inflammatory responses[62]. During FV3 infection, the virus blocks pro-inflammatory responses from the host cell for successful replication to occur[120]. It is possible that CKs activate inflammatory responses leading to inhibition of FV3. In addition, CKs are known to activate AMP-activated protein kinase (AMPK), an enzyme responsible for balancing energy levels in a cell during times of stress[167,168]. The pathways involved in regulating FV3 replication are still unknown. Jang et al in 2023 inhibited AMPK with compound C resulting in inhibition of SARS-Cov-2 replication *in vitro*[169]. The authors tested various compound C concentrations (1, 5, and 10 μM) and found that compared to the control, viral RNA levels decreased

significantly at increasing compound C concentrations[169]. These studies raise the question of whether FV3 uses the AMPK pathway for successful replication and whether CK inhibition of FV3 could be the result of inhibiting this pathway. Future studies could inhibit AMPK's activity and determine if FV3 replication is inhibited. For example, compound C, a known AMPK inhibitor could be applied to cells for 24 hours prior to FV3 infection. If compound C decreases FV3 replication, it suggests that the AMPK pathway is involved in FV3 replication. Next, we can test whether CKs affect the AMPK activity during a viral infection and compare it to AMPK activity when infected cells are treated with compound C. Using a western blot, we would determine whether AMPK protein levels decrease when CKs are present. A decrease of AMPK concentration compared to the AMPK levels when compound C is applied to infected cells could suggest one mechanism that CKs inhibit viral replication is by inhibiting the AMPK pathway.

Overall, the results from both plaque formation assays show that iP, iPR, kinetin and KR act as anti-viral agents at a specific concentration whether as a concurrent treatment or pre-treatment (excluding KR). The results show that concurrent treatment of these CKs causes a greater decrease in plaque formation compared to pretreatment of the CKs. This could be due to the length of time the CKs are on the cells for the concurrent treatment (24 hours) versus 30-60 minutes of pretreatment. Nonetheless, it is quite notable that pre-treatment of CKs for such a short period of time, and prior to addition of FV3 decreases plaque formation. Aside from length of treatment on cells, the difference in decrease of plaque formation between the two treatments could also be attributed to the difference in the uptake and metabolism of CKs.

Aoki et al observed in HeLa cells that in the culture supernatants, iP was highest in concentration; in cell pellets, iPRP was highest in concentration. iPR was lowest in concentration in the supernatant[20]. In both the Aoki and Tran study, exogenous application of

BAR increased the concentration of iPRP in the cell pellets. Based on these studies, there is a possibility that the free base and riboside forms have to be converted to the nucleotide to have an effect on FV3 replication. It may be possible that although exogenous application of both the free base and riboside converts to the nucleotide form, there could be a difference in the concentration of the nucleotide form between the two different treatments. The treatment of iPR/KR could be causing a higher decrease in FV3 activity because there is a higher concentration of the nucleotide form. To determine if this theory is true, high-performance liquid chromatography-mass spectrometry (HPLC-MS) would be done to analyze and quantify CKs in EPC cell pellets during concurrent treatment.

During FV3 infection, there are different morphological changes that occur in the host cell[125]. These morphological changes can indicate the viability of cells[158]. With this knowledge, the morphology of cells during concurrent treatment was assessed. In the Aoki et al 2024 study, the authors assessed the morphology and quantity of mitochondria in *D. discoideum* when there is a deletion/overexpression of the *iptA* gene. During vegetative growth in *D. discoideum*, high CK levels are detected. In an *iptA*-deficient state, the number of mitochondria increase significantly compared to the control. Additionally, the mitochondria in cells with *iptA* deficiency increases in circularity compared to the control. Circularity in mitochondria is indicative of oxidative stress. Therefore, *iptA* deficiency which decrease CK levels proves that CKs are vital to vegetative growth in *D. discoideum*. During FV3 infection, the cell undergoes significant stress. To assess cell viability during concurrent treatment with iP and iPR, morphological changes in the host cell's nuclei were investigated. iP or iPR treatment at 20 µM, showed no significant difference in nuclei area at any of the times tested compared to mock treated cells (**Figure 10**). This confirmed two things: (i) at 20 µM, iP and iPR had no cytotoxic effects on EPC cells and (ii) any changes seen when investigating CK-treated and infected cells would mean that the difference in size is due to

CKs ability to inhibit viral activity. iP and iPR's effect on FV3-infected cell morphology were determined next. Between 12 and 24 hours, the nuclei area for iPR/FV3-infected cells decreased significantly compared to FV3 infected control cells. At 24 hours, the nuclei area decreased significantly with iP/FV3- infected cells compared to FV3 infected cells alone (**Figure 11**).

Altered nuclei size is often associated with pathology in the cells. In a study done Miyazakia et al in 1996 observed that human immunodeficiency virus type 1 (HIV-1) alters nuclear morphology during its life cycle[171]. During viral infections, the interruption of host cell processes eventually leads to cell death, apoptosis[171,172]. One major characteristic of the nucleus during apoptosis is shrinking[173]. Future studies could determine if apoptosis is occurring in the FV3-infected and CK treated cells. In a study done by Orlicka in 2020, KR was shown to induce apoptosis in HeLa and mouse melanoma B16F-10 cells[174]. The authors believed that CKs helped to modulate the mitochondrial membrane potential and stimulating the release of cytochrome c and activating caspase 3. To determine if CKs trigger an apoptotic response faster, cells will be treated with iP or iPR and infected with FV3. At 6, 12, 18 and 24 hours, the levels of Annexin V protein will be determined via western blotting. Annexin V is a marker for early-stage apoptosis and will demonstrate if apoptosis occurs faster in the iP or iPR/FV-3infected cells vs the FV3-infected cells alone[175].

In the study by Seegobin et al, al one-step growth curve shows that iP reduces viral load after 24 hours and iPR reduces viral load after 16 hours compared to FV3 alone[120]. The reduction in viral activity done in the one-step growth curve assay agrees with the results seen in my study with nuclear morphological changes: iPR begins to show decrease in nuclei area of FV3-infected cells at 12 hours and iP shows decrease in nuclei area at 24 hours. This leads to the question, do CKs trigger an apoptotic response faster than FV3 alone? Future studies should investigate this using apoptotic markers. These studies would present another

946 possible mechanism of how CKs are able to inhibit FV3 activity. By removing infected cells
947 faster, it may allow for better chance of survival for uninfected cells compared to FV3-
948 induced apoptosis.
949 We are seeing the effect of lower viral activity and nuclear changes much earlier in
950 the riboside form (iPR) vs the free base form (iP). These results are contrary to studies done
951 in plants where the free base is considered the most active form during cell processes. Future
952 studies could determine why this is happening. The entry of the CKs into the host cell could
953 be reason for difference in timing of effect. Two possibilities for the data are (i) the CK
954 receptor on the host cell has a higher binding affinity for the riboside form versus the free
955 base form and therefore, more active.
956 It is confirmed that in plants, the riboside form of CKs is more abundant as they
957 convert into the free base form which is active form used for plant processes. The riboside
958 form is only converted into the free base form when it is needed by the plants (CK
959 homeostasis)[176]. Contrasting what we see in plants, other systems appear to show opposite
960 where the free base form is possibly not the most active form. In the study by Souza et al,
961 they show that the exogenous application of the free base kinetin had to be converted to its
962 triphosphate form in order to inhibit SARS-Cov-2 activity[119]. In this study's results,
963 compared to the control, there is a decrease in plaque formation during iP concurrent
964 treatment. There is no evidence of the free base being the most active form in vertebrate
965 systems especially considering our results demonstrate that the riboside form (iPR and KR)
966 decreases FV3 replication significantly more compared to the free base form (iP and kinetin).
967 Additionally, Mauir et al's study demonstrates that the free base, kinetin, converts to its
968 nucleotide, KTP which repairs DNA damage in a Huntington's disease model. Based on the
969 Aoki study and this study, one explanation could be that although the exogenously applied iP

may not be taken up at all or as much as iPR, the sensing of the free base extracellularly, prompts a defensive mechanism to activate in the cell leading to decrease in FV3 replication.

To date, there has been no agreement of a CK receptor in vertebrate systems[177]. Adenosine receptors, however, have been targeted in a few studies to demonstrate CK's ability as a neuroprotectant[62]. These studies have confirmed the possibility of CKs interacting with, A_{2A} and A_3 receptors. Future studies should use radioligand binding to determine if there are adenosine receptors present in EPC cells. If this is confirmed, inhibitors like the commercially available ZM 241385 and KR (O4-{[3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl] carbonyl}-2-methyl-1,3-thiazole-4-carbohydroximamide), could be used to block the A_{2A} and A_3 receptors during concurrent treatment with iP and iPR and asses FV3 activity[105,178]. If we do not see a decrease in FV3 activity compared to samples with no adenosine inhibitor, then iP and iPR could be using the adenosine receptors to enter into the cell.

5. CONCLUSION

The aim of this study was to investigate the ability of selected CKs to inhibit FV3 replication. The results show that a 30-minute pre-treatment with iP, iPR, or kinetin at a concentration of 20 μM significantly inhibited FV3 replication. In comparison, concurrent treatment of iP, iPR, kinetin and KR were able to significantly inhibit FV3 replication. To begin answering the question of how CKs are able to inhibit viral replication, morphological changes in the host cell's nuclei was assessed. The results show that FV3-infected nuclei area was reduced after 12 hours with iPR and at 24 hours with iP. This study was the first to assess the morphological changes that occur in the host cell during CK-treatment of FV3 infected cells. Future studies should explore the mechanism of CK inhibition including determining whether apoptosis and the AMPK pathway are involved. Additionally, future studies should focus on the metabolism of CKs to determine which CK from is active during FV3 infection. These future studies will contribute greatly to the study of CKs in vertebrate systems.

6. Appendix

Appendix A: Data points for the Pre-treatment and Concurrent Treatment Plaque Formation Assay

Table 1: **Pre-treatment of iP, iPR and kinetin; and concurrent treatment of all tested CKs inhibit FV3 infection.**

iP				iPR				kinetin				KR			
Pre-treatment		Concurrent Treatment		Pre-treatment		Concurrent Treatment		Pre-treatment		Concurrent Treatment		Pre-treatment		Concurrent treatment	
Control	iP (20 ŒºM)	Control	iP (20 ŒºM)	Control	iPR (20ŒºM)	Control	iPR (20ŒºM)	Control	KIN (20ŒºM)	Control	KIN (20ŒºM)	Control	KINR (20ŒºM)	Control	KINR (20ŒºM)
362	247	259	201	165	131	148	97	319	275	157	124	253	278	254	150
303	232	266	200	140	130	118	89	290	232	187	127	290	269	204	162
315	294	237	183	215	151	137	103	340	258	181	110	313	283	203	159
295	253	299	192	134	154	132	60	319	275	113	71	299	281	211	176
315	248	219	239	220	132	111	75	290	232	122	80	311	296	251	169
327	247	220	149	147	157	63	101	340	258	108	90	324	290	196	157
255	266	261	192	179	155	129	110	228	206	162	88	300	291	268	176
302	286	270	171	166	152	131	83	237	239	156	85	395	270	205	161
	282		164	185	142	154	113	244	192		85	263	270	200	167
								219	182		128				
									182						
									187						
									191						

Appendix B: Non-parametric tests for pre-treatment and concurrent treatment plaque formation assay

Table 2:Statistical analysis for the pre-treatment and concurrent treatment of iP compared to the control (FV3).

Treatment	Summary	P value
Pre-treatment	***	0.001
Concurrent	***	0.0005

Table 3:Statistical analysis for the pre-treatment and concurrent treatment of iPR compared to the control (FV3).

Treatment	Summary	P value
Pre-treatment	*	0.0399
Concurrent	**	0.004

Table 4: Statistical analysis for the pre-treatment and concurrent treatment of Kinetin compared to the control (FV3).

Treatment	Summary	P value
Pre-treatment	**	0.0075
Concurrent	**	0.006

Table 5: Statistical analysis for the pre-treatment and concurrent treatment of KR compared to the control (FV3).

Treatment	Summary	P value
Pre-treatment	ns	0.0809
Concurrent	****	< 0.0001

Appendix C: Data points for the Nuclei Area Measurement

Table 6: **Nuclei Area for concurrent iP/iPR treated and FV3-infecfted cells decrease compared to nuclei area for FV3 infected cells.**

6 hours						12 hours						18 hours						24 hours					
mock	iP	iPR	FV3	FV3 + iP	FV3 + iPR	mock	iP	iPR	FV3	FV3 + iP	FV3 + iPR	mock	iP	iPR	FV3	FV3 + iP	FV3 + iPR	mock	iP	iPR	FV3	FV3 + iP	FV3 + iPR
1269.479	1108.352	881.964	896.473	941.586	1011.182	1391.975	1528.889	1652.079	1218.841	837.307	775.269	975.797	991.333	879.537	738.333	522.533	584.411	906.917	775.194	730.902	818.573	628.132	613.368
996.837	1264.358	1086.75	1091.509	1069.752	802.125	1595.371	1321.586	1333.969	860.145	789.655	467.341	977.258	894.489	843.872	868.089	601.825	490.405	819.59	744.639	715.561	799.366	602.506	553.707
965.955	1114.698	1071.265	1305.453	984.273	1053.579	1286.281	1354.757	1044.857	755.735	728.745	722.752	921.162	1024.413	844.041	671.343	514.969	652.399	804.431	723.722	682.215	821.503	716.93	669.396
1225.716	1183.804	1162.254	969.602	1112.394	841.061	1148.36	2682.379	3288.88	750.767	750.958	468.712	1039.089	991.21	769.486	604.89	583.048	481.441	903.181	817.864	715.397	798.258	699.113	762.192
1025.614	1247.156	857.556	1128.73	1000.725	911.009	1117.333	2985.826	2202.87	1042.448	746.708	549.932	802.351	1094.724	928.612	680.925	725.062	605.921	3807.571	679.095	2630.44	844.736	736.168	755.061
1712.565	1560.787	2423.962	913.321	949.04	932.654	2109.316	3572.762	2569.094	923.44	899	808.032	2576.381	2751.867	3017.714	989.971	705.613	700.966	3372.824	1521.69	2687.129	931.65	690.533	783.333
2366.392	2033.9	2015.433	927.757	1058.507	966.119	2188.125	2323.568	1886.481	912.015	894.909	643.877	2991.207	2464.45	1211.732	898.429	733.382	461.349	2715.471	1156.448	2481.75	889.688	778	671.4
1949.86	1721.615	2021.771	1101.412	1312.211	861.612	1846.086	1688.619	2331.086	848.259	889.74	704.02	2990.756	2309.742	2025.325	912.61	569.111	627.271	2930.667	1894.189	2125.2	914.5	587.224	586.511
2517.677	2090.611		965.877	932.075	1152	2133.172	1884.532	1974.36	720.118	890.948	649.118	4587.038	3074.75	2148.826	686.365	469.1	522.491	2758.149	3545.667	2001.292	894.091	677.983	518.058
2928.69	2158.966		1141.875	1168.721	802.24	2109.316	2463.167	2178.741	748.973	839.835	517.091	2846.129	1886.012	2305.048	743.787	615.984	528.127	1454.418	2439	2483.048	1897.316	645.644	533.741
			1234.312	1098.786	1381.083	2082.37	2699.592	2477.477	824.687	1064.043	706.913	1371.718	2547.381	1845.207	647.825	615.978	435.392	1791.705	2482.905	1960.104	1470.684	544.956	582.386
			1129.73	1177.439	923.839	1974.366	2197.37	2188.407	892.229	1053.667	850.595	2114.029	1855.283	2416.795	765.771	626.36	1945.129	1908.59	2502.435	1360.836	2641.938	605.909	1182.676
			1037.039	1148	848.182	2758.34		2002.636	1107.885	1004.019	885.292	2498.419	2392.645	2035	1380.1	2608.727	1865.653	1934.029	2309.675	1149.08	4199	1430.231	1785.167
			1193.065	937.324	1140.686	1704.963			700.889	1119.565	841	1885.064	1535.585	1777.025	1354.455	1847.207	2209.619	1804	1463.446	1343.839	1144.737	2375	1885.739
			1961.314	1578.177	1218.896	1758.747			810.213	759.305	882.262	2606.743	1889.652	2140.971	1944.5	1410.25	1389.171		2245.054		1655	1213.341	1744.902

Appendix D: One-way Anova (Kruskal-Wallis test) followed by Dunn's post hoc analysis.

Table 7: Statistical analysis all treatments at 6 hours for nuclei area measurements

Treatment	Summary	Adjusted P Value
mock vs. iP	ns	> 0.9999
mock vs. iPR	ns	0.6413
FV3 vs. FV3 + iP	ns	> 0.9999
FV3 vs. FV3 + iPR	ns	0.2602

Table 8: Statistical analysis all treatments at 12 hours for nuclei area assay

Treatment	Summary	Adjusted P Value
mock vs. iP	ns	0.1595
mock vs. iPR	ns	0.3076
FV3 vs. FV3 + iP	ns	> 0.9999
FV3 vs. FV3 + iPR	*	0.0187

Table 9: Statistical analysis all treatments at 18 hours for nuclei area assay

Treatment	Summary	Adjusted P Value
mock vs. iP	ns	> 0.9999
mock vs. iPR	ns	0.3641
FV3 vs. FV3 + iP	ns	0.0903
FV3 vs. FV3 + iPR	*	0.0394

Table 10: Statistical analysis all treatments at 24 hours for nuclei area measurements

Treatment	Summary	Adjusted P Value
mock vs. iP	ns	0.5883
mock vs. iPR	ns	0.5308
FV3 vs. FV3 + iP	**	0.0017
FV3 vs. FV3 + iPR	**	0.0014

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