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IDENTIFYING A NOVEL FERROCENE DERIVATIVE AS A K-RAS INHIBITOR

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

KRISTEN MARIE REHL

B.S. The Ohio State University, 2018

2023
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Kristen Marie Rehl ENTITLED Identifying a novel ferrocene derivative as a K-Ras inhibitor BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Rehl, Kristen Marie. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2023. Identifying a novel ferrocene derivative as a K-Ras inhibitor.

Ras proteins are small GTPases that regulate cell proliferation, differentiation and survival at the plasma membrane (PM). There are three Ras isoforms ubiquitously expressed in mammalian cells: H-, N- and K-Ras. Constitutively active Ras mutations are found in ~19% of all human cancers, with ~75% of those being in K-Ras. There are K-Ras inhibitors in clinic but they only target the oncogenic K-RasG12C mutant, which only makes up a small sub-set of K-Ras-driven cancers. Thus, there still exists a need for a pan anti-K-Ras drug. Ferrocene derivatives are a class of compounds that have been shown to inhibit the growth of a lung cancer cell line harboring an oncogenic mutant K-Ras via their elevation of cellular reactive oxygen species (ROS). Given that lung cancer cells harboring an oncogenic mutant K-Ras require K-Ras signaling for their survival, the effect of a novel ferrocene derivative, C$_{16}$H$_{20}$FeClNO, on K-Ras signaling was tested. C$_{16}$H$_{20}$FeClNO was found to specifically disrupt the K-Ras/MAPK signaling pathway and inhibit the growth of K-Ras-dependent human pancreatic and lung cancer cell lines. Given that localization to the PM is essential for K-Ras signaling, the effect of C$_{16}$H$_{20}$FeClNO on K-Ras PM localization was tested. C$_{16}$H$_{20}$FeClNO was found to dissociate K-Ras, but not other Ras isoforms, from the PM. Furthermore, other ROS-elevating drugs had a similar effect on K-Ras PM localization and K-Ras signal output. C$_{16}$H$_{20}$FeClNO was shown to elevate cellular ROS levels and supplementation of N-acetylcysteine (NAC), a general antioxidant, reversed these effects, suggesting that C$_{16}$H$_{20}$FeClNO disrupts K-Ras PM localization.
localization and K-Ras signaling through an ROS-mediated mechanism. Lastly, K-Ras histidine 95 (His95) residue in the G-domain was identified as the oxidative residue involved in $\text{C}_{16}\text{H}_{20}\text{FeClNO}$-induced K-Ras dissociation from the PM. Overall, $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ disrupts K-Ras PM localization and signal output through oxidation of His95. Taken together, this dissertation identifies $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ as a novel K-Ras inhibitor and provides the groundwork for $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ to be developed into an anti-cancer drug.
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1. Literature Review

1.1 K-Ras Background

1.1.1 History of Ras

In 1964, JJ Harvey noticed the induction of sarcoma in rats that were infected with the Moloney’s leukemogenic virus, a transforming retrovirus from rats with leukemia [1-3]. The virus was named Harvey-Murine Sarcoma Virus (Ha-MSV) [2]. In 1967, Werner Kirsten had a similar discovery with a murine erythroblastosis virus, later named Kirsten-Murine Sarcoma Virus (Ki-MSV), that also induced sarcoma in rats [1, 2, 4]. Studies showed that the transforming ability of both the Ha-MSV and Ki-MSV originated from a recombination of their murine retroviral genome with sequences from the rat genome [2, 5-7]. The genes transformed by these murine retroviruses became known as viral-Harvey- and Kirsten-Ras genes, with “Ras” being short for “rat sarcoma” [1, 5]. In 1979, it was discovered that the genes encode a 21 kDa protein, “p21”, that binds to guanine nucleotides and associate with the plasma membrane (PM) [1, 2, 8, 9]. Homologues of these viral-Harvey and -Kirsten-Ras genes were found in the human genome and the proteins became known as Ha-Ras and Ki-Ras, shortened to what we know call H-Ras and K-Ras [2, 5, 10]. H-Ras and K-Ras have shown to be involved in regulating cellular growth [11]. In the early 1980s, scientists were taking DNA from human cancer cell lines to transform mouse fibroblasts [5]. The transforming genes were identified as H-RAS and K-RAS, comprising of a single mutation at codon 12, similar to the viral genes [5]. This made Ras the first human oncogene discovered [2]. In 1983, another Ras transforming gene was discovered in neuroblastoma-derived DNA, known as N-RAS [2, 5, 12]. The human genes of H-RAS, K-RAS and N-RAS are located onto chromosomes 11 (11p15.1-p15.5), 12 (12p12.1-pter), and 1 (1p22-p32), respectively [2, 13, 14].
1.1.2 Structure and Function of Ras

Ras proteins are members of the Ras superfamily of small GTPases, which is composed of more than 150 members [15]. As mentioned previously, there are three isoforms of Ras: K-Ras, H-Ras and N-Ras, each encoded by their own gene, four exons in total [13, 14]. K-Ras undergoes alternative splicing on the fourth exon, generating two splicing variants: K-Ras4A and K-Ras4B (Fig. 1) [13, 14]. Studies have shown that while K-Ras4A and K-Ras4B have similar transcript levels in human cell lines, K-Ras4B (hereafter K-Ras) is the predominant protein expressed in mammalian cells, especially in human cancer [16-18].

Figure 1. Functional domains of the Ras isoforms. The G-domain, containing switch I (SI) and switch II (SII), is involved in Ras’ catalytic activity and effector binding. Amino acid differences in the HVR for each Ras isoforms are shown. All isoforms possess a CAAX motif at the C-terminus of the HVR. Bold cysteines (C) are sites of farnesylation and underlined cysteines are sites of palmitoylation. Lysine (K) residues are in red and serine (S) residues that get phosphorylated (P) are in green. Figure adapted from [19].

H-Ras, N-Ras and K-Ras4A encode a 189-residue protein while K-Ras4B encodes a 188-residue protein (Fig. 1) [18, 20]. All Ras isoforms share ~95% homology in their G-domain at the N-terminus, residues 1-166 (Fig. 1), which contains Ras’ catalytic activity and binding sites for
effector proteins [13, 21-25]. The Ras isoforms differ in their hypervariable region (HVR) located in the C-terminus (Fig. 1), residues 166-188 and 189 for K-Ras4B and H-/N-/K-Ras4A, respectively [13, 21, 23, 26]. The HVR region is responsible for post-translational modifications that dictate Ras trafficking and membrane localization [23, 24, 27, 28].

Ras proteins are membrane-bound GTPases. Ras proteins function as molecular switches, cycling between a GDP-bound inactive state and a GTP-bound active state (Fig. 2A) [22, 24, 27]. Since Ras proteins have a picomolar affinity for guanine nucleotides and GTP/GDP exist in millimolar concentration in the cell, Ras proteins remain bound to a nucleotide, either GTP or GDP, throughout their lifespan [18, 29]. Normally, Ras proteins are bound to GDP (Fig. 2A). In response to upstream signaling, guanine exchange factors (GEFs) bind Ras, which facilitates the release of GDP and subsequent binding of GTP (Fig. 2A), activating Ras. GEFs induce the conformational change of the nucleotide-binding site of Ras, which decreases the affinity for the nucleotide, leading to nucleotide release [29, 30]. Since GTP exists in ~10-fold concentration in the cytosol, it will bind to Ras [18, 21, 22, 24, 27, 29]. The binding of Ras to GTP induces a conformational change in the G-domain, specifically, switch I (residues 30-40) and switch II (residues 60-76), which creates a binding site for effector proteins [18, 21, 25, 27]. Well-studied Ras-GEFs are son of sevenless 1 (SOS1) and 2 (SOS2) and phospholipase Cε (PLCε) and they contain the CDC25 homology domains and the Ras exchange motif (REM) [20, 27, 29, 31].
Figure 2. The RasGTPase cycle. (A) Ras cycles between a Ras-GDP inactive state and a Ras-GTP active state. Inactive Ras is activated by GEFs, which promotes exchange of GDP for GTP. Active Ras is inactivated by GAPs, enhancing intrinsic GTPase hydrolysis and converting GTP to GDP. (B) A schematic of intrinsic Ras-GTP hydrolysis. T35 and Q61 interact with the nucleophilic water, while Y32 forms a hydrogen bond with the β and γ phosphate of GTP. Q61 extracts a hydrogen from the water, making it a hydroxyl ion (OH) which hydrolyzes the γ phosphate of GTP, resulting in GDP. Figure adapted from [32].

In intrinsic Ras-GTP hydrolysis, a conformational change in Switch I and Switch II regions constitute the active site for GTP hydrolysis [29, 33]. A threonine residue (T35) and the glutamine (Q61) residue on Ras interacts with a nucleophilic water molecule (Fig. 2B) [32, 33]. The glycine residue (G13) and the tyrosine residue (Y32) form a hydrogen bond with the β-γ bridging oxygen atom of GTP (Fig. 2B) [32, 33]. The side carbonyl chain of Q61 takes a hydrogen from the water, leaving it a hydroxyl ion (Fig. 2B) [32-34]. This hydroxyl ion then hydrolyzes the γ-phosphate of GTP, resulting in GDP (Fig. 2B) [32, 33]. However, this process occurs very slowly, taking approximately 8-17 hours in vitro [34, 35]. To accelerate this process,
Ras interacts with a GTPase-activating protein (GAP) (Fig. 2A) [29, 30]. In GAP-mediated Ras-GTP hydrolysis, GAP provides an arginine finger (R789) that interacts with the active site of Ras proteins by forming a salt bridge interaction with the α- and γ-phosphates of GTP, stabilizing Ras proteins [33]. This allows the water molecule to attack the GTP, hydrolyzing it back to Ras-GDP. GAP speeds up this reaction 100,000 x fold, taking less than 1 second in vitro [36, 37]. Examples of RasGAPs are p120GAP (RasGAP) and neurofibromin (NF1) [37, 38].

1.1.3 Ras Localization and Trafficking

As mentioned previously, the Ras isoforms differ in their HVR, which dictate how Ras gets trafficked and localized within the cell. The HVR contains two signals that regulate Ras trafficking to the PM. The first signal is the last 4 C-terminal residues, the CAAX motif (C=Cys, A= aliphatic amino acid, X = Met or Ser), which is seen in all isoforms (Fig. 1) [13, 21, 23-25, 27, 39, 40]. The Cys residue will undergo farnesylation (Fig. 3), addition of a 15-carbon prenyl moiety, by the cytosolic farnesyltransferase to translocate Ras from the cytosol to the outer leaflet of endoplasmic reticulum (ER) (Fig. 3) [13, 21, 23, 27, 39, 41]. In the ER, Ras converting CAAX endopeptidase 1 (Rce1) removes the -AAX and isoprenylcysteine carboxyl methyltransferase (Icmt) methylates the now-C-terminal Cys (Fig. 3) [13, 21, 23, 27, 39, 42]. While the farnesyl moiety allows Ras proteins to bind to cellular membranes, the second signal confers Ras proteins the stable PM interaction, which is essential for Ras signaling [18, 23, 43-46].
Figure 3. Ras post-translational modifications and PM trafficking. Ras proteins get farnesylated (red line) at the Cys residue in their CAAX motif (1) and transported to the ER (2) where Rce1 cleaves the -AAX (3) and Icmt methylates the now terminal Cys (4). For H- and N-Ras, they are sent to the Golgi (5) to get palmitoylated (gold line) and traffic to the PM via the classical secretory pathway (6). Depalmitoylated H- and N-Ras are sent back to the Golgi (7) and cycle back to the PM. For K-Ras, it contains a PBD (++++) that targets it to the PM but K-Ras trafficking is less understood (8). Figure adapted from [47] and created using BioRender.com.

In H-, N- and K-Ras4A, the second signal is palmitoylation in the ER and Golgi [13, 18, 22, 25, 39, 45, 48]. This occurs at Cys181 and 184 in H-Ras, Cys 181 in N-Ras and Cys180 in K-Ras4A (Fig. 1) [13, 21, 23, 27, 39]. N- and H-Ras get palmitoylated by palmitoyl acyl transferases in the ER and trafficked to the PM via the classical secretory pathway through the Golgi complex (Fig. 3) [13, 23, 27, 39, 41, 48]. Cellular thioesterases, such as APT1/APT2, depalmitoylate H- and N-Ras, which translocates them from the PM to the Golgi (Fig. 3) [48,
Palmitoylation, unlike farnesylation, is a reversible modification and shows rapid cycling in Ras proteins which establishes a consistent H- and N-Ras distribution between the Golgi and PM [41, 48]. Depalmitoylated H- and N-Ras translocate back to the Golgi, where it can be repalmitoylated and trafficked back to the PM (Fig. 3) [13, 39, 50, 51]. H-Ras does have a stronger enrichment at the PM when compared to N-Ras, due to having two palmitoyl groups instead of one [48, 49]. Additionally, H- and N-Ras may localize to the recycling endosomes [48, 52]. For K-Ras4A, its palmitoyl moiety together with two polybasic domains (PBDs) (residues 167-170 and 182-185) (Fig. 1) allow K-Ras4A to transport and interact with the PM [16, 48].

In K-Ras4B, the second signal is a PBD of six lysines at residues 175-180 (Fig. 1) [13, 18, 23, 25, 27, 39]. This positively-charged PBD forms an electrostatic interaction with anionic phospholipids, like phosphatidylserine in the inner leaflet of the PM (Fig. 4) [13, 22, 23, 25].

![Figure 4. The PBD of K-Ras forms an electrostatic interaction with PtdSer in the inner leaflet of the PM. K-Ras (black line) contains a polybasic domain (PBD), a string of six lysine residues (green). The PBD of K-Ras forms an electrostatic interaction with the negatively-charged headgroup of PtdSer (red), which is the second PM targeting signal for K-Ras.](image-url)
This PBD, along with the farnesyl tail, are necessary for K-Ras4B trafficking to the PM. However, the trafficking occurs through a Golgi-independent mechanism that is less understood [13, 21, 23, 27, 39, 41, 43] (Fig. 3). One mechanism of K-Ras transport to the PM involves the use of a chaperone protein, rod specific cyclic GMP phosphodiesterase 6 delta subunit (PDE6δ) [13, 25, 53]. It is a cytoplasmic protein that is expressed widely in various tissues [53, 54]. PDE6δ contains a hydrophobic pocket that binds the farnesyl moiety of K-Ras and depalmitoylated H- and N-Ras [46, 54]. In the cytosol, PDE6δ binds to K-Ras, specifically at residues 180-184 prior to farnesylated cysteine, after dissociation from any membrane (Fig. 5) [46, 55]. PDE6δ has an allosteric site that binds to the small GTPase Arl2 in the GTP-bound state and Arl2 catalyzes the dissociation of K-Ras from PDE6δ to the perinuclear membranes [46, 56]. K-Ras then binds to the recycling endosome (RE) through an electrostatic interaction with the RE’s negatively charged membrane, and returns to the PM (Fig. 5) [46, 54]. PDE6δ knockdown or inhibition by small molecules (Fig. 5) redistributes K-Ras to all endomembranes in the cell,
suggesting PDE6δ plays a critical role in K-Ras enrichment at the PM [46, 57, 58].

Figure 5. Role of PDE6δ as a chaperone protein involved in K-Ras trafficking to the PM. After K-Ras dissociates from the PM, PDE6δ binds to K-Ras via the farnesyl moiety and releases it to the perinuclear region. K-Ras translocates to the recycling endosome (RE) through electrostatic interaction and returns to the PM via RE-mediated transport. Figure adapted from [13].

Yet, PDE6δ binding is not specific to K-Ras, as it binds to many farnesylated proteins, such as H-Ras and N-Ras [13, 48, 54]. K-Ras may also be trafficked via a microtubule-dependent mechanism. Prenylated proteins have been shown to bind to microtubules and it was shown that K-Ras, but not H-Ras, binds to microtubules [39, 59]. Despite both proteins being farnesylated, K-Ras is proposed to bind to microtubules through its PBD [28, 59]. When treated with paclitaxel, a drug that stabilizes microtubules formation, K-Ras, but not H-Ras, was redistributed
from the PM to endosomes, suggesting microtubules may be involved in K-Ras targeting to the PM [39, 41, 43, 59]. Some studies have suggested that calcium (Ca\textsuperscript{2+})-bound calmodulin (CaM) can serve as a chaperone protein as well (Fig. 3) [27, 41, 56, 60]. CaM binds K-Ras, but not to other Ras isoforms, inducing K-Ras to translocate to endosomes and decreases K-Ras signaling [28, 61, 62].

**1.1.4 Ras Signaling Pathway**

Ras activates a variety of signaling pathways that are involved in cell differentiation, proliferation and survival. One of the most studied pathways is the Ras/Raf/MEK/ERK-mediated or mitogen-activated protein kinase (MAPK) pathway. A growth factor will bind to a receptor tyrosine kinase, resulting in its dimerization and autophosphorylation (Fig. 6). The adaptor protein growth factor receptor-bound protein 2 (Grb2) recruits the Ras-GEF, SOS1 or SOS2, to the PM, which facilitates Ras GTP binding and activates Ras proteins (Fig. 6) [20, 31, 63, 64]. One of the well-studied downstream effectors is the rapidly accelerated fibrosarcoma (Raf) kinase, a serine/threonine kinase, which gets recruited to the membrane by Ras [64-66]. The three isoforms of Raf are A-Raf, B-Raf and C-Raf, and activated Raf phosphorylates and activates mitogen-activated protein kinase (MEK), which then activate the extracellular signal-regulated kinase (ERK) [18, 63, 67, 68]. Activated ERK translocates to the nucleus for activating numerous transcription factors, such as c-myc, Ets-1 and c-Jun (Fig. 6), which promote cell cycle
progression, proliferation and differentiation [20, 63, 64, 67, 68].

Figure 6. The Ras signaling pathway. Ras is activated by a growth factor binding to upstream receptor tyrosine kinases (RTK). Grb2, the adaptor protein, recruits Ras-GEFs to the PM, to facilitate Ras GTP binding and activate Ras. Ras activates numerous pathways involved in cell proliferation, differentiation, survival and motility. Figure adapted from [69].

Another well-studied Ras signaling pathway is the Ras/PI3K/Akt/mTOR pathway. Phosphatidyl-inositol-3-kinase (PI3K) is a lipid kinase phosphorylating the 3’-OH group on phosphatidylinositols at the PM [66, 70]. The active Ras-GTP bound form is able to interact with the p110 of PI3K, the catalytic subunit, recruiting it to the PM, which further recruits the p85-regualtory subunit for phosphorylating its target lipids [70, 71]. PI3K also can be activated through Ras-independent mechanisms, such as interacting directly with extracellular growth factor receptor (EGFR) or via adaptor molecules such as insulin receptor substrate (IRS-1) at the
PM [72]. Then, PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP_3) [66, 71]. Akt is then recruited to the PM and binds to PIP_3 via the pleckstrin homology (PH) domain, which activates Akt [66, 71]. Akt then translocates to the cytosol and activates the kinase mammalian target of rapamycin (mTOR) [66]. K-Ras has shown to be a better recruiter of Raf to the PM, thereby a more potent activator of the MAPK pathway, while H-Ras is a more potent activator of the PI3K pathway [73]. This seems to stem from the differences in how H-Ras and K-Ras attach to the PM or the differences in the structure and sequences of their HVR [73]. The PI3K/Akt/mTOR pathway (Fig. 6) promotes cell survival by inhibiting Bad and BAX, pro-apoptotic proteins, and by upregulating protein synthesis, phosphorylating eukaryotic initiation factor 4E and ribosomal S6 protein [71, 74-76]. In addition, PI3K can bind to the PH domain of 3’-phosphoinositide-dependent kinase 1 (PDK1) (Fig. 6), recruiting it to the PM and activating serine/threonine kinases including protein kinase C (PKC) isoforms and p70 ribosomal protein S6 kinase (p70S6K), a kinase that is involved in translating the mRNA that codes for ribosomal proteins and elongation factors [66, 71, 77-80]. Lastly, PI3K can activate Ras-related C3 botulinum toxin substrate (Rac) (Fig. 6), by stimulating Rac-GEFs activity [20, 71, 81, 82]. Rac is involved in cytoskeletal assembly and actin remodeling, which can promote cell motility, migration and invasion [71, 83].

Ras has also been shown to activate Ras-like (Ral)-specific GEF (Ral-GDS) (Fig. 6), which activates Ral, a small GTPase part of the Ras family [20, 84]. Ral’s effector proteins include Ral binding protein 1 (RalBP1), which is a Rac-GAP, and Sec5/Exo84 proteins involved in exocytosis [84, 85]. Ral also activates phospholipase D1 (PLD1), which is involved in vesicle
trafficking and transport, and conversion of phosphatidylcholine (PC) to phosphatidic acid (PA) [20, 86].

Ras signaling is also proposed to be involved in Ca\(^{2+}\) signaling via activation of PLC\(\varepsilon\) (Fig. 6). PLC\(\varepsilon\), an isoform of phospholipase C, has been shown to be an effector protein of Ras, having both GEF and Ras-binding domains, and it cleaves PIP\(_2\) in the membrane into secondary messengers’ diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)) [87, 88]. DAG and IP\(_3\) lead to increase in intracellular Ca\(^{2+}\) and PKC activation, which are involved in cell proliferation and differentiation [20, 88, 89].

1.1.5 Ras in Cancer

As mentioned previously, constitutively active Ras mutants stem from a single amino acid substitution at residues glycine 12 (G12), glycine 13 (G13) and glutamine 61 (Q61) [18, 21, 24, 25, 27]. These mutations impair intrinsic or GAP-mediated Ras ability for GTP hydrolysis [33, 34, 90].

Q61 mutants impairs intrinsic RasGTPase hydrolysis activity. The glutamine at position 61 has an amide group side chain that forms hydrogen bonds with the GAP arginine finger (R789), stabilizing the active site, and its carbonyl side chain hydrogen bonds with the water molecule [32-34, 90]. Mutating the glutamine to an arginine (Q61R) or leucine (Q61L) not only impairs the alignment of the water molecule to the active site, it is also unable to stabilize the active site, resulting in reduced GTP hydrolysis [33, 90].

G12 and G13 mutants prevent the ability of Ras to interact with the GAP. Through van der Waals interactions, the R789 of the GAP interacts with the C\(\alpha\) atom of G12 [90]. Mutating the glycine to a valine (G12V), aspartate (G12D) or cysteine (G12C) increases the size of the amino
acid at this residue, causing them to take up the space that is meant for the R789 of the GAP [33, 90]. The oncogenic G13 mutant, G13D, also behaves in a similar way, creating steric hinderance to prevent interaction with the GAP [33, 90]. Since R789 cannot access the GTP, the GAP does not bind and the process of GTP-hydrolysis is delayed.

There are 19 different activating codon mutations at 12, 13, or 61 that can be found in each isoform [91]. While \(~70\%\) of Ras mutants in cancer contain the following five codon changes: G12D, G12V, G12C, G13D and Q61R, each Ras isoform does exhibit frequencies toward a specific codon switch (Fig. 7) [91].

![Figure 7. The frequency of codon changes found in Ras isoforms.](image)

K-Ras codon changes are mostly G12D, G12V and G12C, followed by G13D and G12S [90]. H-Ras mutants occur more evenly with \(~38\%\) at codon 61, \(~26\%\) at codon 12 and \(~23\%\) at codon 13 (Fig. 7) [90, 91]. H-Ras codon changes are mostly G12V, followed by Q61R and Q61L [90]. N-Ras mutants occur predominantly at codon 61 (\(~62\%\)) followed by codon 12.
(~23%) and codon 13 (~11%) (Fig. 7) [90, 91]. N-Ras codon changes are mostly Q61R and Q61K, followed by G12D and G13D [90].

Constitutively active mutations in Ras occur in ~19% of all human cancer [13, 24, 91]. Out of all the constitutively active Ras mutations, ~75% of them are K-Ras mutations (Fig. 8) [91]. N-Ras mutants make up ~17% and H-Ras makes up ~7% of the remaining constitutively active Ras mutations in cancer (Fig. 8) [91]. Oncogenic K-Ras mutations frequently occur in pancreatic (~88%), colorectal (~50%) and lung cancer (~30%) (Fig. 8) [13, 91]. K-RasG12C mutants are more frequent in lung cancer while K-Ras G12V and G12D are found more in pancreatic and colorectal (Fig. 8) [90]. Oncogenic mutations of H-Ras frequently occur in head and neck squamous cell (~86%) and bladder cancer (~57%) (Fig. 8) [18, 90]. H-Ras G12V mutants are found more in bladder cancer while Q61L are found more in head and neck squamous cell (Fig. 8) [90]. Oncogenic mutations of N-Ras frequently occur in skin melanoma (~94%) and leukemia (~59%) (Fig. 8) [18]. N-Ras Q61K and Q61R are found more in skin melanoma while
G12V and G12D more found in leukemia (Fig. 8) [90].

![Ras Mutations Diagram](image)

**Figure 8. Ras mutations in cancer.** Constitutively active mutations of Ras occur predominantly in K-Ras, followed by N-Ras and H-Ras. The presence of oncogenic mutations, and the specific activating mutation, of K-Ras, N-Ras and H-Ras vary among different cancer tissues.

Cancer cells undergo “oncogene addiction”, where they become dependent on a single mutated gene for continued growth and survival, despite accumulating other defects [92-94]. This stems from the re-wiring cancer cells undergo that differs from normal cells, focusing on pathways that emphasize growth [92]. When oncogenic mutant K-Ras is knocked down in a range of cancer cells expressing oncogenic mutant K-Ras, their growth and signaling was inhibited, revealing a dependency on K-Ras signaling [95]. Thus, cancer cells harboring oncogenic mutant K-Ras undergo a “K-Ras addiction”, where they become dependent on K-Ras...
signaling for survival (i.e., K-Ras-driven cancers) [13, 92, 95-98]. Therefore, inhibiting K-Ras activity would serve as an attractive method to treat K-Ras-driven cancer.

1.1.6 Current Approaches for Targeting K-Ras

1.1.6.1 Direct Inhibitors

Many approaches are being used to target K-Ras activity in cancer (summarized in Fig. 12). In the past, direct inhibition of K-Ras has been deemed impossible due to the picomolar affinity Ras has for GTP and the high concentration of GTP that exists within the cell, ~0.5 mM [18, 22, 24-26]. SCH-53239 and SCH-54292 bind to the hydrophobic pocket near the switch II region and compete with GDP for nucleotide binding to Ras [18, 99, 100]. While the compounds inhibited Ras-dependent cell growth, they lacked potency and contained a hydroxylamine, which had high toxicity [18]. Additionally, K-Ras does not have other deep hydrophobic pockets, making high-affinity allosteric inhibitors ineffective [18, 99].

Other ways to directly target Ras would be to prevent Ras interaction with GEFs or prevent Ras-GTP interaction with Raf. DCAI binds to the pocket in the α2 helix and β sheet of GDP-bound K-Ras, blocking Ras-SOS interaction and inhibiting Ras activation [101, 102]. However, DCAI lacked potency and weakly bound to K-Ras and despite improvements made with Bay-293, it is unclear if these inhibitors would serve as beneficial for mutated Ras constitutively bound to GTP [18, 24]. Kobe 0065 and Kobe 2602 bind to a site on the switch II region, preventing the interaction with Raf, thereby blocking the MAPK-induced proliferation and the growth of H-Ras-driven cancer cell lines [18, 24, 103]. Similarly, neither compound is potent enough for clinical use and contained a toxic thiosemicarbazide scaffold [18].
There have been FDA-approved inhibitors for the K-RasG12C mutation, sotorasib (AMG-510) and adagrasib (MRTX849) (Fig. 9). Initially, a screening was performed by Shokat and colleagues to identify molecules that tether to the GDP-bound-K-RasG12C [104]. In addition to containing reactive thiol groups, the mutant cysteine at position 12 resides near both the nucleotide binding pocket as well as the switch regions where effectors bind [104]. They discovered compounds that extends from the Cys12 to the switch II-pocket (S-IIP), a newly discovered allosteric pocket near the switch II region between α2 and α3 helices [26, 104]. S-IIP is only accessible in the GDP-bound form of K-RasG12C [104, 105]. Additionally, the compounds selectively and irreversibly bind to the cysteine of K-RasG12C [18, 26, 104]. The compounds exhibited a preference for GDP over GTP, diminished interactions with effectors, and decreased viability of K-RasG12C-driven cancer cell lines over other K-Ras-driven cancers [26, 104]. These compounds were modified for increased potency and for use in vivo applications, ARS-1620 and ARS-853 [26, 99]. ARS-853 binds only to GDP-bound-K-RasG12C and locks it in the inactive state, thus preventing GTP-binding and reducing signaling in K-RasG12C-mutant cancer cell lines [26, 106]. In addition to S-IIP binding, ARS-1620 forms an interaction with the histidine 95 (His95) to form a more stable and favored interaction with K-RasG12C, and has increased potency [26, 105]. The discovery of this His95 interaction was exploited to further optimize the compound, leading to the discovery of sotorasib, which selectively and irreversibly binds to the Cys12 in K-RasG12C, locking it in the inactive state [99]. In addition to the His95, Tyr96 and Gln99 formed the binding pocket of sotorasib, causing increased potency and selectivity [24, 26, 99, 107, 108]. In 2021, sotorasib was FDA-approved for non-small cell lung cancer (NSCLC)-patients with a K-RasG12C mutant, becoming the first
FDA-approved K-Ras inhibitor (Fig. 9A) [26, 109]. Adagrasib (MRTX849) was FDA-approved for NSCLC as well (Fig. 9B) [110, 111].

Despite the existence of these inhibitors, the K-RasG12C oncogenic mutant is found only in a small fraction of K-Ras-driven cancers; ~3% of pancreatic, ~4 of colorectal and ~13% of NSCLC that harbor oncogenic mutations in K-Ras, leaving them ineffective against other K-Ras mutations [13]. Additionally, NSCLC patients have started to exhibit resistance to sotorasib and adagrasib, due to acquired mutations in the compound-binding pocket, acquiring additional K-Ras activating mutations or by-passing activation of the MAPK or PI3K pathway [110, 112, 113]. Thus, inhibitors for pan-oncogenic mutant K-Ras would have great clinical utility.

**Figure 9. K-RasG12C inhibitors.** The chemical structures for the K-RasG12C inhibitors that have been FDA-approved for NSCLC patients: (A) Sotorasib, figure adapted from [99], and (B) Adagrasib, figure adapted from [111].

### 1.1.6.2 Targeting Downstream Effectors
Another approach is to inhibit the downstream signaling effectors of Ras (Fig. 12). In the Raf/MEK/ERK pathway, Raf is the most important effector for oncogenic mutant Ras-dependent cancer growth [18, 114, 115]. The first Raf inhibitor approved was sorafenib; however, it functions by blocking the activity of tyrosine kinases in general, making it less specific to Raf [18, 66, 116, 117]. Vemurafenib and dabrafenib were approved for B-Raf-driven metastatic melanoma, but in K-Ras- or N-Ras-driven cancer cells, they paradoxically increased the MAPK signaling through hyperactivation of C-Raf [24, 118-120]. Inhibition of C-Raf or co-inhibiting it with EGFR has shown success in patient-derived xenograft models with K-Ras mutations [24, 121]. Similarly, trametinib, a MEK inhibitor, is approved for B-Raf-driven metastatic melanoma, but has little effect on K-Ras-driven cancers [18, 24, 66, 122]. For treatment of K-Ras driven cancers, MEK inhibitors are often used in combination with other inhibitors [18, 24, 66]. ERK inhibitors, MK-8353 and AZD0364, are currently being evaluated for B-Raf-or/K-Ras-driven cancers [18, 24, 123]. However, using multiple inhibitors in combination with each other seems to be the most effective way to combat resistance [18]. Additionally, since Ras promotes multiple pathways, inhibition of the MAPK pathway can be compensated by other pathways [3, 5, 11, 35, 37].

As previously mentioned, K-Ras is a stronger activator of the MAPK pathway over the PI3K/Akt/mTOR [73]. Furthermore, inhibiting PI3K activity in K-Ras-driven cancers did not impact their growth, suggesting inhibition of the PI3K/Akt pathway alone may not be enough to block K-Ras-driven cancer growth [18, 124, 125]. Thus, the combination of inhibitors for the MAPK and PI3K pathways have been under evaluation. Co-treatment of a MEK inhibitor with pan-PI3K and mTOR inhibitors slowed the growth of K-Ras-driven lung cancers, and co-treatment of K-RasG12C inhibitor and PI3K inhibitors was effective against K-RasG12C
NSCLCs in vivo and in vitro [24, 124, 126]. However, these combinational therapy treatments caused high toxicity and limited efficiency during clinical trials, limiting their clinical use [24, 25, 127-129].

Silencing Ral and Rac have shown to inhibit the growth of K-Ras-driven pancreatic and lung cancer cell lines [130, 131]. However, given that they are small GTPases themselves, it is unclear if direct inhibitors can be made or if they need to be targeted indirectly through upstream kinases or downstream effectors [18].

Synthetic lethality is an approach to target genes whose loss would be lethal only in the presence of a certain driver oncogene, in this case, oncogenic mutant Ras (Fig. 12) [18, 25]. A successful synthetic lethality is the identification of PARP inhibitors to treat BRCA-mutant breast cancers [18, 25, 132]. A genome wide RNA-interference (RNAi) screen in human cancer cell lines has identified genes important for K-Ras-driven cancer survival; however, there was a lack of overlap and the inhibitors for the identified genes were not effective against K-Ras-driven cancers [18, 25, 132, 133]. Thus, an improved screening approach must be taken, such as using CRISPR-Cas9 system libraries for better screening [18, 134].

1.1.6.3 Targeting PM Association

Another approach is to inhibit Ras association with the PM (Fig. 12), since PM localization is essential for Ras proteins to recruit and activate their effectors [13, 23, 24, 26].

Farnesyltransferase Inhibitors (FTIs)

Studies have reported that mutations in the CAAX motif block Ras post-translational modifications, preventing it from interacting with the PM and thus, blocking its activity [135]. The first attempt was farnesyltransferase inhibitors (FTIs), which block the addition of the
farnesyl moiety to Ras, which is necessary for membrane binding and subsequent biological activity [136, 137]. Some FTIs showed success in vitro and in vivo, with some advancing into phase I and II clinical trials [1, 13, 138-141]. However, FTIs were only effective against H-Ras-driven cancers and had no effect on K-Ras- or N-Ras-driven cancers [13]. It is because in the presence of FTIs, K-Ras and N-Ras are able to undergo alternative prenylation, geranylgeranylation, the addition of a 20-carbon fatty chain, by geranylgeranyltransferase (GGTase), allowing it to bypass the farnesylation and still interact with the PM [1, 28, 138, 142]. Inhibition of Rce-1 and Icmt, enzymes involved in CAAX post-translational modification, is cytotoxic since they modify other essential proteins in the cell [18].

Depleting PtdSer from the PM

Phosphatidylserine (PtdSer) is an anionic phospholipid that is concentrated on the inner leaflet of the PM [143, 144]. In addition to being involved in apoptosis and the recruitment of proteins to the PM, PtdSer in the inner PM leaflet generates the negative electrostatic potential of the PM [143-145]. The positively-charged PBD of K-Ras forms a strong electrostatic interaction, specifically with the negatively-charged head group of PtdSer, and this, in conjugation with the farnesyl moiety, localizes K-Ras to the PM [145, 146]. Studies have shown that depleting the PtdSer content from the PM, weakens the electrostatic interaction, resulting in dissociation of K-Ras from the PM and disrupted K-Ras signaling [23, 147, 148]. One way is to disrupt proteins involved in the PtdSer/phosphatidylinositol 4-phosphate (PI4P) exchange at the PM/ER that regulate PtdSer abundance at the PM (Fig. 10) [13, 149]. PI is phosphorylated by PI 4-kinases into PI4P, and PI4P at the PM is exchanged for PtdSer synthesized in the ER by oxysterol-
binding protein-related proteins 5 and 8 (ORP5/8) (Fig. 10) [150-153].

Figure 10. PtdSer PM enrichment is regulated by ORP5 and 8. ORP5 and 8 are lipid transporters that exchange ER PtdSer with PM PI4P. PI4P levels at the PM are kept high by PI 4-kinases (PI4KA) and PI4P levels at the ER are kept low by Sac1. This establishes a PI4P concentration gradient, which drives the PI4P/PtdSer exchange by ORP5 and 8. Figure adapted from [13].

Silencing PM PI 4-kinases or ORP5 or 8 depletes PM PtdSer contents, resulting in K-Ras dissociation from the PM [154, 155]. Another way is to disrupt recycling endosome (RE) activity because the RE can return endocytosed PtdSer back to the PM [13, 25, 143]. Acylpeptide hydrolase (APEH) is a cytosolic enzyme that removes N-terminal acylated amino acids from acetylated proteins and regulates ubiquitin-mediated degradation [156]. The knockdown of APEH impairs endocytic recycling and both PtdSer and K-Ras were mis-localized from the PM.
(Fig. 11), suggesting that PtdSer abundance at the PM is maintained by the RE [148, 157].

**Figure 11. Mechanisms to disrupt K-Ras association with the PM.** Inhibition of APEH perturbs RE activity, resulting in the mis-localization of both PtdSer and K-Ras from the PM. Phosphorylation of K-Ras at Ser181 weakens the electrostatic interaction K-Ras has with the PM, resulting in its dissociation. The metabolism and distribution of sphingomyelin (SM) and ceramide (Cer) regulates PtdSer content at the PM and inhibiting sphingomyelinases (ASM) mis-localizes PtdSer and K-Ras from the PM. Inhibition of PDE6δ (Deltarasin) results in K-Ras mis-localization from the PM. Figure adapted from [25].

Lastly, PtdSer can be depleted from the PM through disrupting sphingomyelin (SM)/ceramide balance (Fig. 11) [13, 25]. Ceramide, from the ER, gets converted to SM in the Golgi complex, and SM traffics to the PM and lysosome, where sphingomyelinases convert it to ceramide [158].
Inhibiting acid or neutral sphingomyelinases (ASM and NSM, respectively), or by disrupting their activity (Fig. 11), has shown to reduce PtdSer content at the PM, leading to dissociation of K-Ras and reduced K-Ras signaling [147, 148, 159-161].

**Inhibiting K-Ras interaction with PDE6δ**

As mentioned previously, PDE6δ is a chaperone protein involved in K-Ras trafficking to the PM and disrupting this interaction results in K-Ras dissociation from the PM [46, 54]. Deltarasin, a small molecule binding to the hydrophobic pocket of PDE6δ, prevents the binding of the farnesyl tail of K-Ras (Figs. 5 and 11) [57]. Deltarasin, and other PDE6δ-inhibitors, mis-localize K-Ras from the PM and inhibit the signaling and growth of K-Ras-driven cancer cell lines [57, 58, 162, 163]. New PDE6δ inhibitors, with more hydrogen bonds for tighter binding, are more potent for blocking the growth of K-Ras-dependent, but not -independent, cancer cell lines [162, 163]. In addition to K-Ras, deltarasin elevates cellular autophagy and co-treatment with an autophagy inhibitor leads to cell death through elevation of cellular reactive oxygen species (ROS) [164]. While PDE6δ-inhibitors are attractable compounds to target K-Ras, their specificity still needs to be determined because, PDE6δ binds to other farnesylated proteins, like H-Ras and N-Ras.

**K-Ras Phosphorylation at Ser181**

K-Ras contains a serine at residue 181 (Ser181), directly adjacent to the PBD. Ser181 is able to be phosphorylated by protein kinase C (PKC) and protein kinase G (PKG) [165, 166]. K-Ras phosphorylation at Ser181 disrupts the electrostatic interaction of K-Ras with the PM, resulting in K-Ras dissociation and reduced signaling (Fig. 11) [25, 165-168]. Bryostatin-1, a PKC agonist, was shown to translocate K-Ras from the PM to the ER, Golgi and mitochondria
via Ser181 phosphorylation and when present at the mitochondria, the translocated K-Ras can induce apoptosis via its interaction with BcL-XI, a pro-apoptotic protein [165]. In addition, bryostatin-1 can inhibit the growth of oncogenic mutant K-Ras tumors in mice [165]. Similarly, a chalcone derivative dissociates K-Ras from the PM, inhibits oncogenic mutant K-Ras signaling and K-Ras-driven cancer growth via PKC phosphorylation of Ser181 [167]. PKG is activated through AMPK activation of endothelial nitric oxide synthase (eNOS) signaling and activated PKG dissociates K-Ras from the PM and inhibits the growth of K-Ras-driven cancers [166]. Thus, compounds that reduce K-Ras signaling through Ser181 phosphorylation would be attractive for treating human K-Ras-driven cancers [166-168].

1.1.6.4 Metabolism

Given their unregulated proliferation, cancer cells alter their metabolism to increase their nutrient uptake and sustain their growth [169, 170]. Cancer cells, compared to normal cells, have an increased glucose uptake and lactate production, even in the presence of oxygen [18, 169]. This is known as the Warburg effect, which is one of the hallmarks of cancer [18, 94, 169]. Additionally, cancer cells use anabolic processes to provide the macromolecules, like amino acids and lipids that are required for their growth [18, 170, 171]. Oncogenic mutations of K-Ras are capable of driving this metabolic rewiring, but the exact processes depend on the specific tumor type [18, 25, 26]. Oncogenic mutant K-Ras upregulates expression of the GLUT1 transporter and hexokinase 1 and 2 to increase glucose uptake and glycolytic activity [26, 172]. It also promotes pathways involved in nucleic acid synthesis and lipid metabolism [26, 97, 98, 173, 174]. In pancreatic cancer, K-Ras increases the metabolism of glutamine, to generate pyruvate and NADPH, which is necessary to maintain reduced glutathione levels and keep ROS levels low [18]. Additionally, constitutively active K-Ras/MAPK signaling promotes autophagy and
macropinocytosis to meet their metabolic needs [18, 26, 175-177]. Autophagy occurs when intracellular substrates are sequestered in autophagosomes before entering the lysosome where they are broken down and recycled back to the cytoplasm [98, 178]. Macropinocytosis is a process, where extracellular molecules are engulfed by the PM and then brought to the lysosome for degradation [179].

Given the dependence on Ras signaling for the growth of tumor cells, but not normal cells, targeting metabolic pathways in tumor cells is an attractive target (Fig. 12) [18]. Co-treatment of autophagy inhibitors with the MAPK inhibitors blocked pancreatic tumor growth [24, 25, 176]. Chloroquine and hydroxychloroquine inhibit autophagy by inhibiting lysosomal acidification and are currently undergoing clinical trials for co-treatment with MEK inhibitors [176, 177, 180, 181]. Inhibition of enzymes involved in glycolysis, like hexokinase 2 and lactate dehydrogenase, have shown to inhibit pancreatic cancer growth in vitro and in xenografts [97, 182, 183]. Inhibitors of glutaminase, an enzyme involved in the glutamine metabolism, elevate cellular ROS levels to inhibit pancreatic cancer growth and are in early phases of clinical trials [18, 98, 184].
Figure 12. Current approaches for targeting oncogenic mutant K-Ras. A summary of approaches that are currently being used to target oncogenic mutant K-Ras: (I) directly inhibiting K-Ras or disrupting its interaction with Ras-GEFs, (II) inhibiting its association with the PM, (III) inhibiting the activity of its downstream effectors, (IV) inhibiting genes that would be synthetically lethal in the presence of oncogenic mutant K-Ras, and (V) inhibition of proteins involved in metabolism. Figure adapted from [125] and created using BioRender.com.

1.2 Reactive Oxygen Species

1.2.1 Generation of Reactive Oxygen Species

Reactive oxygen species (ROS) are a broad range of molecules derived from oxygen [185]. The reduction of oxygen into ROS was first discovered in the 1950s by Gerschmann and Denham Harman [186-188]. All ROS possess oxidizing abilities but they can be organized into two groups: radicals, containing unpaired electrons, and non-radicals, with no unpaired electrons [189-191]. Radicals include superoxide anion (O$_2^-$) and hydroxyl radical (OH•), while non-
radicals include hydrogen peroxide (H$_2$O$_2$) (Table 1) [185, 189]. Given that O$_2^-$ is formed after one electron transfer from oxygen, it is known as a primary ROS, which further gets broken down directly or indirectly through enzyme or metal-catalyzed reactions [192, 193]. OH• has a high reactivity, more than O$_2^-$ and H$_2$O$_2$, but has a short half-life [191, 192]. H$_2$O$_2$ is stable and is heavily involved in a variety of signaling pathways [191, 194]. Also grouped into ROS are reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite (ONOO•) (Table 1) [189]. NO is very reactive, readily diffuses across membranes and is more stable in the presence of low oxygen [192].

ROS is produced endogenously, mostly by the mitochondrial electron transport chain (ETC) during aerobic respiration (Table 1) [185, 189, 191, 194]. Located on the inner mitochondrial membrane, the ETC is composed of four complexes, I-IV [185, 189]. To generate ATP, electrons shuffle through the ETC to oxygen, the final electron acceptor [189]. ~1-2% of oxygen used during this process ends up as O$_2^-$, mostly due to leakage from complexes I and III [189, 192, 194, 195]. O$_2^-$ either stays in the intermembrane space or diffuses into the cytosol through the mitochondrial permeability transition pore [189, 191, 196]. Or, O$_2^-$ can be reduced to H$_2$O$_2$ by superoxide dismutase (SOD) in either the cytosol or mitochondria (Table 1) [189, 191]. In the ER, redox reactions are involved in protein folding and maturation, leading to the reduction of oxygen to H$_2$O$_2$ (Table 1) [185, 191, 197]. Additionally, peroxisomes are known to be major producers of H$_2$O$_2$ (Table 1) [192, 194, 198].

NADPH oxidases (NOXs) are a family of membrane-bound enzymes that are known to produce ROS [189, 191, 194, 199]. NOX uses NADPH to transfer an electron to oxygen, generating O$_2^-$ (Table 1) [191, 194, 200]. Initially known to be present in phagocytic cells for host defense, the NOX enzymes are ubiquitously expressed and are activated in response to a
variety of signals, such as growth factors [192, 201-204]. Nitric oxide synthases (NOS) are enzymes that generate NO (Table 1), through the single electron reduction of oxygen and addition of nitrogen [185, 205]. Xanthine oxidase is involved in the breakdown of purine nucleic acids and during so, reduces oxygen into O$_2^-$ and H$_2$O$_2$ (Table 1) [185, 192, 194, 206]. Cytochrome P450, an enzyme involved in the metabolism of xenobiotics and fatty acid biosynthesis, generates O$_2^-$ and H$_2$O$_2$ (Table 1) [185, 192, 194, 207]. The Fenton reaction, a metal-catalyzed reaction, is one of the biggest generators of OH• (Table 1) [192, 208, 209]. Iron in the +2 oxidation state (Fe$^{2+}$) reacts with H$_2$O$_2$ to form Fe$^{3+}$, OH• and hydroxide ion (OH$^-$) [192, 209, 210]. In addition to iron, copper, chromium or cobalt have been suggested to serve as the metal used in this reaction [192]. ROS can also be exogenous through drugs, UV radiation or exposure to toxins [186, 211].

| Table 1. The types of ROS molecules and where in the cell they are generated. |
|------------------------|--------------------------------------------------------------------------------|
| **Type of ROS**        | **Generation of ROS**                                                            |
| O$_2^-$                | Leakage from ETC in mitochondria during oxidative phosphorylation               |
|                        | Cytochrome P450 enzymes                                                         |
|                        | NOX enzymes                                                                      |
|                        | Xanthine oxidase                                                                |
| H$_2$O$_2$             | Reduced from O$_2^-$ by SOD in cytosol or mitochondria                           |
|                        | Cytochrome P450 enzymes                                                         |
|                        | Generated from peroxisomes and ER                                               |
|                        | Xanthine oxidase                                                                |
| OH•                    | Metal-catalyzed reactions                                                        |
| NO                     | NOS enzymes                                                                      |

1.2.2 Functions of ROS at Different Cellular Levels

When ROS is present at physiological levels, they serve as secondary messengers for signaling pathways [185, 190, 212, 213]. In fact, the Nobel Prize in Physiology or Medicine in 1998 was awarded for the discovery of the role of NO as a signaling molecule for vasodilation
H$_2$O$_2$ is produced in response to a variety of upstream stimulus like cytokines and growth factors [215, 216]. H$_2$O$_2$ can mimic insulin in adipocytes, transporting glucose through the pentose phosphate pathway and enhancing lipid synthesis [217, 218]. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) signaling generates H$_2$O$_2$ which can lead to activation of the PI3K and/or MAPK signaling, and phospholipase-C phosphorylation [215, 219-221]. Additionally, H$_2$O$_2$ reversible oxidizes the SH2 domain on protein tyrosine phosphatases, inhibiting their activity and thus, increasing the MAPK signaling [222]. Similarly, H$_2$O$_2$ and O$_2^-$ can inactivate phosphate and tensin homolog (PTEN) through reversible oxidation of cysteines, leading to increased PI3K signaling [185, 219, 223, 224]. Nuclear factor kappa B (NF-κB) can be activated by tumor necrosis factor (TNF) and interleukin-1 (IL-1) through the production of H$_2$O$_2$ [213, 219, 225, 226]. H$_2$O$_2$ also leads to DNA binding of activator protein 1 (AP-1), a transcription factor involved in cell growth and proliferation [213, 227].

When ROS levels increase past physiological levels, they are no longer beneficial to the cell but rather, become toxic and cause cellular damage. In both nuclear and mitochondrial DNA, ROS can lead to the addition of a hydroxyl radical to the C8 position on the guanine ring, converting to 8-oxoguanine (8-oxo-guanine) [228, 229]. Guanine has a low oxidation potential, making it the most susceptible base for oxidation [229]. 8-oxo-guanine leads to mis-match pairing and transversions [228-231]. If oxidized bases are unable to be repaired by base excision repair, double-strand DNA breaks can be generated and genomic instability occurs [231, 232]. Additional, double-strand breaks can occur from hydroxyl radicals damaging the sugar backbone of DNA [231]. ROS induces lipid peroxidation of polyunsaturated fatty acids (PUFAs) in lipid membranes, which increases membrane fluidity and permeability [189, 228, 233, 234]. PUFAs oxidize to lipoperoxyl radical (LOO$^-$), which gets further oxidized to the unstable, lipid
hydroperoxide (LOOH) [189, 235, 236]. LOOHs lead to the generation of new radicals and breakdown into by-products that become secondary messengers [189, 235, 236]. The most reactive by-product, 4-hydroxynonenal (HNE), can covalently modify other macromolecules to impact gene expression, cell proliferation and apoptosis [189, 234, 235]. ROS, such as OH•, oxidize the polypeptide backbone of proteins, cleaving the peptide bond and resulting in fragmentation [237]. Additionally, ROS can oxidize certain amino acid residues on proteins, which alter their structure and disturb their functions (Fig. 13) [189, 194, 228, 238]. Cysteine (Cys) is perhaps the most reactive amino acid, due to its thiol (-SH) group [238]. Cys initially gets oxidized to sulfenic acid, which is reversible, and further oxidized to other products [239, 240]. Irreversible modifications include s-sulfenation by H2O2 leading to protein damage and subsequent degradation (Fig. 13A) [239, 240]. Reversible modifications include S-nitrosylation by NO, s-glutathionylation by glutathione, and disulfide bonds with a Cys of another protein.
(Fig. 13A), all of which are known to negatively affect protein function [237-240].

Figure 13. Examples of oxidation of amino acids by ROS. (A) Cysteine undergoes irreversible modifications by s-sulfenation, or reversible modifications by s-nitrosylation or disulfide bond formation. (B) Methionine gets oxidized to methionine sulfoxide. (C) Histidine gets oxidized to 2-oxo-histidine. (D) Tyrosine gets oxidized irreversible to nitrotyrosine. (E) Tryptophan and (F) phenylalanine get oxidized to form hydroxyderivatives of themselves. Figures adapted from [240, 241].

Methionine (Met) is modified, similarly to Cys, in that its thioester group gets reversibly oxidized to methionine sulfoxide (MeSOX) (Fig. 13B) [237, 238, 240, 242]. Histidine (His) gets oxidized to 2-oxo-histidine (Fig. 13C) by hydroxyl radicals through a metal-catalyzed reaction or by NHE, lipid peroxidation by-product [237, 238, 240]. Tyrosine can be irreversible modified into 3-nitrotyrosine by NO or can form tyrosine-tyrosine crosslink (Fig. 13D) [238-240].

Tryptophan (Trp) and phenylalanine (Phe) get oxidized by hydroxyl radicals, leading to the formation of hydroxyderivatives of Trp and Phe (Figs. 13E and F) [237, 238, 240]. All amino acids, but Lysine, Arginine, Proline and Threonine with greater sensitivity, undergo
carbonylation, where ketones or aldehydes are introduced into the protein, causing detrimental effects on the protein [237-240].

1.2.3 Antioxidants

In order to prevent the cellular damage caused by toxic levels of ROS, cells have antioxidant proteins and enzymes to keep ROS levels in check. Glutathione is perhaps the most abundant antioxidant protein, with intracellular concentrations ranging from 1-10 mM [243-245]. Glutathione exists in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG), with GSH being the predominant form [189, 246, 247]. GSH, containing a thiol group, serves as a scavenger, directly interacting with ROS and RNS [246-248]. Most of the GSH in the cell functions as co-factor for the redox enzymes glutathione peroxidases (GPxs), which detoxifies H₂O₂ into water (Fig. 14) [189, 247, 249, 250]. During the process, GSH gets oxidized to GSSG (Fig. 14), which can be harmful to the cell [251, 252]. Glutathione reductase (GRx), along with NADPH co-factor, converts GSH back to its reduced active state (Fig. 14) [189, 247, 253].

Figure 14. Cellular antioxidant systems. O₂⁻ gets broken down by SOD enzymes to create H₂O₂. H₂O₂ gets broken down into water by catalase or GPx and GSH. GRx, with NADPH co-factor, restores GSSG to its active form, GSH. Trx removes disulfide bonds of oxidized proteins (P-S-S-P), restoring their structure (P-SH). TrxR, with NADPH co-factor, restores oxidized Trx (Trxo) to its active form (Trxred).
Thioredoxin (Trx) is another antioxidant protein with two reactive cysteines in the active site [254-256]. Trx restores oxidatively modified proteins back to their active, normal state (Fig. 14) [257, 258]. The two cysteines of Trx are involved in breaking the disulfide bonds of oxidized proteins, restoring them to their active state [255-257]. However, Trx gets oxidized in the process and forms a disulfide bond [255]. Thioredoxin reductase (TrxR) is the redox enzyme that, along with NADPH, reduces Trx to its active form (Fig. 14) [245, 257, 259]. There are two isoforms of Trx/TrxR: Trx1/TrxR1 exists in the cytosol while Trx2/TrxR2 exist in mitochondria [255, 259]. As mentioned previously, SOD enzymes convert O$_2^-$ into H$_2$O$_2$ (Fig. 14) [260, 261]. SOD enzymes differ based on their localization and the metal needed to catalyze the reaction. SOD1 or Cu/Zn SOD is localized to the cytosol and mitochondrial intermembrane space and uses copper or zinc as a catalysis [185, 191, 262, 263]. SOD2 or MnSOD is localized to the mitochondria matrix and uses manganese as a catalysis [185, 191, 263]. SOD3 is present in the extracellular matrix and also uses copper/zinc as a catalysis [191, 263]. Catalase is an enzyme localized to the cytosol and peroxisomes, that converts H$_2$O$_2$ into water (Fig. 14) [264-266]. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor involved in modulating antioxidant gene expression [244]. NRF2 is localized to the cytosol where it interacts with Kelch-like ECH-associated protein 1 (KEAP1), a E3 ubiquitin ligase that targets NRF2 for proteasomal degradation [267, 268]. In response to oxidative stress, NRF2 dissociates from KEAP1, translocates to the nucleus and binds to the antioxidant-response element (ARE) of genes to promote transcription of antioxidant proteins such as the ones previously mentioned [185, 244, 269].
Exogenous antioxidants, such as N-acetylcysteine (NAC), vitamin E (alpha-tocopherol) and vitamin C (ascorbate) are often taken as supplements [270]. NAC is thought to serve as a direct scavenger of ROS due its thiol group, serve as a source of cysteine for GSH biosynthesis, and breaks down disulfide bonds [271]. Vitamin E can prevent lipid oxidation and serve as scavenger for radicals, while Vitamin C serves as a scavenger for ROS and peroxyl radicals generated by lipid peroxidation and enhancing the effects of vitamin E [272, 273].

1.2.4 Role of ROS in Cancer Development

As mentioned previously, ROS at physiological levels serve as signaling molecules and are kept in check by cellular antioxidants (Fig. 15). However, if ROS levels increase past the antioxidant capacity, they can promote tumorigenesis (Fig. 15) [18, 185, 186, 274, 275]. Increased ROS levels can damage DNA, resulting in mutations that lead to inactivation of tumor suppressor genes, like p53, or activation of oncogenes, such as FLT3-ITD in leukemia [194, 276-278]. Additionally, ROS can impair proteins involved in DNA repair [194, 276]. ROS promote cellular proliferation by inhibiting PTEN or MAPK phosphatases, leading to increased MAPK and PI3K/Akt/mTOR signaling [223, 244, 266, 279]. Also, ROS increases the mRNA level of cyclins involved in the progression of G1 to S phase, promoting cell cycle progression [266, 280]. ROS further promote cell survival by activating NRF2 and NFκB, leading to upregulation of antioxidant proteins and anti-apoptotic proteins like BCL2 [244, 266, 269]. Angiogenesis is the process by which new blood vessels are formed in order to provide the tumor with the nutrients and oxygen it needs for their growth [186, 244, 266]. ROS, specifically H2O2, stabilizes hypoxia-inducible factor-α (HIF-1α), which is the upstream activator of vascular endothelial growth factor (VEGF), the growth factor for angiogenesis [186, 244, 266]. Additionally, ROS-induced activation of PI3K leads to VEGF secretion [186, 244, 266, 281]. Epithelial to
mesenchymal (EMT) is a process cancer cells must undergo to promote invasion and metastasis, and ROS promote the expression of transcription factors involved in EMT, such as Snail, Twist and ZEB1 [186, 266, 276, 282]. Moreover, ROS activate matrix metalloproteinases (MMPs), which degrade the extracellular matrix, allowing tumors cells to migrate [189, 244, 276]. ROS also regulate cytoskeleton remodeling, leading to the formation of invadopodia and cell migration [244, 266, 276]. Lastly, ROS inhibit the maturation and activation of immune cells, preventing them from attacking tumor cells [276, 283].

Figure 15. The effects that different levels of ROS have on cells. In a healthy cell, ROS exists at physiological levels and cells undergo normal growth and survival. In tumor cells, ROS levels are elevated to generate genomic instability and promote growth. However, tumor cells also increase their antioxidant capacity and shift their metabolism to prevent further ROS elevation. However, if ROS levels become too high, they become toxic and induce cell death via apoptosis. Figure adapted from [194].
Compared to normal cells, cancer cells exhibit higher oxidative stress, due to the activation of oncogenes and their altered metabolism [192]. To balance this out, tumor cells upregulate the expression of antioxidant enzymes and proteins to elevate their cellular antioxidant capacity (Fig. 15) [194]. Expression of NRF2 has shown to be elevated in a variety of cancers, including K-Ras-driven cancers, and is associated with chemoresistance [185, 191, 194, 284-286]. Increased expression of SOD enzymes has been observed in breast, stomach and colorectal cancers [191, 194, 287, 288]. Moreover, TrxR, GPx and GRx enzymes have shown to be upregulated in numerous cancers, such as colorectal, breast, lung and liver [289-292]. Additionally, NAC or vitamin E supplementation promotes K-Ras-driven lung cancer metastasis [293, 294]. As described previously, the Warburg effect, or altered metabolism, is another way for tumor cells to keep ROS levels in check [18, 94, 169]. By shifting their metabolism towards glycolysis, they bypass the ETC and prevent the formation of additional ROS molecules (Fig. 15).

However, if cancers cells are unable to maintain the redox balance, an increase of cellular ROS to toxic levels leads to inhibition of tumor growth (Fig. 15) [186, 275]. In contrast to physiological levels, toxic levels of ROS, such as H$_2$O$_2$, can decrease the expression of EGF/EGFR, leading to inhibition of the MAPK and PI3K/Akt signaling pathways and cell proliferation [276, 295]. Toxic ROS levels also induce cell cycle arrest via downregulation of cyclins, cyclin-dependent kinases and cell cycle regulatory molecules, leading to inhibition of tumor growth [275, 276, 296, 297]. Moreover, toxic ROS levels impair the ability of tumor cells to synthesize nucleotides and ATP [276]. ROS has been shown to reverse a specific mutation of p53, restoring its function and leading to p53-induced suppression of growth [276, 298]. Toxic levels of ROS can also stimulate intrinsic apoptosis pathway (Fig. 15). In mitochondria, toxic
levels of ROS oxidize cardiolipin on the inner membrane, causing cytochrome-c to be detached [276]. Cardiolipin moves to the outer membrane to recruit pro-apoptotic proteins Bcl-2 associated X protein (BAX) and Bcl-2-antagonist killer (BAK), leading to the opening of mitochondrial permeability pore and release of cytochrome-c to the cytosol [186, 276, 299]. The released cytochrome-c interacts with apoptotic peptidase activating factor 1 (Apaf-1) and procaspase-3 to form an apoptosome [186, 275, 299]. Then, cleavage of procaspase 9 activates cleavage of caspase-3, resulting in induction of apoptosis [186, 275, 299]. In addition, ROS accumulation in the ER leads to activation of pro-apoptotic C/EBP homologous protein (CHOP), causing mitochondrial-mediated apoptosis [276, 300]. ROS can also induce apoptosis by activating the p38 MAPK and JNK pathways [275, 276, 299]. Another ROS-induced cell death pathway is ferroptosis, a distinct form of cell death that is caspase-independent and stems from iron-dependent ROS production and lipid peroxidation [276, 301, 302].

Tumor cells exhibit increased oxidative stress, when compared to normal cells, due to their oncogenic addicted signaling and metabolic shift [274]. This makes them more sensitive to changes in ROS levels than normal cells [274]. Thus, elevating cellular ROS levels to cytotoxic levels and causing cell death is seen as a mechanism for chemotherapeutic drugs. One approach is through inhibition of antioxidant systems. Inhibition of SOD1 has been shown to decrease the growth of lung cancer and leukemia cell lines [185, 186, 262]. Auranofin, a known rheumatoid arthritis drug, is a TrxR inhibitor that is currently in clinical trials to be repurposed for chronic lymphocytic leukemia [255, 274, 303, 304]. Motexafin gadolinium, a Trx inhibitor, is in clinical III trials for malignant myeloma, non-Hodgkin lymphoma and leukemia cells [186, 299, 305]. Phenethyl isothiocyanate (PETIC) blocked GPx, reducing GSH levels, to target H-Ras-driven ovarian cancer cells [186, 299, 306]. Additionally, buthioninesulfoximine (BSO) and imexon
that reduce GSH levels to induce apoptosis, are used for leukemia cancer and are currently in phase I trials for non-Hodgkin lymphoma [186, 244, 299]. The other approach to elevate cellular ROS levels in cancer cells is to produce ROS directly in the cell. One of the first pro-oxidant drugs was procarbazine, that was approved for glioma and lymphoma in the 1960s [186, 299, 307]. Sulindac, an FDA-approved anti-inflammatory drug, was shown to elevate H$_2$O$_2$ in colon and lung cancers, inducing apoptosis [266, 308]. Arsenic trioxide (ATO) and elesclomol are mitochondrial ETC inhibitors used for leukemia and in clinical trials for melanoma, respectively [244]. Doxorubicin and pirarubicin, who elevate cellular ROS to trigger mitochondrial apoptosis, are used for leukemia, bladder cancer, lymphoma and breast cancer [186, 276, 299, 309, 310]. Combined treatment of ROS-inducing drugs with chemotherapeutic drugs have shown to be more efficient in targeting cancer cells over normal cells [186, 311].

1.3 Ferrocene Derivatives

1.3.1 Discovery of Ferrocene

In 1965, Dr. Barnett Rosenberg discovered that cisplatin, a metal-based drug previously discovered in 1845 by Dr. Michele Peyrone, was able to inhibit cell division [312, 313]. The active component of cisplatin is the platinum, which directly binds to DNA, specifically the N7 position of guanine [312]. This results in the formation of DNA adducts and cross-links, resulting in DNA damage and if left un-repaired, cells undergo apoptosis [312, 314, 315]. In 1978, cisplatin received FDA-approval for bladder and testicular cancers and has since been used to treat a variety of cancers, such as cervical, ovarian and small cell lung cancer [312, 315-317]. ~50\% of all cancer patients receive cisplatin for their treatment [315, 318]. However, the efficiency of cisplatin has decreased due to its toxic side effects and increased resistance to it. Cisplatin exhibits nephrotoxicity, hepatotoxicity, gastrointestinal and neurotoxicity [315, 317,
Cisplatin resistance stems from a variety of mechanisms, such as decreased drug intake, increased drug efflux, increased detoxification of the drug, or increased DNA damage repair [312, 315, 320]. Resistance can be intrinsic or can be acquired after treatment [315, 316, 320]. Additionally, relapse has been shown in small-cell lung cancer patients treated with cisplatin [315, 321]. Carboplatin and oxaliplatin, two cisplatin analogs, have been FDA-approved but do not exhibit significant advantages over cisplatin [315].

Due to the limitations of cisplatin, this began a search for other metal-based drugs that have similar anti-tumor ability but lower toxicity. Metallocenes, or sandwich compounds, are compounds that contain a metal atom between two π-bonded cyclopentadienyl ligands [316, 318]. Metallocenes exhibit great versatility and have been used for a variety of applications, including as anti-tumor agents [312]. In 1979, Köpf and Köpf-Maier discovered titanocene dichloride I (Cp₂TiCl₂), which was the first metallocene to undergo clinical trials for colon, breast and lung cancers [316, 322, 323]. While Cp₂TiCl₂ was less toxic than cisplatin, it had a low response in metastatic cancers and the clinical trials were discontinued [316, 322]. Despite this, other metallocenes, such as ferrocene, were being investigated. Originally discovered in 1951, ferrocene contains an iron atom (Fe) between two π-bonded cyclopentadienyl ligands (Fig. 16) [318, 324, 325]. Ferrocene exhibits low toxicity, stability in water and due to its unique structure, serves as a building block for a variety of molecules [317, 318, 326, 327]. In 1984, Köpf and Köpf-Maier discovered cytotoxic properties of ferrocene-containing compounds [328,
Figure 16. The general mechanism of action for ferrocene derivatives. By itself, ferrocene (Fe$^{2+}$) is insoluble and inactive. Functional groups are added to aid its entry inside the cell. Once inside the cell, ferrocene undergoes a one electron oxidation to generate the active ferrocenium cation (Fe$^{3+}$). This reversible reaction leads to continuous generation of cellular ROS via the Fenton reaction, figure adapted from [330]. The elevated cellular ROS levels cause cellular damage and induce cell death via apoptosis.

1.3.2 Ferrocene’s Mechanism of Action

Ferrocene itself, while stable, is insoluble in water (Fig. 16). Because of this, the cyclopentadienyl ring of ferrocene undergoes the addition of a variety of functional groups, such as picrate, trichloroacetate and polyaspartamide (Fig. 16) [318, 326, 328, 331, 332]. These substituted ferrocene compounds are collectively known as ferrocene salts or ferrocene derivatives. Ferrocene derivatives increase the solubility of ferrocene in water and allow it to enter cell [318, 329]. The entrance of ferrocene derivatives into the cell is essential for its activity since unmodified ferrocene itself is inactive [328, 329]. Once inside the cell, the iron atom in ferrocene undergoes a reversible, one electron oxidation, Fe$^{2+}$ to Fe$^{3+}$ (Fig. 16) [317, 318, 325].
This leads to the formation of the ferrocenium cation, the stable and active form [317, 318]. While the exact mechanism remains unclear, the general mechanism of action for ferrocene derivatives involves the generation of ROS (Fig. 16) [210, 317, 328, 333]. Ferrocene derivatives generate OH• from the Fenton reaction, which as previously mentioned, generates Fe³⁺ and OH• from Fe²⁺ reacting with H₂O₂ (Fig. 16) [210, 318, 326]. Given that the ferrocene/ferrocenium exchange is reversible, this leads to continuous ROS generation [274, 326]. The elevated ROS levels generated by ferrocene can interact with DNA or lipids, resulting in cellular damage (Fig. 16) [210, 317, 318, 333, 334]. The accumulation of this damage leads to the induction of cell death via intrinsic apoptosis in mitochondria (Fig. 16) [210].

1.3.3 Medicinal Effects of Ferrocene Derivatives

Ferrocene salts have shown to exhibit medicinal effects, stemming from their ability to elevate ROS and cause cellular damage. Malaria is mosquito-borne disease caused by plasmodium falciparum (P. falciparum) [335]. The main anti-malarial compound used is chloroquine, due to its low toxicity and cost [336]. However, due to the growing resistance to chloroquine, other drugs are needed [326, 336]. In the 1990s, ferroquine was created, a hybrid chloroquine that contains a ferrocene instead of a methylene group [326, 337]. Given the avidity of P. falciparum for free iron, the addition of ferrocene to chloroquine served as an effective way to target chloroquine-resistance [336]. In fact, ferroquine showed to be active towards both chloroquine-sensitive and -resistant strains of P. falciparum [318, 326]. Ferroquine elevates cellular ROS to inhibit the formation of hemozoin, a pigment produced by P. falciparum to avoid the toxicity of free heme, and cause cell death [338-340]. Ferroquine is still undergoing clinical trials for malaria, after passing phase II [318, 340]. The addition of ferrocene to other known anti-malarial
drugs, like artemisinin, have shown to be effective in combating drug-resistant strains of *P. falciparum* [326, 340].

As mentioned previously, ferrocene derivatives were shown to exhibit cytotoxic effects and have been investigated in a variety of different cancers. In the 1990s, ferrocifen was generated by fusing ferrocene to hydroxytamoxifen, the active metabolite of tamoxifen, (Fig. 17) an FDA-approved selective estrogen modulator for ER+ breast cancer [317, 318, 322]. Thus, ferrocifen functions as both an ROS-inducer and estrogen receptor inhibitor [316-318]. Ferrocifen-, and its derivatives, have shown to be effective in both hormone-dependent or -independent breast cancer cell lines, and are currently in pre-clinical trials [210, 317, 322, 341-343]. Ferrocene attached to steroid groups have shown anti-proliferative activity against colorectal, breast and prostate cancer cell lines [317, 326, 341, 344]. While ferrocene attached to amino groups have shown anti-proliferative effects in osteosarcoma, prostate and leukemia cancer cell lines, ferrocene attached to nucleoside analogs have shown to inhibit the growth of lymphoma and leukemia cancer cell lines [210, 318, 326, 332, 345, 346].

![Chemical structure of Ferrocifen](image)

**Figure 17. Chemical structure of Ferrocifen.** Ferrocifen contains a ferrocene moiety and hydroxytamoxifen, the active metabolite of tamoxifen, an FDA-approved selective estrogen modulator, and is currently in clinical trials for breast cancer. Figure adapted from [341].
2. **Project Objective**

2.1 **Role of ROS in K-Ras-driven Cancers**

Elevated cellular ROS levels play a role in promoting tumorigenesis, specifically in K-Ras-driven cancers. In pancreatic cancer, oncogenic mutant K-Ras can promote cellular transformation by elevating cellular ROS through the Rac1/NOX4 signaling or through the p38/MAPK/PKC/NOX1 activation [69, 347-350]. Additionally, the ROS generated by oncogenic mutant K-Ras can lead to increased activation of the MAPK and PI3K signaling pathways [350].

In order to sustain tumor growth, oncogenic mutant K-Ras upregulates antioxidants, such as NRF2 and SOD enzymes, to increase their antioxidant capacity [69, 350, 351]. Given that K-Ras-driven cancers are more sensitive to changes in cellular ROS levels, perturbing the ROS levels was investigated as a potential therapeutic target [185, 192].

The use of antioxidants, to prevent cellular ROS from reaching tumor-promoting levels, was investigated. However, studies showed that NAC or vitamin E treatment increased cell proliferation and accelerated tumor progression in lung cancer cell lines and mice expressing oncogenic mutant K-Ras, suggesting that reducing cellular ROS levels can promote K-Ras-driven cancer progression [293, 294]. The other side, elevating cellular ROS to reach toxic levels that overwhelm tumor cells’ antioxidant capacity and induce cell death, has also been investigated. Lanperisone is a small molecule that specifically inhibits the growth of K-Ras-driven cancer cells via elevating cellular ROS levels to induce ferroptosis [185, 352, 353]. LCS-1, a small molecule inhibitor of SOD1, was also shown to inhibit the growth of K-Ras-driven lung cancer cell lines [185, 262]. Benzyl isothiocyanate induces apoptosis of K-Ras-driven pancreatic cancer cell lines, but not normal pancreatic cell lines, through elevation of cellular ROS and reduced GSH levels [353, 354]. Piperlongumine induces apoptosis in K-Ras-driven
colorectal and pancreatic cancer cells via simultaneously reducing GSH levels and inhibiting TrxR [353, 355]. Gold(I) N-heterocyclic carbene (Au(I)-NHC) complexes inhibit TrxR to elevate cellular ROS levels and induce apoptosis in K-Ras-driven pancreatic and lung cancer cell lines [274, 303, 356]. Additionally, administering a ROS-elevating drug along with current chemotherapeutic drugs have shown to be effective in targeting chemo-resistant K-Ras-driven cancer cell lines [353, 357]. Overall, this suggests that elevation of cellular ROS levels may be an attractive strategy to target K-Ras-driven cancers.

2.2 Previous Work

As mentioned previously, Au(I)-NHC complexes are compounds that inhibit TrxR. This occurs via the gold atom interacting with the thiol group present in the active site of TrxR, locking it in the inactive form [274, 303]. Given that TrxR is overexpressed in lung cancer cell lines, its inhibition seemed like an attractive approach [303, 358]. However, inhibition of TrxR alone did not cause significant effects on lung cancer cell lines, suggesting additional redox pathways would need to be targeted [303, 358]. This is the approach that our collaborator, Dr. Arumugam at Wright State, takes in his lab. His lab works on investigating the anticancer effects of adding redox active ligands to Au(I)-NHC complexes. He generated an Au(I)-NHC complex that contains a ferrocene moiety (Fig. 18A). As previously mentioned, ferrocene generates continuous cellular ROS production via the reversible oxidation of ferrocene to the ferrocenium cation. Thus, the general cellular ROS produced by ferrocene, when combined with the selective inhibition of TrxR by Au(I)-NHC complex, could overwhelm tumor cell’s redox balance, inducing apoptosis [274, 359]. His worked showed that this ferrocene derivative were able to elevate cellular ROS levels to inhibit proliferation of a lung cancer cell line harboring an
oncogenic mutant K-Ras [274]. Overall, this suggests that ferrocene derivatives may be able to inhibit the proliferation of K-Ras-driven cancers.

Figure 18. Compounds previously published from the Arumugam lab and our lab. (A) Au(I)NHC-ferrocene derivative synthesized by Dr. Arumugam’s lab (Wright State) that has been shown to inhibit the growth of a lung cancer cell line harboring an oncogenic mutant K-Ras. Figure adapted from [274]. (B) A chalcone derivative synthesized in collaboration with Dr. Ketcha (Wright State), and that our lab has shown can disrupt K-Ras association with the PM via PKC-induced phosphorylation of Ser181. The α,β-unsaturated carbonyl system is labeled in red. Figure adapted from [167].

Chalcones, or 1,3-diaryl-2-propen-1-one, are naturally derived compounds that contain two aromatic rings joined together by an α,β-unsaturated carbonyl [167, 360]. Similar to ferrocene, chalcones are a scaffold structure which allows the addition of groups for generating a variety of compounds [360]. Chalcone derivatives have exhibited anti-cancer, anti-inflammatory and antioxidant properties [361, 362]. These properties stem from the reactive α,β-unsaturated carbonyl (Fig. 18B) system, which serves as a Michael reaction acceptor, or an electrophile [167, 360]. The α,β-unsaturated carbonyl can interact with thiol groups on proteins, such as KEAP1, resulting in NRF2 activation and subsequent expression of its target genes [360, 363]. In our lab,
the research interest involves identifying compounds that disrupt K-Ras association with the PM, since K-Ras localization to the PM is essential for its signaling [13, 23, 24, 26]. Previously in our lab, in a collaboration with Dr. Ketcha’s lab at Wright State, a screening identified chalcone derivatives as potential compounds for blocking K-Ras PM binding [167, 364]. These chalcone derivatives (Fig. 18B) dissociated K-Ras, but not H-Ras, from the PM through phosphorylation of Ser181 by PKC [167]. Additionally, these compounds specifically disrupted oncogenic mutant K-Ras signaling and inhibited the growth of K-Ras-driven human pancreatic and lung cancer cell lines [167]. Overall, this suggests that chalcone derivatives can be used to target K-Ras-driven cancers.

2.3 Novel Compound: $\text{C}_{16}\text{H}_{20}\text{FeClNO}$

$\text{C}_{16}\text{H}_{20}\text{FeClNO}$ is a novel ferrocene derivative (Fig. 19) that was created by Dr. Arumugam. Looking towards the right of the structure, $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ contains a charged amino group and Cl (Fig. 19A). These charges enhance the water solubility of the compound, allowing it to enter the cell. In the middle of the compound, $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ contains an $\alpha,\beta$-unsaturated carbonyl
Lastly, on the left, \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) possesses a ferrocene moiety (Fig. 19C).

\[
\begin{align*}
\text{O} & \quad B \\
\text{Fe} & \quad \text{N} \\
\text{Cl} & \quad \text{H}
\end{align*}
\]

\( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) possesses three unique characteristics: (A) a charged amino group and Cl to increase water solubility and enhance entry into the cell, (B) the \( \alpha,\beta \)-unsaturated carbonyl system, and (C) a ferrocene moiety. (Rehl, K.M. et al, manuscript submitted for publication).

\textbf{Figure 19. The structure of \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \), the novel ferrocene derivative.} \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) possess three unique characteristics: (A) a charged amino group and Cl to increase water solubility and enhance entry into the cell, (B) the \( \alpha,\beta \)-unsaturated carbonyl system, and (C) a ferrocene moiety. (Rehl, K.M. et al, manuscript submitted for publication).

\( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) is a novel compound, which has not been characterized for its cellular activity. In collaboration with Dr. Arumugam, \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) was provided to our lab to ascertain its biological activity, specifically towards K-Ras. The reason for this was that \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) contains an \( \alpha,\beta \)-unsaturated carbonyl system, just like chalcones, and our lab has shown that chalcone derivatives can inhibit oncogenic mutant K-Ras signaling and cancer cell growth [167]. Thus, this suggested that the \( \alpha,\beta \)-unsaturated carbonyl system in \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) may also be able to target oncogenic mutant K-Ras. Furthermore, \( \text{C}_{16}\text{H}_{20}\text{FeClNO} \) contains a ferrocene moiety, which is involved in generation of cellular ROS. K-Ras-driven cancers, like lung cancer, are sensitive to the elevation of cellular ROS, inhibiting their growth [262, 352, 354, 355]. The Arumugam lab has shown that ferrocene derivatives can inhibit the growth of lung cancer cell lines through production of ROS [274]. Given that lung cancer cells expressing oncogenic
mutant K-Ras require K-Ras signaling for their growth, it suggests that C_{16}H_{20}FeClNO may be able to block the growth of K-Ras-driven cancers through elevation of cellular ROS.

2.4 Hypothesis and Specific Aims

As previously mentioned, constitutively active mutations of Ras are present in ~19% of all human cancer, with ~75% of those being K-Ras [91]. Despite the development of sotorasib and adagrasib, they only target the K-RasG12C mutant, which makes up a small sub-set of K-Ras-driven cancer [13]. Thus, a need exists for a pan-K-Ras inhibitor. C_{16}H_{20}FeClNO is a newly developed ferrocene derivative but its mechanism, specifically its anti-cancer effects, remain unknown. K-Ras-driven cancers are sensitive to changes in cellular ROS levels and previous ferrocene derivatives have shown to block lung cancer cell growth through elevation of cellular ROS [274]. Thus, I propose that C_{16}H_{20}FeClNO will behave in a similar manner and could target K-Ras-driven cancer cells. Taken together, the central hypothesis of this dissertation is that C_{16}H_{20}FeClNO blocks the growth of K-Ras-driven cancers by disrupting K-Ras signaling through elevating cellular ROS levels.

The hypothesis will be tested via the following specific aims:

Specific Aim #1: Identify the effect of C_{16}H_{20}FeClNO on the K-Ras signaling pathway. The hypothesis of this aim is that C_{16}H_{20}FeClNO disrupts the signaling of oncogenic mutant K-Ras and inhibits K-Ras-driven cancer growth. Aim 1-1 will identify the effect of C_{16}H_{20}FeClNO on the K-Ras signaling pathway. Aim 1-2 will identify the effect of C_{16}H_{20}FeClNO on the growth of human cancer cells harboring oncogenic mutant K-Ras.

Specific Aim #2: Identify the effect of C_{16}H_{20}FeClNO on K-Ras PM localization. The hypothesis of this aim is C_{16}H_{20}FeClNO dissociates K-Ras from the PM through an ROS-
mediated mechanism. Aim 2-1 will identify the effect of C_{16}H_{20}FeClNO on K-Ras PM localization. Aim 2-2 will assess the role of ROS in K-Ras localization to the PM. Aim 2-3 will assess the effect of antioxidants on K-Ras PM localization.

**Specific Aim #3:** Dissect the mechanism of C_{16}H_{20}FeClNO-induced K-Ras PM dissociation. The hypothesis of this aim is that C_{16}H_{20}FeClNO dissociates K-Ras from the PM by directly oxidizing K-Ras. Aim 3-1 will assess the effect of C_{16}H_{20}FeClNO on the phosphatidylserine distribution at the PM. Aim 3-2 will assess the effect of C_{16}H_{20}FeClNO on K-Ras phosphorylation at Ser181. Aim 3-3 will assess the effect of C_{16}H_{20}FeClNO on K-Ras oxidation.

Completion of this project will provide insight on how C_{16}H_{20}FeClNO inhibits the growth of K-Ras-driven cancers. Specifically, it will characterize the ability of C_{16}H_{20}FeClNO to elevate cellular ROS levels and its effects on oncogenic mutant K-Ras signaling, and the growth of K-Ras-driven cancers. Overall, this project will provide a foundation for C_{16}H_{20}FeClNO to be further developed as an anti-tumor drug, specifically as a K-Ras specific inhibitor.
3. **Materials and Methods**

3.1 **Cell Culture**

Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s modified eagle medium (DMEM, Gibco; Cat #10569-010). Human pancreatic ductal adenocarcinoma (PDACs) cells: Bx-PC3, Panc10.05 and AsPC-1 were maintained in RPMI-1640 (ATCC; 30-2001), Mia- PaCa2 and Panc-1 were maintained in DMEM, HPAC were maintained in DMEM-F12 (Gibco; Cat # 11320033) and HPAF-II were maintained in EMEM (ATTC; 30-2003). Human non-small cell lung cancer (NSCLCs) cells: H522, H1975, H1299, A549, H23, H441, H1703 and H358 were maintained in RPMI-1640. All cancer cell lines were maintained in media supplemented with 10% Fetal Bovine Serum (Gibco; Cat #16000-069) and 2 mM L-glutamine (GenDEPOT; Cat # CA009-010). Cells were tested for mycoplasma (MycoAlert PLUS Mycoplasma Detection Kit; Cat# LT07-710). All cell lines were maintained in an incubator at 37°C at 5% CO₂.

3.2 **Antibodies**

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): pAkt ((Ser473) (D9E) XP; Cat #4060L), total Akt ((pan) (40D4); Cat # 2920S), ppERK (p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E)) XP; Cat #4370L), total ERK (p44/42 MAPK (ERK1/2) (L34F12); Cat # 4696S), cleaved caspase-3 ((Asp175) (5A1E); Cat #9664) and cleaved PARP-1 ((Asp214) (D64E10) XP; Cat # 5625). The following antibodies were purchased from Proteintech (Rosemont, IL): β-actin (Cat # 66009-1-Ig) and GFP-tag (Cat # 60002-1-Ig).

3.3 **Compounds**
C₁₆H₂₀FeCl₅O was provided in collaboration with Dr. Arumugam (Wright State University, Dayton, OH). Mitomycin C was purchased from Alfa Aesar (Cat # J63193). Carmustine was purchased from MedChemExpres (Cat # HY-13585/CS-2935). N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (Cat #A17250-10G). Hydrogen peroxide (H₂O₂) was purchased from Sigma Aldrich (Cat #H1009).

3.4 Western Blotting

3.4.1 Ras Signaling

MDCK cells stably expressing GFP-tagged oncogenic mutant K-RasG12V (KG12V), -H-RasG12V (HG12V) or -N-RasG12V (NG12V) were grown to 100% confluency, washed x2 with 1X phosphate-buffered saline (PBS), trypsiniized with 2 mL 0.25% trypsin (Gibco; Cat #25200-072) and then resuspended in 2 mL complete growth medium. 2 mL of cell solution was diluted into 8 mL complete growth medium, spun down at 1,500 xg for 3 minutes and the pellet was resuspended in 5 mL complete growth medium. Cells were counted using the Countess II Automated Cell Counter (LifeTechnologies; Thermo Fisher Scientific; Rockford, IL) with Trypan Blue Dye (Gibco; Cat # 15250061). Cells were seeded at 3x10⁵ in 2 mL complete growth medium on a 6-well plate.

The next day, cells were washed x2 with 1X PBS and treated either with 1% DMSO (control), increasing concentrations of C₁₆H₂₀FeCl₅O (0.1, 0.5, 1, or 2 µM) or mitomycin C (0.01, 0.1, or 1 nM) prepared in 2 mL complete growth medium for 48 hours. These mixes either contained the compound themselves or co-mixed with 500 µM NAC.

Then, cells were washed x2 in cold 1X PBS and harvested on ice in 400 µL lysis buffer consisting of 50 mM Tris pH 7.5, 25 mM NaF, 5 mM MgCl₂, 75 mM NaCl, 5 mM EGTA, 3.3
µg/µl aprotinin, 0.1 mg/mL leupeptin, 1 mM DTT, 100 µM NaVO₃ and 1% NP-40. Lysates were incubated on ice for 10 minutes, vortexed every 5 minutes, and then spun down at 15,000 rpm for 10 minutes in 4°C centrifuge. Protein concentration of the lysates was determined using the BCA protein assay (Thermo Fisher Scientific; Reagent A Cat #23228; Reagent B Cat # 1859078; Rockford, IL). 20 µg protein samples were prepared and denatured at 95°C for 5 minutes.

Samples were run on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were transferred onto PVDF membranes (BioRad; Cat # 1620177) using a semi-dry transfer system (Trans-Blot SD Semi-Dry Transfer Cell; BioRad; model 221BR) for 45 minutes at 15V. Membranes were incubated for 1 hour on room temperature rocker in 5% BSA in 1X TBS-T for phosphorylated proteins or 5% milk in 1X TBS-T for non-phosphorylated proteins. Membranes were incubated overnight on 4°C rocker in primary antibody. The following dilutions were used: pAkt (1:1,000 anti-rabbit), ppERK (1:3,000 anti-rabbit), total Akt (1:1,000 anti-mouse), total ERK (1:1,000 anti-mouse), GFP (1:4,000 anti-mouse) and β-actin (1:4,000 anti-mouse).

Membranes were washed x3 in 1X TBS-T for 5 minutes each on room temperature rocker. Membranes were incubated in secondary antibody for 1 hour on room temperature rocker. The following dilutions were used: 1:5,000 anti-rabbit (Goat anti-Rabbit IgG (H+L) HRP conjugate, Cat # G21234; Invitrogen) and 1:2,000 anti-mouse (Goat anti-Mouse IgG (H+L) HRP conjugate; Cat # G21040; Invitrogen). 1X TBS-T washes were repeated. pAkt, ppERK, total Akt and total ERK membranes were developed for 4 minutes in a 4:1 dilution of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific; Cat # 34580) with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific; Cat # 34075). GFP and β-actin membranes were developed for 4 minutes in SuperSignal West Pico PLUS chemiluminescent
Substrate only. Membranes were imaged using chemiluminescence on the Amersham Imager 600 (GE Healthcare Life Sciences; Marlborough, MA).

Membranes were quantified using ImageJ software (version 1.52a) to quantify the intensity of each protein bands. Each lane was normalized to the DMSO-treated (control) lane. Further statistically analysis was performed using the GraphPad Prism software (version 8.0.2).

3.4.2 Apoptosis

MDCK cells stably expressing GFP-K-RasG12V were trypsinized, counted and seeded at 3x10^5 on a 6-well plate as previously described in section 3.4.1. The next day, cells were washed x2 with 1X PBS and treated either with 1% DMSO (control) or 2 μM C_{16}H_{20}FeClNO in 2 mL complete growth medium for 48 hours, or 2 μM staurosporine in 2 mL complete growth medium for 6 hours. After treatment, the media was removed from the wells, spun down at 15,000 rpm for 10 minutes in 4°C centrifuge and the pellet was kept on ice. In the meantime, cells adherent to the well were washed x2 in 1X PBS and then harvested in the same lysis buffer, ~300 μL based on pellet size, as described in section 3.4.1. The harvested cells were added to the same tube containing the media pellet, incubated on ice for 10 minutes, and spun down at 15,000 rpm for 10 minutes in 4°C centrifuge. Protein concentration of the lysates were determined using the BCA protein assay. 20 μg protein samples were prepared and denatured at 95°C for 5 minutes.

Samples were run on 8%, 12%, and 15% SDS-PAGE gels. Proteins were transferred, blocked in 5% milk in 1X TBS-T for 1 hour on room temperature rocker and incubated in primary antibodies overnight on 4°C rocker. The following dilutions were used: cleaved-caspase 3 (1:1,000 anti-rabbit), cleaved PARP-1 (1:1,000 anti-rabbit) and β-actin (1:3,000 anti-mouse). Membranes were washed x3 in 1X TBS-T for 5 minutes each and incubated in secondary
antibody for 1 hour on room temperature rocker. The secondary antibody dilutions were used: 1:5,000 anti-rabbit and 1:2,000 anti-mouse. Cleaved-caspase-3 and actin membranes were developed for 4 minutes in 4:1 dilution of SuperSignal West Pico PLUS Chemiluminescent Substrate, while cleaved-PARP-1 was developed with the addition of SuperSignal West Dura Extended Duration substrate. Membranes were imaged using chemiluminescence on the Amersham Imager 600.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type of Cancer</th>
<th>K-Ras Signature</th>
<th>Cell Seeding #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bx-PC3</td>
<td>PDAC</td>
<td>WT</td>
<td>3x10^5</td>
</tr>
<tr>
<td>Panc10.05</td>
<td>PDAC</td>
<td>G12D</td>
<td>3x10^5</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>PDAC</td>
<td>G12D</td>
<td>3x10^5</td>
</tr>
<tr>
<td>MIA-PaCa2</td>
<td>PDAC</td>
<td>G12C</td>
<td>3x10^5</td>
</tr>
<tr>
<td>Panc-1</td>
<td>PDAC</td>
<td>G12D</td>
<td>3x10^5</td>
</tr>
<tr>
<td>HPAC</td>
<td>PDAC</td>
<td>G12D</td>
<td>3x10^5</td>
</tr>
<tr>
<td>HPAF-II</td>
<td>PDAC</td>
<td>G12D</td>
<td>2x10^5</td>
</tr>
<tr>
<td>H522</td>
<td>NSCLC</td>
<td>WT</td>
<td>1x10^5</td>
</tr>
<tr>
<td>H1975</td>
<td>NSCLC</td>
<td>WT</td>
<td>1x10^5</td>
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<tr>
<td>H1299</td>
<td>NSCLC</td>
<td>WT</td>
<td>1x10^5</td>
</tr>
<tr>
<td>A549</td>
<td>NSCLC</td>
<td>G12S</td>
<td>1x10^5</td>
</tr>
<tr>
<td>H23</td>
<td>NSCLC</td>
<td>G12C</td>
<td>1x10^5</td>
</tr>
<tr>
<td>H441</td>
<td>NSCLC</td>
<td>G12V</td>
<td>1x10^5</td>
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<td>H358</td>
<td>NSCLC</td>
<td>G12C</td>
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</tr>
<tr>
<td>H1703</td>
<td>NSCLC</td>
<td>G12C</td>
<td>1x10^5</td>
</tr>
</tbody>
</table>

**3.5 ROS assay**

Wild-type (WT) MDCK cells were grown to 100% confluency, washed, trypsinized and counted as previously described in section 3.4.1. WT-MDCK cells were seeded at 2x10^4 on a clear bottomed 96-well plate in 100 µL complete growth medium. Cells were washed x2 with 1X PBS and treated with either 1% DMSO (control), 100 µM H_2O_2, 2 µM C_{16}H_{20}FeClNO, or 2 µM C_{16}H_{20}FeClNO with 500 µM NAC in 100 µL complete growth medium for 48 hours. All
concentrations were incubated with 25 µM of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Thermo Fisher Scientific; Cat # D399) dye during the treatment. After 48 hours, fluorescence was read using BioTek Synergy H1 microplate reader at excitation of 495nm and emission of 527nm. The fluorescence of drug-treated cells was normalized to the DMSO-treated (control) cells and further statistical analysis was performed using the GraphPad Prism software.

3.6 Proliferation Assay

Human pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC) cell lines (Table 2) were grown to 100% confluency, washed x2 with 1X PBS and trypsinized with 0.05% trypsin (Gibco; Cat # 25300062), and counted as previously described in section 3.4.1. PDAC and NSCLC were seeded on a clear bottomed 96-well plate in 100 µL of corresponding media. Table 2 includes the list of cell seeding numbers for each cell line and the type of K-Ras present in each cell line [95, 147, 167].

Next day, cells were washed x2 with 100 µL of 1X PBS. Cells were treated with either 1% DMSO (control), increasing concentrations of C16H20FeClNO (0.01, 0.1, 1, 10, 50, 100, 500 or 1000 µM) or mitomycin C (1, 10, 100, 500, 1000, 5000, 10000 or 50000 nM) in 100 µL of corresponding media. Cells were treated for 72 hours, changing the growth media every 24 hours. Cells were washed x2 with 1X PBS and were quantified in 100 µL of 1X dye binding solution (CyQUANT NF Cell Proliferation Assay Kit; Molecule Probes; Cat # 35006). Plates were incubated for 30 minutes at 37°C and fluorescence was read using the BioTek Synergy H1 microplate reader at excitation of 480nm and emission of 530nm. Plates were incubated for an additional 30 minutes and read again, and analyzed as described in section 3.5.

3.7 Confocal Imaging
3.7.1 Ras PM Localization

MDCK cells stably co-expressing GFP-K-RasG12V, -H-RasG12V or -N-RasG12V with mCherry-CAAX endomembrane marker, or stably expressing GFP-K-Ras4AG12V only, were grown, trypsinized and counted as described in section 3.4.1. Cells were seeded at 2.75x10^5 (K-RasG12V), 2.50x10^5 (H-RasG12V and N-RasG12V), and 1.25x10^5 (K-Ras4AG12V) onto glass coverslips on a 12-well plate in 1 mL complete growth medium.

The next day, cells were washed x2 with 1X PBS and treated either with 1% DMSO (control), increasing concentrations of C_{16}H_{20}FeClNO (0.01, 0.05, 0.1, 0.5, 1, 1.5 or 2 μM), mitomycin C (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25 or 50 nM), carmustine (1, 2.5, 5, 10, 25, 50 or 100 μM) or H_{2}O_{2} (0.01, 0.1, 0.5, 1, 10, 25, 50 or 100 μM) prepared in 2 mL complete growth medium for 48 hours. These mixes either contained the compound themselves or co-mixed with 500 μM NAC.

After the treatment, cells were washed x2 with cold 1X PBS and then fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Services, Cat #15710) for 30 minutes in the dark. Cells were then washed again and incubated with 50 mM NH_{4}Cl for 10 minutes in the dark. Coverslips were washed with 1X PBS and miliQ dH2O and mounted using 7 μL of Vectashield mount agent (Vector Laboratories; Cat # H-1000). Slides were stored at 4°C. Images were taken using the Olympus Fluoview FV1000 in the Wright State University Microscope core at 60X objective. Three representative fields were taken per coverslip.

Co-localization was measured using the Manders’ coefficient from the ImageJ software. For every treatment, the images were averaged to generate a Manders’ coefficient. Manders’ coefficient is the total number of red pixels co-localized with the total number of green
pixels/total number of red pixels [365].

\[
Manders' \ coefficient = \frac{\text{total # of red pixels co-localized with total # of green pixels}}{\text{total # of red pixels}}
\]

Manders’ coefficients for various concentrations tested were plotted to generate a dose-response plot curve and a IC$_{50}$ was generated from it using GraphPad Prism.

### 3.7.2 Organelle Markers

The RFP-tagged organelle markers were purchased from Invitrogen (Carlsbad, CA): CellLight Golgi-RFP BacMam 2.0 (Cat# C10593), CellLight Lysosomes-RFP BacMam 2.0 (Cat # C10597), CellLight Mitochondria-RFP BacMam 2.0 (Cat # C10601), CellLight Late Endosome-RFP BacMam 2.0 (Cat #C10589), CellLight Early Endosome-RFP BacMam 2.0 (Cat #C10587), and ER-Tracker Red ((BODIPY TR Glibenclamide) (Cat #E34250)). Mechanism for each organelle marker is described in Table 3.

MDCK cells stably expressing GFP-K-RasG12V were seeded at 2.50x10$^5$ onto glass coverslips on a 12-well plate in 1 mL complete growth medium. Cells were co-treated with either 1% DMSO (control) or 2 µM $C_{16}H_{20}FeClNO$ and either early endosome (50 µL), late endosome (100 µL), lysosome (50 µL), mitochondria (50 µL) or Golgi (100 µL) for 48 hours. For the ER tracker, cells were co-treated with 4 µM of ER tracker for 1 hour prior to fixation. Cells were fixed, imaged and analyzed as previously described in section 3.7.1.

### 3.7.3 LactC2 PtdSer Probe

MDCK cells stably expressing GFP-LactC2 probe were seeded at 2.75x10$^5$ onto glass coverslips on a 12-well plate in 1 mL complete growth medium. Cells were treated with 1%
DMSO (control), C_{16}H_{20}FeClNO or mitomycin C, fixed, imaged and analyzed as described in section 3.7.1.

3.7.4 S181A Mutant

MDCK cells stably expressing GFP-K-RasG12V-S181A mutation were seeded at 2.25x10^5 onto glass coverslips on a 12-well plate in 1 mL complete growth medium. Cells were treated with 1% DMSO (control) or C_{16}H_{20}FeClNO, fixed, imaged and analyzed as described in section 3.7.1.

3.7.5 GFP-CTK

MDCK cells stably expressing GFP-C-terminus (residues 165-188) truncated K-RasG12V (CTK) were seeded at 2.75x10^5 onto glass coverslips on a 12-well plate in 1 mL complete growth medium. Cells were treated with 1% DMSO (control) or C_{16}H_{20}FeClNO, fixed, imaged and analyzed as described in section 3.7.1.
3.8 Subcellular Fractionation Assay

MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were washed, trypsinized and counted as described in section 3.4.1. Cells were seeded at 17x10^5 onto 10 cm dishes in 5 mL complete growth medium. Then, cells were washed x2 with 1X PBS and treated with either 1% DMSO (control), C_{16}H_{20}FeClNO (0.5 or 2µ M), mitomycin C (1 or 5 nM), carmustine (10 or 50 µM) or H_{2}O_{2} (10 or 50 µM) in 5 mL complete growth medium for 48 hours.

Dishes were washed x2 with cold 1X PBS and lysates were harvested in 400 µL lysis buffer A containing 10 mM Tris pH 7.5, 25 mM NaF, 5 mM MgCl₂, 1 mM EGTA, 1 M DTT, 10 µg/µL aprotinin, 0.1 mg/mL leupeptin, 100 µM NaVO₄ and 1 mM DTT. Harvested cells were incubated on ice for 10 minutes, vortexed every 5 minutes. Cells were further broken by passing them x30 through a 1 mL syringe (BD; Cat # 1029400) with a 23G-1 needle (Exel International Disposable...
Hypodermic Needles; Exilent; Cat #14-840-88). Lysates were spun at 1,500 xg for 10 minutes in 4°C centrifuge. The supernatant was spun using the Sorvall Discovery MX120SE Ultracentrifuge (Thermo Scientific; Waltham; MA) at 100,000 xg for 1 hour at 4°C to isolate the supernatant, the cytosolic fraction or S100. The pellet was washed in 1 mL lysis buffer, resuspended in 120 µL lysis buffer and sonicated x10 by a Sonic Dismembrator (model FB120; Fisher Scientific) at 4°C to isolate the membrane-bound fraction or P100. Protein concentrations of the lysates were determined using the BCA assay. Protein samples were prepared, with 20 µg for cytosolic-fraction proteins and 5 µg for membrane-fraction proteins.

Samples were run on 10% SDS page gel and transferred as described in section 3.4.1. Membranes were blocked in 5% milk in 1X TBS-T and incubated in primary antibody overnight at 4°C rocker. The primary antibodies dilutions used were: GFP (1:4,000 anti-mouse) and β-actin (1:4,000 anti-mouse). The next day, membranes were washed x3 in 1X TBS-T for 5 minutes each, incubated with 1:2,000 dilution of anti-mouse secondary antibody and imaged as described in section 3.4.1. The cytosolic and membrane fraction were imaged on the same blot. Membranes were quantified and analyzed as described in section 3.4.1.

3.9 Electron Microscopy

MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were seeded onto fibronectin-coated, gold EM grids (IGG200, Ted Pella Inc). Next day, cells were incubated with either 1% DMSO (control), 2 µM C\textsubscript{16}H\textsubscript{20}FeCl\textsubscript{NO} or 25 nM mitomycin C in complete growth media for 48 hours. The apical membrane was removed by placing PBS-soaked Whatman filter paper onto cells for 5 minutes, applying pressure using a silicon bung and then removing the filter paper. This procedure leaves the cytosolic surface of the adherent basal PM exposed. Basal PM sheets that remained attached to the gold EM grid were washed, fixed and the cytosolic
leaflet was labeled with anti-GFP antibody conjugated to 4.5-nm gold particle. Digital images of the immunogold-labeled PM sheets were taken in a transmission electron microscope at 100,000x (Wright State University Microscope core). Intact μm² areas of the PM sheet were identified using ImageJ software, and the (x,y) coordinates of the gold particles were determined [375, 376]. K-functions were calculated and standardized on the 99% confidence interval for univariate functions [375, 376]. [375-377]. In the case of univariate functions, a value of $L(r) - r$ greater than the CI indicates significant clustering, and the maximum value of the function ($L_{max}$) estimates the extent of clustering. Differences between replicated point patterns were analyzed by constructing bootstrap tests as described previously [377, 378], and the statistical significance against the results obtained with 1,000 bootstrap samples was evaluated.

3.10 His95 Mutations

3.10.1 Site-Directed Mutagenesis

Forward and reverse primers for GFP-K-RasG12V-H95A, GFP-K-RasG12V-H95C and GFP-H-RasG12V-Q95H mutants were designed and ordered from Agilent QuikChange Primer Design (Agilent; Santa Clara, CA). cDNA of H-RasG12V-Q95H, K-RasG12V-H95A and H95C were cloned into a pEF6 vector. Correct mutation and sequence alignment was ensured by Sanger Sequencing by Genewiz (South Plainfield, NJ) and Basic Local Alignment Search Tool (BLAST; NIH National Library of Medicine; Bethesda, MD). The cDNA of those pEF6 vectors were cloned into pBLST vectors. Correct mutation and sequence alignment was ensured in the same way.

3.10.2 Transfection
150 µL of WT-MDCK cells were seeded onto a 6-well plate to ensure 80% confluency for the following day. The next day, the plasmids were transfected into the cells using the Lipofectamine3000 kit (Invitrogen; Cat #L3000150). Transfected cells were selected and grown in complete growth medium containing 10 µg/µl blastocidin (Research Products International; Cat # B12200-0.01) for 6 days. Transfection efficiency was checked using the lab fluorescent microscope (Zeiss AxioVision; SN 3833000578). All cell lines were maintained in an incubator at 37°C at 5% CO₂. During experiments, transfected cells were maintained in complete growth medium containing 4 µg/µL blastocidin.

3.10.3 Confocal Imaging

MDCK cells expressing GFP-K-RasG12V-H95A, GFP-K-RasG12V-H95C and GFP-H-RasG12V-Q95H were grown to 100% confluency, washed, trypsinized and counted as described in section 3.4.1. Cells were seeded at 1.5x10⁵ (K-Ras H95A and H95C) or 2.5x10⁵ (H-RasQ95H) onto glass coverslips on a 12-well plate in 1 mL complete growth medium. The next day, cells were washed and treated with either 1% DMSO (control), 2 µM C₁₀H₂₀FeClINO or 2 µM C₁₀H₂₀FeClINO with 500 µM NAC in 2 mL complete growth medium. Cells were washed, fixed, and imaged as described in section 3.7.1.

3.10.4 Electron Microscopy

MDCK cells expressing GFP-K-RasG12V-H95A, GFP-K-RasG12V-H95C or -H-RasG12V-Q95H were seeded onto fibronectin-coated, gold EM grids (IGG200, Ted Pella Inc). Next day, cells were incubated with either 1% DMSO (control), 2 µM C₁₀H₂₀FeClINO or 2 µM C₁₀H₂₀FeClINO with 500 µM NAC for 48 hr. Basal PM sheets preparation and EM analysis were performed as described in section 3.9.
3.11 Statistics

GraphPad prism software was used for one-way ANOVA and Student $t$-test for statistical analysis.
4. Results

4.1 Specific Aim #1: Identify the effect of $C_{16}H_{20}FeClNO$ on the K-Ras signaling pathway

Rationale

Ferroene derivatives have shown to inhibit the growth of lung cancer cell lines that expresses oncogenic mutant K-Ras [274]. In a subset of human cancers that harbor oncogenic mutant K-Ras, they undergo re-wiring to become dependent on K-Ras signaling or “K-Ras-addicted” for their growth and survival [13, 92, 95-98]. Thus, one possible mechanism of ferrocene derivatives’ anti-cancer effects in these lung cancer cells is through disrupting oncogenic mutant K-Ras signaling. Ferrocene derivatives inhibit cancer cell growth, in part, by elevating cellular ROS levels, and K-Ras-driven cancers exhibit higher oxidative stress, when compared to normal cells, due to their metabolic reprogramming and oncogene activation [185, 192, 341-343, 379-381]. Because of this, K-Ras-driven cancers are more sensitive to changes in cellular ROS levels. ROS-elevating agents have shown to be effective in slowing the growth of K-Ras-driven cancers [262, 352, 354, 355]. Thus, it is possible that $C_{16}H_{20}FeClNO$ can inhibit the growth of K-Ras-driven cancers via elevating cellular ROS levels. Taken together, the hypothesis of this aim is that $C_{16}H_{20}FeClNO$ disrupts the signaling of oncogenic mutant K-Ras and inhibits K-Ras-driven cancer growth.

4.1.1 Aim 1-1: Identify the effect of $C_{16}H_{20}FeClNO$ on the K-Ras signaling pathway

Immunoblotting was performed on MDCK cells stably expressing GFP-tagged oncogenic mutant K-Ras (K-RasG12V) to determine whether $C_{16}H_{20}FeClNO$ had an effect on the K-Ras signaling pathway. Lysates were probed for phosphorylated ERK (ppERK) and Akt (pAkt S473), the two most well-studied Ras downstream effectors [28, 63, 66, 76, 147]. $C_{16}H_{20}FeClNO$
treatment on K-RasG12V-expressing cells significantly decreased the level of ppERK in a dose-dependent manner (Figs. 20A and B), but it had no effect on the level of pAkt (Figs. 20 A and B). C_{16}H_{20}FeClNO treatment had no effect on total Akt nor total ERK levels, suggesting it has no effect on the total protein expression of Akt and ERK (Fig. 20A). Also, C_{16}H_{20}FeClNO had no significant effect on GFP-K-RasG12V protein level, suggesting it has no effect on the stability of K-Ras protein (Figs. 20A and B). I repeated the immunoblot experiment using cell lysates from MDCK cells stably expressing oncogenic mutant H-Ras (H-RasG12V) or N-Ras (N-RasG12V) after C_{16}H_{20}FeClNO treatment. My data show that C_{16}H_{20}FeClNO did not alter levels of ppERK,
pAkt and GFP-RasG12V protein expression in these cells (Figs. 20A and B).

**Table 20.** C16H20FeCINO disrupts the K-Ras/MAPK signaling. (A) MDCK cells stably expressing GFP-K-RasG12V, -H-RasG12V or -N-RasG12V were treated with DMSO (control) or various concentrations of C16H20FeCINO for 48 hours. Harvested cell lysates were blotted for ppERK, pAkt (S473), total ERK, total Akt and GFP-RasG12V expression. An actin blot served as a loading control. Representative blots from three independent experiments are shown. (B) Quantification of ppERK, pAkt and GFP-RasG12V are shown. The graphs show the mean ± standard error of the mean (S.E.M.) from three independent experiments. One-way ANOVA was performed to assess statistical differences between DMSO-treated and C16H20FeCINO-treated cells (*p<0.05, ****p<0.0001).
Taken together, my data suggest that C16H20FeClNO specifically inhibits the K-Ras/MAPK signaling pathway.

Next, we examined if C16H20FeClNO does, in fact, elevate cellular ROS levels. Briefly, wild-type (WT) MDCK cells were treated with C16H20FeClNO in the presence of H2DCFDA dye. The cell-permeable H2DCFDA dye gets cleaved by intracellular esterases into non-fluorescent DCFH [382]. Once exposed to ROS, DCFH undergoes electron oxidation to form the fluorescent, DCF, which is the output for this assay [343, 382]. My data shows that C16H20FeClNO significantly increased DCF fluorescence while co-treatment with N-acetylcysteine (NAC), a general antioxidant that has been shown to lower general ROS levels, reversed it (Fig. 21A), suggesting
that C_{16}H_{20}FeClNO elevates cellular ROS levels [271, 383].

**Figure 21.** C_{16}H_{20}FeClNO disrupts the K-Ras/MAPK signaling through an ROS-mediated mechanism. (A) WT-MDCK cells were treated with DMSO (control), 100 µM H_{2}O_{2}, 2 µM C_{16}H_{20}FeClNO (Fe) or 2 µM C_{16}H_{20}FeClNO with 500 µM NAC, in the presence of 25 µM H_{2}DCFDA dye, for 48 hours. Fluorescence was read on a plate reader at excitation of 495 nm and emission of 527 nm. The graph shows the mean + S.E.M. from three independent experiments. One-way ANOVA was performed to assess statistical differences between DMSO-treated and drug-treated cells (*p<0.02, ****p<0.0001, ns = not significant). (B) MDCK cells stably expressing GFP-K-RasG12V were treated with DMSO (control), 2 µM C_{16}H_{20}FeClNO (Fe) or 2 µM C_{16}H_{20}FeClNO with 500 µM NAC for 48 hours. Harvested cell lysates were blotted for ppERK and total ERK. An actin blot served as a loading control. Representative blots from three independent experiments are shown. (C) Quantification of ppERK is shown. The graph shows the mean + S.E.M. from three independent experiments. One-way ANOVA was performed to assess statistical differences between DMSO-treated and drug-treated cells (***p<0.0005, ns = not significant).
Given that $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ elevates cellular ROS levels, I examined if $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ disrupts the K-Ras/MAPK signaling through an ROS-mediated mechanism. I studied K-Ras signaling after co-treating cells with NAC. My data show that co-treatment of $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ with NAC rescued the abrogated ppERK level in K-RasG12V-expressing cells (Figs. 21B and C). These data suggest $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ disrupts K-Ras signal output through an ROS-mediated mechanism.

Given that $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ disrupts K-Ras signal output through an ROS-mediated mechanism, it is possible that other ROS-elevating drugs have a similar effect on K-Ras signaling. MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were treated with mitomycin C and cell lysates were blotted for ppERK and pAkt. In addition to its known DNA-alkylating properties, mitomycin C has also shown to exert anti-cancer effects through elevation of cellular ROS via its inhibition of TrxR, an important component for the Trx antioxidant system [384, 385]. In K-RasG12V-expressing cells, mitomycin C significantly decreased both ppERK and pAkt levels in a dose-dependent manner (Figs. 22A and B). In H-RasG12V-expressing cells, mitomycin C had no effect on ppERK level and only reduced pAkt level at a
lower concentration (Figs. 22A and B).

**Figure 22. Mitomycin C specifically disrupts oncogenic mutant K-Ras signaling.** (A) MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were treated with DMSO (control) or various concentrations of mitomycin C for 48 hours. Harvested cell lysates were blotted for ppERK, pAkt, total ERK, total Akt and GFP-RasG12V expression. An actin blot served as a loading control. Representative blots from three independent experiments are shown. (B) Quantification of ppERK, pAkt and GFP-RasG12V are shown. The graphs show the mean + S.E.M. from three independent experiments. One-way ANOVA was performed to assess statistical differences between DMSO-treated and mitomycin C-treated cells (*p<0.05, **p<0.0001).
Thus, these data suggest that mitomycin C inhibits the K-Ras/MAPK signaling while it blocks Akt signaling in a Ras isoform-independent manner. Together, these data suggest that C_{16}H_{20}FeClNO specifically inhibits the K-Ras/MAPK signaling, likely via cellular ROS elevation.

4.1.2 Aim 1-2: Identify the effect of C_{16}H_{20}FeClNO on the growth of human cancer cells harboring oncogenic mutant K-Ras

Given that oncogenic mutant K-Ras signaling is essential for K-Ras-driven cancer growth, proliferation assays were performed on a panel of human pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC) cell lines to determine if C_{16}H_{20}FeClNO and mitomycin C inhibit their growth [147, 167]. Pancreatic and lung cancer cell lines were chosen due to the high occurrence of oncogenic mutant K-Ras present in both cancer tissues [13, 91]. Both the PDAC and NSCLC panels included cancer cell lines expressing WT K-Ras or oncogenic mutant K-Ras (Table 2). Cell proliferation was measured using a fluorescent dye that binds to nuclear DNA, indicative of cell number [386]. In the PDAC panel, C_{16}H_{20}FeClNO did not inhibit the growth of WT K-Ras cancer cell line, but it significantly inhibited the growth of the oncogenic mutant K-Ras cancer cell lines (Fig. 23A). Mitomycin C, on the other hand, showed significant inhibition of oncogenic mutant K-Ras PDACs as well as WT K-Ras cancer cell line (Fig. 23B). In the NSCLC panel, C_{16}H_{20}FeClNO had minimal effects on the growth of WT K-Ras cancer cell lines while it significantly inhibited the growth of lung cancer cell lines harboring oncogenic mutant K-Ras, with the exception of A549 cell line (Fig. 23C). Mitomycin
C showed significant inhibition of most NSCLCs, with the exception of A549 (Fig. 23D).

Figure 23. C$_{16}$H$_{20}$FeCINO and mitomycin C inhibit the growth of K-Ras-dependent human pancreatic and lung cancer cell lines. A panel of pancreatic ductal adenocarcinoma (PDAC) cell lines and non-small cell lung cancer (NSCLC) cell lines were treated with (A and C) 50 µM C$_{16}$H$_{20}$FeCINO and (B and D) 1000 nM mitomycin C for 72 hours, replacing complete growth medium containing each drug every day. Cell growth was measured by the CyQUANT NF Cell Proliferation Assay. The graphs show the mean cell proliferation + S.E.M. from three independent experiments. Gray bars represent cancer cell lines expressing wild-type K-Ras. Black bars represent cancer cells expressing oncogenic mutant K-Ras. A Student’s t-test for each
cell line was performed to assess statistical differences between DMSO-treated and drug-treated cells (*p=0.05, **p<0.001, ***p=0.0002, ****p<0.0001, n.s. = not significant).

A previous study has reported that although A549 cell line expresses oncogenic mutant K-Ras, its growth is independent of K-Ras signaling [95]. Overall, these data suggest that while mitomycin C non-specifically inhibits the growth of human PDAC and NSCLC cells, C\textsubscript{16}H\textsubscript{20}FeClINO inhibits the growth of K-Ras-dependent PDAC and NSCLC.

Given that ferrocene derivatives have shown to elevate cellular ROS levels to induce cell death via apoptosis, I decided to assess the apoptosis induced by C\textsubscript{16}H\textsubscript{20}FeClINO [274, 329, 343, 345]. MDCK cells stably expressing GFP-K-RasG12V were treated with C\textsubscript{16}H\textsubscript{20}FeClINO or staurosporine, and immunoblotted for apoptotic markers, cleaved caspase-3 and cleaved poly [ADP-ribose] polymerase 1 (PARP-1) [148, 150]. Staurosporine is a pan-kinase inhibitor, inducing apoptosis in both caspase-dependent and -independent manners [387, 388]. During the caspase-dependent apoptosis, cleavage and activation of caspase-3 results in cleavage and inactivation of PARP-1, the DNA repair protein [389, 390]. My data show that C\textsubscript{16}H\textsubscript{20}FeClINO treatment had no detectable level of cleaved caspase-3 nor cleaved PARP-1 (Fig. 24), suggesting
it does not initiate apoptosis at the concentration that blocks the K-Ras/MAPK signaling.

**Figure 24. C$_{16}$H$_{20}$FeClNO does not induce apoptosis at the concentration that inhibits the K-Ras/MAPK signaling.** MDCK cells stably expressing GFP-K-RasG12V were treated with DMSO (control) or 2 µM C$_{16}$H$_{20}$FeClNO (Ferro) for 48 hours, or 2 µM staurosporine (STS) for 6 hours. Harvested cell lysates were blotted for cleaved caspase-3 and cleaved PARP-1. An actin blot served as a loading control. Representative blots from three independent experiments are shown.

Overall, this suggests that C$_{16}$H$_{20}$FeClNO disrupts the K-Ras/MAPK signaling independent of apoptosis.

**4.2 Specific Aim #2: Identify the effect of C$_{16}$H$_{20}$FeClNO on K-Ras PM localization**

**Rationale**

K-Ras localization to the PM is essential for K-Ras signaling [13, 23, 24]. Previous studies in our lab have shown that inhibition of K-Ras-PM interaction results in a disruption of oncogenic mutant K-Ras signaling [13, 147, 150, 159, 161, 166, 167]. Thus, one possible mechanism for the observed inhibited K-Ras signaling after C$_{16}$H$_{20}$FeClNO treatment is by K-Ras dissociation...
from the PM. Also, C$_{16}$H$_{20}$FeClNO treatment disrupted the signaling of K-Ras, but not other Ras isoforms, through an ROS-mediated mechanism (Fig. 21), suggesting C$_{16}$H$_{20}$FeClNO may affect K-Ras-PM interaction through the ROS-mediated mechanism. Given this, the hypothesis of this aim is that C$_{16}$H$_{20}$FeClNO dissociates K-Ras from the PM through an ROS-mediated mechanism.

4.2.1 Aim 2-1: Identify the effect of C$_{16}$H$_{20}$FeClNO on K-Ras PM localization

Confocal microscopy was performed on MDCK cells stably co-expressing GFP-K-RasG12V and mCherry-CAAX, a generic endomembrane marker, to determine if C$_{16}$H$_{20}$FeClNO mis-localizes K-Ras from the PM [43, 147, 167]. In control cells, K-RasG12V was localized predominantly at the PM, with Manders’ coefficient value of 0.23 (Fig. 25A). Manders’ coefficient was used to measure the fraction of mCherry-CAAX that are co-localizing with GFP-K-RasG12V, a method to quantitate K-Ras dissociation from the PM [365]. After treatment with C$_{16}$H$_{20}$FeClNO, Manders’ coefficient increased to 0.39, suggesting that K-Ras is translocated to endomembranes (Fig. 25A). Manders’ coefficient values were used to plot a dose-response curve
of C$_{16}$H$_{20}$FeClNO to estimate an IC$_{50}$ value of 0.39 µM (Fig. 25B).

Figure 25. C$_{16}$H$_{20}$FeClNO distributes K-Ras to endomembranes. (A) MDCK cells stably co-expressing GFP-K-RasG12V with mCherry-CAAX, an endomembrane marker, were treated with either DMSO (control) or various concentrations of C$_{16}$H$_{20}$FeClNO for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localizing with GFP-K-RasG12V. (B) The graph show mean ± S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$.

To further characterize K-Ras PM dissociation, MDCK cells stably expressing GFP-K-RasG12V were treated with C$_{16}$H$_{20}$FeClNO in the presence of RFP-tagged different organelle markers (Table 3) and Manders’ coefficients were calculated [147]. My data show that after C$_{16}$H$_{20}$FeClNO treatment, K-RasG12V co-localized with organelle markers for the early endosome, late endosome, Golgi, lysosome and ER, but not mitochondria (Figs. 26 and 27). This suggests that C$_{16}$H$_{20}$FeClNO translocates K-Ras to the early endosome, late endosome,
Golgi, lysosome and ER.

**Figure 26.** C$_{16}$H$_{20}$FeClNO translocates K-Ras from the PM to intracellular organelles. MDCK cells stably expressing GFP-K-RasG12V were treated with DMSO (control) or 2 µM C$_{16}$H$_{20}$FeClNO in the presence of modified baculovirus encoding RFP-tagged organelle markers (A-C and E-F) for 48 hours, or (D) 4 µM ER-tracker 1 hour before fixation. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. Selected regions indicated by the white squares are shown at higher magnification. K-RasG12V that is co-localized with organelle markers are indicated by arrows. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localizing with GFP-K-RasG12V.
Figure 27. C₁₆H₂₀FeClNO translocates K-Ras from the PM to various intracellular organelles. Quantification of MDCK cells stably expressing GFP-K-RasG12V treated with DMSO (control) or 2 μM C₁₆H₂₀FeClNO (Fe) in the presence of modified baculovirus encoding RFP-tagged organelle markers (A-C and E-F) for 48 hours, or (D) 4 μM ER-tracker 1 hour before fixation. The graphs show mean ± S.E.M. of Manders coefficients for concentrations tested, with each dot representing a set. n = total number of cells co-expressing both GFP-K-RasG12V and RFP-tagged organelle markers. A Student’s t-test was used to assess statistical differences between DMSO-treated cells and C₁₆H₂₀FeClNO-treated cells (*p<0.05, **p<0.001, ***p<0.0005, ns = not significant).

I further repeated the experiment with MDCK cells stably co-expressing mCherry-CAAX with either GFP-H-RasG12V or -N-RasG12V, or stably expressing GFP-K-Ras4AG12V only, the alternative K-Ras splicing variant, to determine the isoform specificity of C₁₆H₂₀FeClNO. My
data show that the compound had no effect on the PM localization of H-Ras, N-Ras nor K-Ras4A (Fig. 28). Taken together, these data suggest that C_{16}H_{20}FeClNO specifically disrupts the
PM localization of K-Ras.

Figure 28. C$_{16}$H$_{20}$FeClINO does not disrupt the PM localization of other Ras isoforms. MDCK cells stably co-expressing mCherry-CAAX, an endomembrane marker, with (A) -GFP-H-RasG12V and, (B) -N-RasG12V, or (C) MDCK cells expressing-GFP-K-Ras4AG12V only, were treated with either DMSO (control) or various concentrations of C$_{16}$H$_{20}$FeClINO for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative
images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-RasG12V. (D-E) The graphs show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$.

### 4.2.2 Aim 2-2: Assess the role of ROS in K-Ras localization to the PM

In Aim 1, I showed that C$_{16}$H$_{20}$FeClNO disrupts oncogenic mutant K-Ras signaling through an ROS-mediated mechanism. Given that K-Ras localization to the PM is essential for K-Ras signaling, I examined if ROS plays a role in K-Ras binding to the PM. To do this, confocal microscopy was performed on MDCK cells stably co-expressing mCherry-CAAX with either GFP-K-RasG12V or –H-RasG12V after treatment with ROS-elevating drugs mitomycin C (Fig. 29A), carmustine (Fig. 29B) and H$_2$O$_2$. Carmustine is a chemotherapeutic drug that inhibits GRx, leading to elevation of cellular ROS and induction of apoptosis in tumor cells [295, 391].

![Molecules](image)

**Figure 29. Mitomycin C and Carmustine.** The chemical structures of the ROS-elevating drugs used for this dissertation: (A) Mitomycin C, figured adapted from [384], and (B) Carmustine, figured adapted from [392].
H$_2$O$_2$ is a well-known, stable ROS molecule that is involved in a variety of signaling pathways [191, 194]. My data showed that mitomycin C, carmustine and H$_2$O$_2$ translocated K-Ras from the PM to endomembranes with IC$_{50}$ values of 1.1 nM (Figs. 30A and B), 5.7 µM (Figs. 31A and B), and 1.9 µM (Figs. 32A and B), respectively. Furthermore, none of these compounds had an effect on the PM localization of H-RasG12V (Figs. 30-32, B and C).

Figure 30. Mitomycin C specifically distributes K-Ras, but not H-Ras, to endomembranes. MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with (A) GFP-K-RasG12V or (C) -H-RasG12V were treated with various concentrations of mitomycin C, for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-
CAAX co-localized with GFP-RasG12V. (B) The graph show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$.

**Figure 31.** Carmustine specifically distributes K-Ras, but not H-Ras, to endomembranes. MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with (A) GFP-K-RasG12V or (C) -H-RasG12V were treated with various concentrations of carmustine for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-RasG12V. (B) The graph show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$. 
Figure 32. H$_2$O$_2$ specifically distributes K-Ras, but not H-Ras, to endomembranes. MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with (A) GFP-K-RasG12V or (C) -H-RasG12V were treated with various concentrations of H$_2$O$_2$ for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-RasG12V. (B) The graph show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$.

Taken all together, these data suggest that mitomycin C, carmustine and H$_2$O$_2$ specifically mis-localize K-Ras, but not H-Ras from the PM.
Furthermore, electron microscopy (EM) was performed to directly measure the level of K-Ras at the PM [375, 376]. Intact basal PM sheets from MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V treated with C_{16}H_{20}FeClNO or mitomycin C were labeled with anti-GFP conjugated to 4.5 nm gold particles and imaged by EM [147, 375, 376]. In DMSO-treated K-RasG12V cells, there were ~90 gold particles per µm^2 while it was significantly decreased to ~60 particles in both C_{16}H_{20}FeClNO and mitomycin C-treated cells (Fig. 33A), indicative of reduced K-Ras abundance at the PM. In H-RasG12V-expressing cells, C_{16}H_{20}FeClNO had no effect on the amount of gold particles when compared to the control (Fig. 33B), suggesting that C_{16}H_{20}FeClNO specifically mis-localizes K-Ras from the PM.

Figure 33. C_{16}H_{20}FeClNO and mitomycin C specifically decrease the level of K-Ras at the PM. Basal PM sheets prepared from MDCK cells expressing (A) GFP-K-RasG12V or (B) -H-RasG12V were treated with DMSO (control), 2 µM C_{16}H_{20}FeClNO (Ferro/Ferrocene) or 25 nM mitomycin C (Mito C) for 48 hours and labeled with anti-GFP-conjugated 4.5 nm gold particles and visualized with EM. The graphs show mean ± S.E.M. One-way ANOVA test (left panel) and a Student’s t-test (right panel) were used to assess significant differences between DMSO-treated and drug-treated cells (**p<0.001, ****p<0.0001, n.s. = not significant).
Taken all together, these data demonstrate that C_{16}H_{20}FeClNO and other ROS-elevating drugs specifically dissociate K-Ras from the PM.

In addition to microscopy, a subcellular fractionation assay was performed. MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were treated with C_{16}H_{20}FeClNO, mitomycin C, carmustine or H_{2}O_{2} and the cytosolic fraction of GFP-RasG12V proteins were isolated from the membrane-bound fraction [161]. While ~90% of K-Ras was present in the membrane-bound fraction in control cells, C_{16}H_{20}FeClNO treatment significantly decreased the amount of membrane-bound K-Ras to ~60% (Figs. 34A and B). Similar results were seen in K-RasG12V cells treated with mitomycin C (Figs. 34A and B), carmustine (Figs. 35A and B) and a higher concentration of H_{2}O_{2} (Figs. 35A and B). However, none of these compounds had an
effect on the membrane-bound fraction of H-RasG12V (Figs. 34 and 35).

Figure 34. C$_{16}$H$_{20}$FeClNO and mitomycin C specifically decrease the amount of K-Ras bound to the membranes. MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were treated with various concentrations of C$_{16}$H$_{20}$FeClNO (Ferro) or mitomycin C (MC) for 48 hours. Cells were fractionated into membrane-bound (Ras-Mem) and cytosolic fractions (Ras-Cyt) and immunoblotted for GFP-RasG12V. (A) Representative blots from three independent experiments are shown. Actin blots served as a loading control. (B) The graphs show mean + S.E.M. from three independent experiments. One-way ANOVA was used to assess significant differences between DMSO-treated and drug-treated cells (*p=0.02, n.s. = not significant).
Figure 35. Carmustine and H$_2$O$_2$ specifically decrease the amount of K-Ras bound to the membranes. MDCK cells stably expressing GFP-K-RasG12V or -H-RasG12V were treated with various concentrations of carmustine (Carm) and H$_2$O$_2$ for 48 hours. Cells were fractionated into membrane-bound (Ras-Mem) and cytosolic fractions (Ras-Cyt) and immunoblotted for GFP-RasG12V. (A) Representative blots from three independent experiments are shown. Actin blots served as a loading control. (B) The graphs show mean ± S.E.M. from three independent experiments. One-way ANOVA was used to assess significant differences between DMSO-treated and drug-treated cells (*p=0.02 ***p=0.0002, n.s. = not significant).

These data, along with the microscopy data, further support that C$_{16}$H$_{20}$FeClNO, mitomycin C, carmustine and H$_2$O$_2$ specifically mis-localizes K-Ras, but not H-Ras, from the PM.
4.2.3 Aim 2-3: Assess the effect of antioxidants on K-Ras PM localization

To determine if the effects of the ROS-elevating agents on K-Ras PM binding were through cellular ROS elevation, I performed confocal microscopy on MDCK cells stably co-expressing GFP-K-RasG12V and mCherry-CAAX after NAC supplementation. I found that NAC co-treatment reversed the $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ (Figs. 36A and B), mitomycin C- (Figs. 37A and B) and $\text{H}_2\text{O}_2$ (Figs. 38A and B)-induced K-Ras PM dissociation.

Figure 36. NAC treatment reverses $\text{C}_{16}\text{H}_{20}\text{FeClNO}$-mediated distribution of K-Ras to endomembranes. (A) MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with GFP-K-RasG12V were co-treated with NAC and various concentrations of $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ (Ferro) for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V. (B) The graph show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$. 

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Figure 37. NAC treatment reverses mitomycin C-mediated distribution of K-Ras to endomembranes. (A) MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with GFP-K-RasG12V were co-treated with NAC and various concentration of mitomycin C (Mito C) for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V. (B) The graph show mean + S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC_{50}. 
Figure 38. NAC treatment reverses H$_2$O$_2$-mediated distribution of K-Ras to endomembranes. (A) MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with GFP-K-RasG12V were co-treated with NAC and various concentrations of H$_2$O$_2$ for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V. (B) The graph show mean ± S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC$_{50}$.

These data suggest that NAC reduces ROS-mediated K-Ras PM mis-localization. Taken together, these data suggest that C$_{16}$H$_{20}$FeClNO specifically mis-localizes K-Ras from the PM in an ROS-mediated mechanism.

4.3 Specific Aim #3: Dissect the mechanism of C$_{16}$H$_{20}$FeClNO-induced K-Ras PM dissociation
4.3.1 Aim 3-1: Assess the effect of C_{16}H_{20}FeClNO on phosphatidylserine (PtdSer) distribution at the PM

_Rationale_

PtdSer is an anionic phospholipid that is concentrated on the inner leaflet of the PM, and generates a strong negative electrostatic potential for the PM [143-145]. The positively charged PBD, string of six Lys, of K-Ras forms a strong electrostatic interaction with the PM, specifically with the negatively-charged head group of PtdSer, and this, in conjugation with K-Ras C-terminal farnesyl moiety located immediately adjacent to the PBD, stably localizes K-Ras to the PM [145, 146].

![Figure 39. Avicin G and Fendiline mis-localize K-Ras from the PM via PtdSer depletion.](image)

Work previously published by our lab showing that (A) Avicin G, figure adapted from [147], and (B) Fendiline, figure adapted from [161], dissociate both GFP-K-RasG12V and GFP-LactC2 probe from the PM, suggesting both compounds dissociate K-Ras from the PM via PtdSer depletion.
Studies have shown that depleting PtdSer from the PM weakens the electrostatic interaction, resulting in disrupted K-Ras PM binding (Fig. 3) and K-Ras signaling [147, 148, 150, 157, 159]. Taken together, the hypothesis of this sub-aim is that C_{16}H_{20}FeClNO reduces PtdSer contents at the PM, resulting in K-Ras dissociation from the PM.

To examine if C_{16}H_{20}FeClNO or mitomycin C redistributes PtdSer from the PM, confocal microscopy was performed on MDCK cells stably co-expressing mCherry-CAAX and GFP-LactC2, a well-studied PtdSer probe [147, 148, 393]. In control cells, LactC2 probe was predominantly present at the PM (Fig. 40A). After C_{16}H_{20}FeClNO treatment, there was no change in the distribution of GFP-LactC2 (Figs. 40A and B), suggesting that C_{16}H_{20}FeClNO does not disrupt PtdSer enrichment at the PM. In addition, mitomycin C treatment only minimally affected the localization of GFP-LactC2 (Figs. 40A and C), suggesting that
mitomycin C does not disrupt the PtdSer distribution at the PM.

Figure 40. C_{16}H_{20}FeClINO and mitomycin C dissociates K-Ras from the PM independent of PM PtdSer enrichment. (A) MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with GFP-LactC2 probe were treated with DMSO (control), various concentrations of C_{16}H_{20}FeClINO or mitomycin C for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V. (B-C) The graphs show mean ± S.E.M. of Manders coefficients for concentrations tested. These values were plotted on a dose-response plot to estimate an IC\textsubscript{50}.

Taken together, these data suggest that both C_{16}H_{20}FeClINO and mitomycin C mis-localize K-Ras from the PM independent of PtdSer distribution.

4.3.2 Aim 3-2: Assess the effect of C_{16}H_{20}FeClINO on K-Ras phosphorylation at Ser181
Rationale

K-Ras, but not the other Ras isoforms, contains a serine at residue 181 (Ser181), which is able to be phosphorylated by PKC and PKG via independent mechanisms [165-167]. Phosphorylation of Ser181, which is located right next to the PBD, disrupts the electrostatic interaction K-Ras has with the PM, resulting in K-Ras PM dissociation [166, 167]. Thus, the hypothesis of this sub-aim is that C$_{16}$H$_{20}$FeClNO induces K-Ras phosphorylation at Ser181, resulting in K-Ras dissociation from the PM.

To test this, MDCK cells stably co-expressing mCherry-CAAX with a GFP-K-RasG12V-S181A, where Ser181 is mutated to alanine (Ala) to prevent it from being phosphorylated, were
treated with $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ and imaged using confocal microscopy [166, 167].

![Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V.](image)

**Figure 41.** $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ dissociates K-Ras from the PM independent of K-Ras Ser181 phosphorylation. MDCK cells stably co-expressing mCherry-CAAX endomembrane marker with GFP-K-RasG12V-S181A mutant were treated with DMSO (control) or 2 µM $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. The inserted values indicate Manders coefficients that were calculated using ImageJ software to measure the fraction of mCherry-CAAX co-localized with GFP-K-RasG12V.

After $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ treatment, K-RasG12V-S181A was mis-localized from the PM (**Fig. 41**), suggesting that $\text{C}_{16}\text{H}_{20}\text{FeClNO}$-mediated K-Ras PM dissociation is independent of K-Ras phosphorylation at Ser181.

**4.3.3 Aim 3-3: Assess the effect of $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ on K-Ras oxidation**

*Rationale*
ROS have shown to oxidatively modify certain amino acid residues on Ras GTPases, altering their activity (Table 4) [394, 395]. H-Ras undergoes palmitoylation at Cys 181 and 184, which is needed for its trafficking from the Golgi complex to the PM [13, 21, 23, 27, 39]. Cys181 and 184 can also undergo s-glutathionylation which prevents the palmitoylation, keeping H-Ras in the Golgi complex and decreases its signaling (Table 4) [394, 396]. Another residue that undergoes oxidative modification is Cys118, which is located in the nucleotide binding region of the G-domain in all Ras isoforms [394]. In H-Ras, Cys118 undergoes S-nitrosylation by NO or oxidation by O$_2^-$, which promotes the exchange of GDP for GTP, resulting in increased activity (Table 4) [397-399]. The interaction of ROS with Cys118 results in the formation of a Ras Cys118-thiol radical [397, 398]. This Cys118-thiol radical converts the GDP bound to Ras to an unstable 5-nitro-GDP, and this modified nucleotide disrupts interactions with the G-domain of Ras, resulting in dissociation of GDP from Ras and promotion of RasGEF-mediated GTP binding [395, 397, 398]. Most studies have been conducted on H-Ras, with little work being done in other isoforms. One study showed that endothelial nitric oxide synthase (eNOS) signaling can activate N-Ras through Cys118 S-nitrosylation (Table 4) [400].

<table>
<thead>
<tr>
<th>Ras Isoform</th>
<th>Amino Acid Residue(s)</th>
<th>Type of ROS</th>
<th>Effect on Ras activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Ras</td>
<td>Cys181 and 184</td>
<td>s-glutathionylation</td>
<td>decrease</td>
</tr>
<tr>
<td></td>
<td>Cys118</td>
<td>S-nitrosylation by NO/O$_2^-$</td>
<td>increase</td>
</tr>
<tr>
<td>N-Ras</td>
<td>Cys118</td>
<td>NO</td>
<td>increase</td>
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In terms of K-Ras, little work has been done on how oxidation directly affects K-Ras and what residues may be involved. Since ROS can modify H-Ras and C$_{16}$H$_{20}$FeClNO-induced K-Ras
dissociation from the PM may be mediated through ROS, the hypothesis of this sub-aim is that $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ directly oxidizes K-Ras, resulting in K-Ras PM dissociation.

Given that little is known about K-Ras oxidation sites, I generated a list of potential oxidative residues. I identified residues that contained amino acids that are able to undergo oxidation: cysteine, methionine, histidine, tyrosine, phenylalanine and tryptophan (Fig. 42) [237, 401].

**Figure 42. Potential oxidative residues present in K-Ras and H-Ras.** A list of potential oxidative residues present in the G-domain and HVR of K-Ras (red) and H-Ras (blue) was generated. Amino acids able to undergo oxidative modifications: M = methionine, C = cysteine, H = histidine, Y = tyrosine, F = phenylalanine. Residues unique to K-Ras are in bold (Rehl, K.M. et al., *manuscript submitted for publication*).

First, I sought to identify if the residue existed in the C-terminal HVR, the region sufficient for Ras PM binding [39, 161]. MDCK cells stably expressing a GFP-tagged truncated K-Ras that only express its C-terminal HVR (GFP-CTK), were imaged to study its cellular localization after $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ treatment [39, 43, 161]. My data show that $\text{C}_{16}\text{H}_{20}\text{FeClNO}$ had no effect the PM localization of GFP-CTK (Fig. 43), suggesting the target region for ROS-mediated mis-
localization of K-Ras is located within the G-domain.

![Image]

**Figure 43. The HVR does not contain the oxidative residue involved in C<sub>16</sub>H<sub>20</sub>FeClNO-induced K-Ras dissociation from the PM.** MDCK cells stably expressing full length GFP-K-RasG12V or GFP-CTK were treated with DMSO (control) or 2 µM C<sub>16</sub>H<sub>20</sub>FeClNO for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown.

Because C<sub>16</sub>H<sub>20</sub>FeClNO dissociates K-Ras, but not H-Ras, from the PM, I sought to identify residues that are unique to K-Ras and able to undergo oxidative modifications. In the G-domain, K-Ras contains a histidine (His) at residue 95 while H-Ras has a glutamine (Gln) (Fig. 42), which is not oxidatively modified [237, 401]. To examine if His95 is involved in the ROS-induced K-Ras PM dissociation, I generated MDCK cells expressing GFP-K-RasG12V-H95A mutant, which does not undergo oxidative modifications, and -H95C mutant, to serve as a positive control since cysteine is known to undergo oxidative modifications, being even more sensitive than histidine [237, 238]. Both confocal and electron microscopy data show that
C$_{16}$H$_{20}$FeClINO had no effect on the PM localization of H95A mutant (Figs. 44A and B), suggesting this may be the oxidative residue involved in C$_{16}$H$_{20}$FeClINO-induced PM dissociation.

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**Figure 44.** Histidine 95 is the oxidative residue involved in C$_{16}$H$_{20}$FeClINO-induced K-Ras dissociation from the PM. (A) MDCK cells expressing GFP-K-RasG12V, -K-RasG12V-H95A or -K-RasG12V-H95C were treated with DMSO (control), 2 µM C$_{16}$H$_{20}$FeClINO or 2 µM C$_{16}$H$_{20}$FeClINO with 500 µM NAC for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. (B-C) Basal PM sheets prepared from these cells were treated with DMSO (control) or 2 µM C$_{16}$H$_{20}$FeClINO (Ferrocene) for 48 hours and labeled with anti-GFP-conjugated 4.5 nm gold particles and visualized with EM. The graph shows a mean ± S.E.M. Students’ t-tests was used to assess significant differences between DMSO-treated and C$_{16}$H$_{20}$FeClINO-treated cells (n.s. = not significant).
Interestingly, C₁₆H₂₀FeClINO also had no effect on the PM localization of the H95C mutant (Figs. 44A and C).

To further examine if His at the 95th position is involved in ROS-induced Ras PM binding in general, I generated MDCK cells expressing GFP-H-RasG12V-Gln95His (Q95H) mutant and imaged them after C₁₆H₂₀FeClINO treatment. My data reveal that while H-RasG12V-Q95H predominantly localizes to the PM in control cells (Fig. 45A), C₁₆H₂₀FeClINO treatment induced dissociation of H-RasG12V-Q95H mutant from the PM (Figs. 45A and B). Quantitative EM also demonstrates C₁₆H₂₀FeClINO reduced the level of H-RasG12V-Q95H mutant at the PM, which was further reversed by NAC supplementation (Fig. 45B). Furthermore, the H-RasG12V-Q95H mutation itself does not dissociate H-Ras from the PM, suggesting the dissociation of H-
RasG12V-Q95H mutant is from C_{16}H_{20}FeClNO treatment (Fig. 45C).

**Figure 45.** C_{16}H_{20}FeClNO dissociates the H-RasQ95H mutant from the PM. (A) MDCK cells expressing either GFP-K-RasG12V, GFP-H-RasG12V or -H-RasG12V-Q95H mutant were treated with DMSO or 2 μM C_{16}H_{20}FeClNO for 48 hours. Cells were fixed with 4% PFA and imaged using a confocal microscope. Representative images from three independent experiments are shown. (B) Basal PM sheets prepared from these cells were treated with DMSO (control), 2 μM C_{16}H_{20}FeClNO (Ferrocene) or 2 μM C_{16}H_{20}FeClNO with 500 μM NAC for 48 hours and labeled with anti-GFP-conjugated 4.5 nm gold particles and visualized with EM. The graphs show a mean ± S.E.M. Students t-tests were used to assess significant differences between DMSO-treated and drug-treated cells (*p<0.02, n.s. not significant). (C) Basal PM sheets
prepared from GFP-H-RasG12V or -H-RasQ95H mutant were treated with DMSO (control) for 48 hours and labeled with anti-GFP-conjugated 4.5 nm gold particles and visualized with EM. The graphs show a mean + S.E.M. Students t-tests were used to assess significant differences between H-RasG12V and H-Ras-Q95H-mutant cells (n.s. = not significant).

Taken together, my data suggest that cellular ROS regulates the stable K-Ras PM binding by oxidatively modifying the His95 residue.

In conclusion, my experimental data demonstrate that C_{16}H_{20}FeClNO blocks the growth of K-Ras-dependent human PDACs and NSCLCs. It also disrupts the PM binding and signaling of K-Ras, but not other Ras isoforms, via elevation of cellular ROS levels. Moreover, I have identified that K-Ras His95 is responsible for the ROS-mediated inhibition of K-Ras PM binding and K-Ras signaling. These data suggest that C_{16}H_{20}FeClNO provide a platform for developing anti-K-Ras therapies for treating K-Ras-driven cancers.
5. **Discussion**

Constitutively active mutations of Ras occur in ~19% of all human cancers, with ~75% of those being in K-Ras [91]. There are FDA-approved direct inhibitors for the K-RasG12C mutation [26, 109, 111]. However, the K-RasG12C oncogenic mutant is found only in a small sub-set of K-Ras-driven cancers, leaving them ineffective against other oncogenic mutations in K-Ras [13]. Additionally, patients have already started to develop resistance to these inhibitors [110, 112, 113]. Thus, a need exists for a universal anti-K-Ras inhibitor. C$_{16}$H$_{20}$FeClNO is a novel ferrocene derivative whose mechanism was unknown. In my dissertation, I have identified that C$_{16}$H$_{20}$FeClNO inhibits the growth of K-Ras-dependent cancer cells and disrupts the signaling of oncogenic mutant K-Ras, but not other Ras isoforms, suggesting it may serve as an anti-K-Ras inhibitor.

**Role of ROS and antioxidants on K-Ras signaling**

In this study, my experimental data demonstrate that C$_{16}$H$_{20}$FeClNO disrupts the K-Ras/MAPK signaling pathway but not the K-Ras/PI3K/Akt signaling pathway. A previous study showed that K-Ras is a more potent recruiter of Raf to the PM and activator of the Raf/MAPK signaling than H-Ras, while, H-Ras is a more potent activator of the PI3K/Akt signaling [73]. This may be explained by how the two isoforms attach to the membrane and the specific sequences present in their C-terminal HVRs, the region required for stable PM binding [73]. K-Ras and Raf, but not H-Ras, co-localize with PtdSer in the PM for their stable PM binding. It is possible that Raf is in closer proximity to K-Ras than H-Ras and thus, more accessible to K-Ras for its activation [73]. Thus, it is plausible that C$_{16}$H$_{20}$FeClNO-induced PM dissociation of K-Ras, but not H-Ras (discussed below), fails to activate the Raf/MAPK signaling without perturbing H-Ras-mediated PI3K/Akt signaling. Additionally, PI3K can be activated independently of Ras, through direct
interaction with G-protein-coupled receptors or insulin growth factor receptor signaling [72].

Thus, it is plausible that the PI3K/Akt signaling is still able to be activated even when C_{16}H_{20}FeClNO inhibits Ras signaling. For future directions, it may be worth testing the efficacy of inhibitors for the PI3K/Akt pathway in conjunction with C_{16}H_{20}FeClNO to further target oncogenic mutant K-Ras signaling. However, co-inhibition of both pathways has shown high toxicity, so that would have to evaluated [24, 25, 127-129].

C_{16}H_{20}FeClNO treatment resulted in no detectable levels of cleaved caspase-3 or cleaved PARP-1, suggesting that C_{16}H_{20}FeClNO disrupts the K-Ras/MAPK signaling pathway independent of apoptosis. Ferrocene derivatives are known to exert their effects via elevation of cellular ROS [274, 329, 343, 345]. Co-treatment of C_{16}H_{20}FeClNO with NAC rescued ppERK levels, suggesting C_{16}H_{20}FeClNO disrupts the K-Ras/MAPK signaling through an ROS-mediated mechanism. Furthermore, mitomycin C, an ROS-elevating drug, disrupted the K-Ras/MAPK and PI3K/Akt signaling pathways. While extensive studies have reported the role of K-Ras signaling on the regulation of oxidative homeostasis, my study reveals the direct effects of redox balance on K-Ras PM binding and K-Ras signaling, which may provide an additional mechanism for the recently reported antioxidant/BACH1/glycolysis-promoted metastasis of K-Ras-driven NSCLC (Fig. 46) [293, 402]. Under oxidative stress, the oxidation of heme-containing proteins releases free heme, which stimulates degradation of BACH1 (BTB and CNC homology 1), a pro-metastatic transcriptional factor [402, 403]. Prolonged supplementation of antioxidants decreases cellular ROS levels, which lowers free heme and stabilizes BACH1 (Fig. 46) [293]. Endogenous antioxidants, via hyperactivation of NRF2 or loss of KEAP1, upregulates heme oxygenase-1, which degrade free heme, resulting in the BACH1-dependent metastasis of K-Ras-driven NSCLC (Fig. 46) [293, 404]. The elevated BACH1 further promotes glycolysis by upregulating
GAPDH and hexokinase-2, resulting in enhanced metastasis in K-Ras-driven NSCLC (Fig. 46) [293]. Thus, my data adds the role of oncogenic K-Ras in this model of antioxidant/BACH1/metastasis signal axis in K-Ras-driven NSCLC. Here, I propose that elevation of cellular antioxidants prevent oxidation of His95 of oncogenic K-Ras, enhancing its binding to the PM (discussed below) and signal output in K-Ras-driven NSCLC (Fig. 46). The elevated K-Ras signaling further enhances the expression of NRF2, and thereby NRF2-mediated antioxidant program (Fig. 46). This in turn, promotes the antioxidant/BACH1/metastasis signal axis [284, 405, 406]. Additionally, oncogenic K-Ras signaling hyperactivates Ras downstream pathways regulating glycolysis and metastasis, further contributing to the BACH1-induced NSCLC metastasis (Fig. 46) [18, 25].

Figure 46. The role of K-Ras in antioxidant/BACH1/glycolysis-induced NSCLC metastasis. Elevation of cellular antioxidants lower cellular ROS levels, which decreases free heme, leading to BACH1 stabilization. BACH1 upregulates transcription of enzymes involved in glycolysis, enhancing NSCLC-metastasis. Elevated cellular antioxidants prevent oxidation of His95, resulting in enhanced K-Ras PM binding and elevated oncogenic K-Ras signaling. The elevated K-Ras signaling elevates NRF2 expression, which not only increases cellular antioxidant levels, but also upregulates heme oxygenase-1 (Ho1), which degrades free heme, promoting BACH1 stabilization. Additionally, oncogenic K-Ras signaling promotes glycolysis and metastasis in K-Ras-driven NSCLC. (Rehl, K.M. et al, manuscript submitted for publication).
\textbf{C}_{16}\text{H}_{20}\text{FeClNO inhibits the growth of K-Ras-dependent cancers}

C\textsubscript{16}H\textsubscript{20}FeClNO inhibits the growth of PDAC and NSCLC cell lines expressing oncogenic mutant K-Ras while having little to no impact on cancer cell lines expressing WT-K-Ras. Thus, C\textsubscript{16}H\textsubscript{20}FeClNO specifically targets K-Ras-driven cancer cells, which are cancer cells that are dependent on K-Ras signaling for their growth and survival [13, 92, 95-98]. The outlier here was A549, a NSCLC cell line, which despite having the oncogenic K-RasG12S mutation, its growth was not impacted by C\textsubscript{16}H\textsubscript{20}FeClNO treatment. An explanation for this is that A549 cell line is actually a “K-Ras-independent” cell line [95, 167]. A study showed that ablation of K-Ras in A549 cell line had no effect on Ras downstream signaling and their growth, suggesting that the growth and survival of A549 cell line is not dependent on K-Ras signaling [95]. This would indicate that C\textsubscript{16}H\textsubscript{20}FeClNO selectively inhibits the growth of K-Ras-driven cancers. Another explanation is that the growth of A549 cell line is very robust and has resistance to numerous drugs such as cisplatin and taxol [407, 408]. Additionally, one study showed that MEK inhibition alone in A549 cells was not enough to impact their growth and required dual targeting of the EGFR receptor, which decreases the PI3K/Akt signaling, for inhibiting their growth [409]. Thus, perhaps other inhibitors that perturb other essential signaling pathways, like the PI3K/Akt signaling, are needed to target A549 cell line, given that C\textsubscript{16}H\textsubscript{20}FeClNO only inhibits the K-Ras/MAPK signaling. Lastly, other mutations present in A549 cells could impact its sensitivity to C\textsubscript{16}H\textsubscript{20}FeClNO. This sentiment can be applied to all the other cancer cell lines tested as well, given they exhibited varying degrees of sensitivity.

Another ROS-elevating agent, mitomycin C, inhibited the growth of both K-Ras-dependent and -independent PDAC and NSCLC cells, suggesting a lack of specificity towards K-Ras-
dependent cancers. This notion has been supported in a study showing that K-Ras mutation status had no impact on cancer patients’ responses to mitomycin C treatment [410]. Thus, it is possible that mitomycin C’s inhibition on Ras signaling is mediated by perturbing multiple mechanisms. In fact, mitomycin C is a known DNA-alkylating agent, forming DNA cross-links to inhibit DNA synthesis [384]. Thus, the inhibited growth observed in both K-Ras-dependent and -independent cancer cells may be a result of DNA-alkylation, together with disrupted K-Ras signaling by elevated ROS production.

In order to develop C_{16}H_{20}FeClNO as an anti-K-Ras drug for treating K-Ras-driven cancers, its toxicity needs to be further explored. In my proliferation assays, BxPC-3, H522, H1975 and H1299 were used as controls, but they still were cancer cell lines, even though they expressed WT K-Ras. Thus, it is necessary to test the effect of C_{16}H_{20}FeClNO on the growth of normal, non-cancerous cell lines; for example, BEAS-2B, a human bronchial epithelial cell line, and HPDE6c7, a human pancreatic duct epithelial cell line [411, 412]. Cancer cells exhibit increased oxidative stress and are more sensitive to increases in cellular ROS levels [192, 274]. In comparison, normal cells have a higher capacity to adapt to changes in cellular ROS levels, and thus, less likely to be affected by ROS elevation [274, 413]. Given this, it would be expected that C_{16}H_{20}FeClNO would have minimal, if any, effects on the growth of normal cell lines at the concentrations that significantly blocked the growth of cancer cells. In the panel of human PDAC and NSCLC cell lines, 50 µM was the concentration of C_{16}H_{20}FeClNO that significantly decreased their growth. However, in assays of K-Ras signaling, K-Ras PM localization and cellular ROS levels, 2 µM of C_{16}H_{20}FeClNO was used. These assays were performed in our model cell line, MDCK cells, which is a canine kidney cell line. When 2 µM C_{16}H_{20}FeClNO was
initially used for the human cancer cell line panel, it had no effect on its growth, suggesting a higher concentration was needed for human cells.

However, the need of 50 µM of any drugs \textit{in vitro}, is too high to be feasible or safe for patient use. For example, in H358 and MIA-PaCa2 cells harboring K-RasG12C mutant, sotorasib almost completely inhibited ppERK levels after 2 hours with IC$_{50}$ ≈ 30 nM and inhibited their growth with IC$_{50}$ ≈ 6 nM and 9 nM, respectively [108]. In those same cell lines, 100 nM adagarsib showed almost complete inhibition of ppERK levels after 6 hours with IC$_{50}$ ≈ 10.5 nM, and inhibited their growth with IC$_{50}$ values ranging between 10 and 1,000 nM [110, 111]. Overall, both sotorasib and adagarsib are more potent when compared to C$_{16}$H$_{20}$FeClNO, which inhibits MIA-PaCa2 and H358 cell growth with IC$_{50}$ ≈ 11.3 µM and 17.5 µM, respectively. Furthermore, ferrocifen, a ferrocene derivative that is currently in pre-clinical trials, was shown to inhibit the growth of MCF-7 and MDA-MB-231 breast cancer cell lines with IC$_{50}$ ≈ 0.5-1 µM [317]. This suggests that 50 µM of C$_{16}$H$_{20}$FeClNO is too high and its potency needs to be increased, at the \textit{in vitro} level, before it can be furthered for \textit{in vivo} work, like patient-derived xenografts.

His95 in K-Ras has been previously reported to play a role in the mechanism of K-RasG12C inhibitors. Sotorasib and adagarsib bind to S-IIP, an allosteric binding pocket that is only accessible when K-RasG12C mutant is bound to the GDP, and extend from there to irreversible bind to the mutant Cys, locking it in the inactive form [26, 104, 105]. In addition to S-IIP pocket, these inhibitors form van der Waals contacts with a groove consisting of His95-Tyr96-Glu99, which helps stabilize the inhibitors and increase their potency [26, 99, 108, 111]. These inhibitors decreased the viability of K-RasG12C-driven cancer cell lines over K-Ras-driven cancers harboring different oncogenic mutations in K-Ras [26, 104]. However, my data show that C$_{16}$H$_{20}$FeClNO inhibited the growth of cancers containing not just K-RasG12C mutation,
but also K-RasG12V and G12D mutation. This lack of specificity suggests that C_{16}H_{20}FeClNO is not a K-RasG12C inhibitor and does not bind to the S-IIP pocket and His95 groove.

NSCLC patients have already started to exhibit resistance to sotorasib and adagrasib [110, 112, 113]. One mechanism of resistance is an adaptive feedback loop, where loss of oncogenic mutant K-RasG12C signaling leads to reactivation of RTK-mediated signaling via wild-type K-Ras or GTP-bound N-Ras and H-Ras [110, 112, 113]. There can also be mutations in the downstream signaling effectors themselves which confer resistance to these K-RasG12C direct inhibitors [112, 113]. Another mechanism is that secondary K-Ras mutations can be acquired in the S-IIP binding pocket, such as Y96D, that impact the binding and stability of the inhibitor [112]. Given that C_{16}H_{20}FeClNO is not a direct K-RasG12C inhibitor, its activity would not be impacted by mutations present in the S-IIP binding pocket and thus, would still be able to target K-RasG12C cancer cells resistant to sotorasib and adagrasib. Additionally, sotorasib and adagrasib exhibited selectivity towards K-RasG12C-driven cancer cell lines [26, 104]. C_{16}H_{20}FeClNO, however, inhibited the growth of not only K-RasG12C-driven cancer cell lines but also K-RasG12D and K-RasG12V. Thus, C_{16}H_{20}FeClNO serves as a pan anti-K-Ras drug for all K-Ras-driven cancers.

C_{16}H_{20}FeClNO dissociates K-Ras from the PM independent of known mechanisms

Given that the PM localization of K-Ras is essential for K-Ras signaling, I tested to see if C_{16}H_{20}FeClNO dissociates K-Ras from the PM. I found that C_{16}H_{20}FeClNO translocates K-Ras, but not other Ras isoforms, from the PM to endomembranes. Furthermore, C_{16}H_{20}FeClNO treatment significantly reduces the membrane-associating of K-Ras, but not H-Ras, while it increases K-Ras present in the cytosol. C_{16}H_{20}FeClNO also specifically reduces the abundance of K-Ras at the PM. Together, my data demonstrate that C_{16}H_{20}FeClNO specifically disrupts K-Ras-PM interaction. After C_{16}H_{20}FeClNO treatment, I showed that K-Ras translocates to the
early endosome, late endosome, ER, Golgi and lysosome, but not the mitochondria. The lack of specificity for cellular organelles to which K-Ras translocates to after PM dissociation has been documented in previous studies [147, 148, 161, 165]. It is unclear whether C_{16}H_{20}FeClNO mis-localizes K-Ras from the PM by directly dissociating K-Ras from the PM and/or disrupting K-Ras forward trafficking from the ER to the PM after the post-translational modifications. To distinguish between the two, live-cell imaging could be performed to study the forward trafficking of newly synthesized K-Ras (unpublished protocol from Dr. Carol Williams from Medical College of Wisconsin). Cells treated with C_{16}H_{20}FeClNO would be transfected with GFP-K-RasG12V and chased after transfection in the continuous presence of C_{16}H_{20}FeClNO. If GFP-K-RasG12V does not reach the PM, it would mean that C_{16}H_{20}FeClNO disrupts K-Ras forward trafficking.

One mechanism to dissociate K-Ras from the PM is to deplete PtdSer content from the PM [147, 148, 150, 157, 159]. I showed that C_{16}H_{20}FeClNO treatment maintained the PM localization of the PtdSer probe, LactC2. Mitomycin C appeared to have no effects on PtdSer PM localization at the concentration that dissociates K-Ras from the PM, and had minimal effects on PtdSer PM localization at higher concentrations. In addition, the immunoblot data showed that C_{16}H_{20}FeClNO, at the concentration that dissociates K-Ras from the PM, does not induce apoptosis, in which PtdSer gets externalized to the outer leaflet of PM [414]. Together, my data suggest that C_{16}H_{20}FeClNO does not redistribute PtdSer from the inner leaflet to the outer PM leaflet and other endomembranes, and that C_{16}H_{20}FeClNO-induced K-Ras PM dissociation is independent of PM PtdSer abundance.

Another mechanism regulating K-Ras PM binding is enhancing K-Ras phosphorylation at Ser181 [165-167]. When K-Ras Ser181 residue is phosphorylated by PKC or PKG, K-Ras
dissociates from the PM and translocates to endomembranes [165-167]. The observation that C$_{16}$H$_{20}$FeClNO dissociates K-RasG12V-S181A mutant from the PM, suggests that C$_{16}$H$_{20}$FeClNO dissociates K-Ras from the PM independent of K-Ras phosphorylation at Ser181. Previous experiments have shown that phosphorylation of K-Ras by PKC induces K-Ras translocation from the PM to mitochondria, as well as the ER and Golgi [165]. However, my data show that C$_{16}$H$_{20}$FeClNO had no co-localization with the mitochondria marker, suggesting it does not translocate to mitochondria. Furthermore, PKC-induced translocation of K-Ras to mitochondria results in the interaction with pro-apoptotic marker Bcl-Xl and subsequent induction of apoptosis [165]. Not only did C$_{16}$H$_{20}$FeClNO not translocate to mitochondria, it also did not induce apoptosis at the concentration that dissociates K-Ras from the PM. Thus, these data support the confocal data that C$_{16}$H$_{20}$FeClNO dissociates K-Ras from the PM independent of Ser181 phosphorylation, at least by PKC. PKG can also phosphorylate K-Ras via the AMPK/eNOS/PKG signaling pathway [166]. However, given that ferrocene derivatives are not known to generate NO, at least not directly, it is unlikely that C$_{16}$H$_{20}$FeClNO activates PKG through its elevation of cellular ROS and thus, does not dissociate K-Ras from the PM via Ser181 phosphorylation by PKG. C$_{16}$H$_{20}$FeClNO also contains an α,β-unsaturated carbonyl system, which was also present in the chalcone derivatives, which inhibit oncogenic mutant K-Ras signaling and cancer cell growth [167]. These chalcone derivatives dissociated K-Ras from the PM via PKC-induced K-Ras phosphorylation at Ser181 [167]. Given that C$_{16}$H$_{20}$FeClNO does not dissociate K-Ras via Ser181 phosphorylation, this suggests that perhaps the α,β-unsaturated carbonyl system is not the reason for PKC activation. The chalcone derivatives also contained a 3,4,5-trimethoxy moiety, which has shown anti-cancer properties [167]. Thus, perhaps this moiety is the reason for the PKC-induced dissociation of K-Ras by the chalcone.
derivatives and the reason why it is not seen with C\textsubscript{16}H\textsubscript{20}FeClNO. Or, perhaps the $\alpha,\beta$-unsaturated carbonyl system is involved, but the ROS generated by C\textsubscript{16}H\textsubscript{20}FeClNO’s ferrocene moiety overpowers it.

**Role of ROS on K-Ras PM Localization**

Given that ROS-elevating agents such as mitomycin C disrupted K-Ras signaling, I tested the effects of other ROS-elevating agents on K-Ras PM localization, which is essential for K-Ras signaling. I found that mitomycin C, carmustine and H\textsubscript{2}O\textsubscript{2} dissociate K-Ras, but not H-Ras, from the PM, and NAC supplementation reversed the PM dissociation of K-Ras, suggesting that C\textsubscript{16}H\textsubscript{20}FeClNO dissociates K-Ras from the PM through an ROS-mediated mechanism. Given that C\textsubscript{16}H\textsubscript{20}FeClNO is a novel ferrocene derivative, it was unknown if it elevated cellular ROS levels. I further found that C\textsubscript{16}H\textsubscript{20}FeClNO significantly elevates cellular ROS levels at 2 $\mu$M, the same concentration at which it disrupts K-Ras signaling and dissociates K-Ras from the PM. Together, these data suggest that the disrupted K-Ras PM binding and signaling by C\textsubscript{16}H\textsubscript{20}FeClNO treatment is via elevated cellular ROS levels.

To measure cellular ROS levels after C\textsubscript{16}H\textsubscript{20}FeClNO treatment, H\textsubscript{2}DCFDA probe was used, which reacts to all cellular ROS. Thus, it is unclear which form of ROS is produced by C\textsubscript{16}H\textsubscript{20}FeClNO. Ferrocene derivatives are known to generate general, non-specific cellular ROS [210, 317, 328, 333]. It occurs through the Fenton reaction, which involves Fe\textsuperscript{2+} + H\textsubscript{2}O\textsubscript{2} turning into Fe\textsuperscript{3+} + OH\textsuperscript{-} + OH• [210, 318, 326]. This reaction is reversible, leading to continuous generation of OH• and H\textsubscript{2}O\textsubscript{2} [274, 326]. Also, my data demonstrate that H\textsubscript{2}O\textsubscript{2} is one of the ROS involved in K-Ras PM dissociation. For a more specific-measurement of H\textsubscript{2}O\textsubscript{2}, imaging with the fluorescent HyPer sensor, a H\textsubscript{2}O\textsubscript{2}-specific probe, could be used [415]. Alternatively, it is possible that OH• is the ROS molecule involved. To detect OH•, the fluorescent probe 3’-(p-
Aminophenyl) fluorescein (APF) could be used as it specifically interacts with hydroxyl radicals and not H$_2$O$_2$ [416].

**Identification of His95 as the oxidative residue involved in C$_{16}$H$_{20}$FeClNO-induced K-Ras dissociation from PM**

ROS have shown to oxidatively modify certain amino acids residues on Ras GTPases, altering their activity [394, 395]. Thus, I sought to identify amino acid residues unique to K-Ras in which C$_{16}$H$_{20}$FeClNO induces K-Ras dissociation from the PM. I identified His95 in the G-domain, a residue unique to K-Ras, as the oxidative residue involved in C$_{16}$H$_{20}$FeClNO-induced dissociation of K-Ras from the PM. C$_{16}$H$_{20}$FeClNO treatment had no effect on the PM localization of K-Ras His95Ala mutant, suggesting an involvement of this residue. Furthermore, C$_{16}$H$_{20}$FeClNO treatment on H-Ras Glu95His mutant resulted in its dissociation from the PM and this was reversed by treatment with NAC. The oxidation of histidine to 2-oxohistidine can occur by hydroxyl radicals generated through a metal-catalyzed reaction [237, 238, 240]. Given that ferrocene derivatives generate ROS via the metal-based Fenton reaction, it is plausible that C$_{16}$H$_{20}$FeClNO can oxidize K-RasHis95 [210, 318, 326]. Further studies are needed to confirm that K-Ras His95 is oxidatively modified by C$_{16}$H$_{20}$FeClNO; for example, performing mass spectrometry on a purified oncogenic mutant K-Ras protein after C$_{16}$H$_{20}$FeClNO treatment [399]. To further validate that His95 is the oxidative residue through which C$_{16}$H$_{20}$FeClNO blocks K-Ras-driven cancer growth, CRISPR/Cas9 could be used to generate endogenous K-Ras His95Ala mutant in K-Ras-dependent human PDAC and NSCLC and cell proliferation assays could be performed. I expect that C$_{16}$H$_{20}$FeClNO will not inhibit the growth of these K-Ras-dependent cancer cells harboring endogenous K-Ras His95Ala mutant.
In the course of identifying the novel residue(s) of K-Ras that are oxidatively modified by ROS, Cys118 was considered after C$_{16}$H$_{20}$FeClNO showed no effect on the PM localization of CTK, suggesting the oxidative residue was present in the G-domain. Cys118 is present in all of the Ras isoforms and is solvent accessible. In H-Ras and N-Ras, oxidation of Cys118 increases their signaling [394, 397-400]. However, my data show that C$_{16}$H$_{20}$FeClNO leads to reduced K-Ras signaling. This suggests that Cys118 is not oxidized by C$_{16}$H$_{20}$FeClNO, or if it is, oxidation of Cys118 differentially regulates signaling activity of Ras isoforms. Thus, this supports the notion that a residue unique to K-Ras, His95, is involved. Another residue that can be considered is Met170, a residue unique to K-Ras that is present in the C-terminal HVR, which is involved in membrane binding. GFP-CTK used for this project contains the HVR of K-Ras (residues 165-188) and GFP at the N-terminus. It is possible that the GFP located in close proximity to Met170 blocks oxidative modification of Met170, thereby maintaining the PM binding of GFP-CTK upon C$_{16}$H$_{20}$FeClNO treatment. This could be investigated in the future by generating a full-length K-RasG12V-M170A mutant and performing confocal imaging after C$_{16}$H$_{20}$FeClNO treatment. If C$_{16}$H$_{20}$FeClNO treatment dissociates the full-length M170A from the PM, this would further support that His95 in the G-domain is the oxidative residue involved in C$_{16}$H$_{20}$FeClNO-induced K-Ras dissociation from the PM.

**Role of K-Ras His95 residue in K-Ras PM Orientation**

Since cysteine is more sensitive to oxidation than histidine, I included K-Ras His95Cys mutant as a positive control that would dissociate from the PM after C$_{16}$H$_{20}$FeClNO treatment [238]. However, my data showed that K-Ras His95Cys did not dissociate from the PM after C$_{16}$H$_{20}$FeClNO treatment. One explanation is that K-Ras has been reported to form a homodimer through hydrogen bonding between lysine residue at 101$^{st}$ position (K101) and glutamate
at 107th (E107) [417]. When both sites were mutated to cysteine, they formed a disulfide bond, which stabilized the dimer, leading to enhanced PM binding [417]. Thus, it is possible that His95Cys mutant forms a dimer with a nearby cysteine of K-Ras, which prevent K-Ras from C_{16}H_{20}FeClNO-induced oxidative modification, resulting in enhanced PM binding. To investigate this possibility, immunoblotting of GFP-K-Ras His95Cys could be performed using native PAGE gels, a technique to detect K-Ras dimer/oligomerization [417]. Furthermore, His95 is located near K101/E107 residues, which are involved in K-Ras dimerization [417]. Due to the close proximity, it is possible that His95 may be involved in stabilizing the dimer and its oxidation prevents this, resulting in K-Ras dissociation from the PM.

Another possible explanation could be the different size of sides chains for histidine and cysteine. Computational modeling performed by our collaborator, Dr. Alex-Gorfe at the University of Texas-Houston, shows that in different membrane-bound orientations of K-Ras protein, the relatively large side chain of His95 is solvent accessible (Figs. 47A and B), suggesting it would be exposed to ROS [418]. By using Swiss modeling software (SIB Swiss Institute of Bioinformatics), substituting the histidine to cysteine resulted in a smaller side chain (Figs. 47C and D). Thus, it is possible that the side chain of cysteine may be buried in the K-Ras
molecule, which prevents it from being oxidatively modified, maintaining the PM binding.

Figure 47. **Location of His95 in the model of the K-Ras structure.** Computational modeling of K-Ras was done by our collaborator Dr. Alex Gorfe (University of Texas-Houston) showing that (A) the side chain of His95 is solvent accessible and (B) in different membrane-bound orientations of K-Ras, His95 can be solvent accessible or close to the PM. Figure adapted from [418]. Swiss modeling was done showing (C) K-Ras His95 and (D) K-Ras His95Cys mutant.

K-Ras binds to the PM via its farnesyl tail and its PBD that forms an electrostatic interaction with the inner PM leaflet. When K-Ras is bound to the PM, it is dynamic, existing in different orientations (Fig. 47B) [418]. A previous study has reported that in these different orientations of oncogenic K-Ras bound to the PM, its catalytic domain, which is present in the G-domain, interacts with the PM [418]. One of the orientation states shows that helices 3 and 4, in which
His95 is located, are involved in the PM interaction [417, 418]. Thus, in certain orientations, His95 of oncogenic K-Ras interacts with the PM. Oxidation of His95 could perturb this interaction, resulting in K-Ras PM dissociation. Also, it is possible that in the other orientation where His95 is solvent accessible, it gets oxidized and then becomes unable to interact with the PM.

**Role of His95 on K-Ras PM transport by PDE6δ**

The exact mechanism by which K-Ras His95 oxidation perturbs the PM binding is unclear. However, since C_{16}H_{20}FeClNO dissociates K-Ras4B, but not K-Ras4A, it suggests it may involve PDE6δ, the chaperone protein for Ras isoforms. PDE6δ contains a hydrophobic pocket that binds the farnesyl moiety of depalmitoylated H- and N-Ras, and K-Ras [46, 54]. After capturing the farnesyl moiety, PDE6δ directly interacts with residues 180-184 of K-Ras, after K-Ras gets dissociated from the PM [46, 55]. PDE6δ has an allosteric site that binds to the small GTPase Arl2 in the GTP-bound state and Arl2 catalyzes the dissociation of K-Ras from PDE6δ to the perinuclear membranes [46, 56]. K-Ras then binds to the recycling endosome (RE) through an electrostatic interaction with the RE’s negatively charged membrane, and returns to the PM [46, 54]. K-Ras4A does not bind to PDE6δ due to steric hinderance caused by two lysine residues immediately prior to the farnesylated cysteine [16, 55]. Although K-Ras residues 180-184 are involved in the PDE6δ binding, and that it is unlikely that His95 residue directly interacts with PDE6δ, it is plausible that oxidatively modified His95 of K-Ras interferes with the Arl2 binding to the K-Ras/ PDE6δ complex, which delays K-Ras release from PDE6δ, and thereby perturbing Ras transport to the PM [46, 53, 55]. Alternatively, the differences in K-Ras and K-Ras4A PM targeting signals may account for the C_{16}H_{20}FeClNO-induced PM dissociation of K-Ras, but not K-Ras4A. While K-Ras has the farnesyl moiety (Cys 185) and PBD in the C-
terminus, K-Ras4A contains farnesyl (Cys 186) and palmitoyl (Cys 180) moieties with two additional PBD (residues 167-170 and 182-185) [13, 16, 21, 23, 27, 39, 48]. This additional palmitoyl moiety may allow stronger interaction of K-Ras4A with the PM even after oxidative modification of His95.

In conclusion, I have demonstrated that a novel ferrocene derivative, C$_{16}$H$_{20}$FeClINO, specifically disrupts the K-Ras/MAPK signaling and inhibits the growth of K-Ras-driven pancreatic and lung cancer cell lines. Furthermore, C$_{16}$H$_{20}$FeClINO dissociates K-Ras, but not other Ras isoforms, from the PM through an ROS-mediated mechanism. I have also identified K-Ras His95 as a novel oxidative residue involved in K-Ras-PM interaction. Taken together, this work identifies that C$_{16}$H$_{20}$FeClINO has anti-cancer properties and provides the groundwork for its development as an anti-K-Ras drug for treating K-Ras-driven human cancers.
6. References


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