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Recent advances and limitations in the application of kahalalides for the control of cancer

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ABSTRACT

Since the discovery of the kahalalide family of marine depsipeptides in 1993, considerable work has been done to develop these compounds as new and biologically distinct anti-cancer agents. Clinical trials and laboratory research have yielded a wealth of data that indicates tolerance of kahalalides in healthy cells and selective activity against diseased cells. Currently, two molecules have attracted the greates level of attention, kahalalide F (KF) and isokahalalide F (isoKF, Irvalec, PM 02734, elisidepsin). Both compounds were originally isolated from the sarcoglossan mollusk *Elysia rufescens* but due to distinct structural characteristics it has been hypothesized and recently shown that the ultimate origin of the molecules is microbial. The search for their true source has been a subject of considerable research in the anticipation of finding new analogs and a culturable expression system that can produce sufficient material through fermentation to be industrially relevant.

1. Introduction

Cancer is recognized as a disease of aging and the second leading cause of death world-wide with the highest prevalence in countries with the highest life expectancy, education, and standard of living. In 2014, the American Society of Clinical Oncology (ASCO) announced that obesity will exceed tobacco use as a number 1 risk factor for cancer. One out of two males and one out of three females will develop cancer within their lifetime. While males have a higher mortality rate than females (189.5 per 100,000 vs. 135.7 per 100,000, respectively), the total cancer death rate is 158.5 per 100,000 for all males and females. Among men, 43% of new cancers diagnosed are composed of prostate, lung, and colorectal cancers with breast, lung, and colorectal cancers accounting for 50% of new diagnoses in females (National Cancer Institute, 2021) [1].

It has been half century since President Nixon declared a war on cancer and since that time 606,520 Americans have still died from cancer in 2021 [2]. A vast search for new therapies has been developed using every imaginable available tool. While new and emerging approaches have generated some successes in cancer treatment. Natural

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products, including biologics, botanicals, semisynthetics, synthetics with natural pharmacophores, and natural product mimics, make up about 71% of newly approved anticancer drugs. These compounds are often derived from microbes and plants and have frequently proven to be highly efficacious [3]. It is noteworthy that docetaxel derived from the Pacific Yew tree remains the most frequently prescribed anticancer drug on the market. This is due in part to evolutionary selection for more potent toxins related to factors such as the sessile nature of some of the more than 700,000 species that live in the ocean [4].

2. Background

Kahalalide F (KF) is a cyclic depsipeptide with a long linear lipopeptide moiety originally isolated in 1993 from the herbivorous sarcoglossan mollusk *Elysia Rufecens* found in Blackpoint Bay, HI. [5] It was later discovered that KF was present in the sea creature's diet of green algae *Bryopsis pennata* and *B. plumosa*. [6] KF is the most active constituent from a class of compounds called kahalalides, a structurally distinct class of compounds exemplified by an endocyclic depsipeptide bond and a variable fatty acid tail of the *N*- terminus, as well as D and modified amino acids such as dehydroaminobutyric acid with ornithine as the single positive residue. KF is a C75 tridecapeptide and has shown high efficacy in various solid tumor cell lines including breast, prostate, non-small cell lung, ovarian, liver, and colon carcinomas [5] In addition, KF displays significant selectivity for cancer vs noncancer human cell lines. This has prompted numerous studies and clinical trials for melanoma, hepatic carcinoma, and others. While KF is considered to be the most active known kahalalide, isokahalalide F (IsoKF) has similar potency and effect [7]. Elisidepsin is a synthetic marine-derived cyclic peptide originally derived from KF and has had notable success in treatment of metastatic or advanced esophageal and xenograph associated cancers [8].

3. Biology

Kahalalides have been found in numerous sarcoglossans around the world. In addition to *Elysia rufescens, E. ornate* and four similar candidate species from the indo-pacific region contain kahalalides. *E. tomentosa* and *E. grandifolia* are other candidate species that may store kahalalides as well. Tilvi et al., [9] Considering the variations in *E. ornate,* it may be beneficial to investigate their diet for signs of kahalalides to find new and possibly more active analogs. Work has been done on better understanding these diverse cryptic species in an effort to resolve their taxonomy and better understand the genetic relationships of this widespread and polymorphic group. Attempts have been made to collect and determine their mitochondrial cytochrome c oxidase I gene (COI). COI is considered the "workhorse" of biological barcoding and species delimitation. Historical oversight in classifying the local taxa were underestimated ten-fold indicating that sea slug diversity could be far higher than originally estimated. Failure to delimit organisms has been an ongoing issue in drug discovery and raises issues about the prospects of biocontrol of invasive green algae (see Fig. 1) [10].

16SrRNA sequencing has been used to determine the bacterial communities that live alongside *Bryopsis* and *Elysia* species with hopes of revealing the origin of kahalalides. Various samples were taken from *E. rufescens*, the surrounding waters, and from *E. rufescens* mucus in an experiment to isolate the bacteria associated with it or to determine the corresponding 16SrRNA gene sequences. The study into *E. rufescens* and its mucus involved a variety of methods and consistently uncovered the same bacterial groups. Tenericutes [*Mycoplasma sp.*) were associated with *E. rufescens* entirety and Gammaproteobacteria (mostly *Vibrio spp.*) were most widely associated with the mucus. *Vibrio* spp. were the most abundant species. The closest relatives of the mycoplasma were uncultured clones from the algal diet of the *Bryopsis* ssp, *E. rufescens* or the gut of mollusks known to forge on the algae. *Vibrio* species are well-known for their symbiotic relations and their ability to produce a wide variety of biologically active secondary metabolites[11].

Fig. 1. An image of *Elysia rufescens* feeding on *Bryopsis pennata*.

Using metagenomic analysis it was later determined that a bacterium with no cultured relatives, *Candidatus Endobryopsis kahalalidefaciens*, lives in symbiosis with *Bryopsis*. This bacterial symbiont harbors an evolutionary reduced genome and lives intracellularly. The closest identified living free relative that has been sequenced is the bacterium *Mangrovimonas* sp. ST2L12. A number of biosynthetic pathways make up a large share of the symbiont's genome, as well as an unusually high number of transposons, transposases, and transposable elements. Additionally, a number of crucial genes have been lost from their original pre-commensal form including DNA helicase, nucleotidyltransferase, some involved in chemotaxis, detoxification, adaptation, and some DNA repair genes. Notably, no complete pathways for the biosynthesis of any of the 20 proteinogenic amino acids have been identified. This is thought to be a trade-off in exchange for symbiosis with the host, although it is not clear how the bioactive molecules are selected for. It has been theorized that KF has anti-predatory activity against fish. Additionally, the combined toxins could act synergistically, or toxins could be selected for individually via unknown selective pressures. Thorough culturing attempts were implemented to determine if the symbiont is cultivable in free form, but this effort was not successful, supporting an obligate symbiont lifestyle. The other kahalalides have shown little to no activity or it may be that their roles have not yet been discovered. It is unclear how *E. rufescens* can sequester, concentrate, and withstand the toxic effects of KF. This is the case for most other marine mollusks as the biological mechanism are often unelucidated. Notably, there is no specific symbiosis organ in *E. rufescens* and the symbiont cells consumed in the algal diet appear to be fully digested [12].

4. Chemistry, SAR, and delivery

Several laboratory syntheses have been developed to make kahalalides A-Z. Most of these are based upon variations of solid-phase peptide synthesis. A single pot method has been developed [7], but because of the expense of rare and D-amino acids, it is often more cost-effective to isolate the naturally occurring material from a biosynthetic source analog studies and development. To date, 16 cyclic depsipeptides have been isolated from either *Bryopsis spp* or *E. rufescens* including kahlalide A-F, K O, P, Q, R, S as well as 3 acyclic peptides kahalalides G, H, and J. Four kahalalides, V, W, X, and Y have been found in exclusively in *E. rufescens* [13]. The KF molecule has been derivatized, creating more than 200 known analogs. Of these, only two modifications of the singular ornithine residue have displayed equal or greater bioactivity than KF. One approach for increasing the activity of the kahalalides has been to extend their half-life, rather than increasing their potency. There are several ways to do this including utilization of the deuterium isotope

effect or adding cationic moieties. The addition of cations has the added effect of increasing water solubility and improving stability and pharmacokinetic profile. Wild harvesting of *Bryopsis* has proven to be a cost-effective source for kahalalides and is competitive compared to solid-phase synthesis [14].

Many novel biologically active compounds of marine source have been identified in the last 50 years. These cyclic peptides generally fall into 4 categories. Cyclic oligopeptides are short chains of amino acids linked via peptide bonds. Cyclic lipopetides are cyclic peptides usually acylated by a lipid, presenting a fatty acid side chain. Cyclic glycopeptides contain covalently linked carbohydrate moieties attached to the side chains of amino acids. Cyclic depsipeptides are peptides in which peptidic bonds are replaced by an ester group. Cyclic peptides have introduced a new option for pharmaceutical development. Jaspamide, a cyclic depsipetide, from the sponge *Jaspis johnstoni* has displayed anticancer and antifungal activity. Discodermin A-H, cyclic oligopeptides, from discodermia, has been used as a gram negative and positive antimicrobial and antifungal. Vancomycin, from the bacteria *Streptomyces fradiae,* has shown efficacy as a cyclic glycopeptide antimicrobial. Several cyclic peptides have made it to clinical trials including Dolastatin's (lipopeptide) for prostate cancer; Soblidotin(a linear lipopeptide derived from dolastatin) for lung cancer; Didemnin B, a cycyclic depsipeptide, for ovarian, renal, breast, prostate, lymphoma, myeloma, and melanoma; Aplidine, a cyclic depsipeptide, has been experimentally used for advanced malignant melanoma, medullary thyroid carcinoma, and small cell lung cancer. Additionally, Brystatin I for malignant melanoma, lymphoma, and ovarian carcinoma is a cyclic depsipeptide [15].

Of the innumerable species of marine life, mollusks have proven to be a trove of bioactive compounds with antimicrobial and anti-cancer properties, among them doliculide, aplyronine A, sphinxolide, hectochlorin, lamellarin D, and spisulosine. It has been hypothesized that natural product-based drugs could be important to multidrug resistant in cancer therapy acquired as a consequence of chromic chemotherapy administrations and tumor microenvironment induced selection pressures. The concept of a "broad spectrum" low toxicity therapeutic approach that could target numerous mechanisms hinges upon the diversity and low toxicity approaches of phytochemicals [16].

Structure- activity relationship has been explored in a successful effort to generate antifungal leads from cyclic and modified peptides including Pneumocanin B0, the cyclic lipopeptide starting material for Caspofungin. Both caspofungin and pneumocandin inhibit the synthesis of 1,3-β-D-glucan, an essential cell wall homopolysaccharide found in many pathogenic fungi. Compounds with this activity display many preferential traits, such as lack of mechanism-based toxicity, potential for fungicidal activity, and activity against strains with resistance mechanisms for existing antifungals. Alkylation of the ornithine group of KF generated several *N*-alkyl derivatives that were also screened against a variety of fungi and bacteria; including: *Escherichia coli*, *Cryptococcus neoformans*, *Mycobacterium intracellulare*, *Aspergillus fumigatus,* and *Fusarium Spp.* Many analogs proved successful in experimentation indicating that ornithine and threonine can help determine target selectivity [17]. Additional screening for antimicrobial activity was published in 2011 which determined that *N*-alkylation provides an important opportunity to modify a pharmacophore at the ornithine group in KF. Determinations were made via testing activity against opportunistic infections, as well as in vitro cytotoxicity, maximum tolerated dose (MTD) assay, and the hollow fiber assay. Monosubstitution of the threonine *N*-amine produced 2 compounds that scored well when challenged against amphotericin, ciprofloxacin, and paclitaxel [17].

Studies regarding hydroxylation of the aliphatic tail have concluded that there is a marked decrease in activity with the addition of polar groups at the alphatic side-chain. To better understand additional activity of kahalalide analogs, 5-OH KF, norKA, and isoKF was tested against the endothelin ET_A and neuropeptide Y Y_1, both of which play a role in vascular remodelling in cardiovascular diseases. The Y_1 is also a promising target for anxiety and other psychiatric disorders. Absolute configuration for the Thr and Ser were found to be L, while the Phe and Leu residues are in the D configuration in norKA as determined using Marfey's method. NorKA inhibited 82% of the binding at the Y1 receptor but showed no binding to the ET_A receptor. Up until this point, no naturally occurring Y_1 ligand had been found [18].

The length of the aliphatic tail has also been shown to have an effect upon the efficacy of other kahalalides such as Kahalalide A (KA). An innovative one pot synthesis of KA utilizing a Kenner's sulfonamide 'safety catch' linker demonstrated total synthesis and antimycobacterial activity of KA derivatives. The structurally simpler KA is devoid of cytotoxicity to various tumor lines and does not present any reactive functional groups, but in order to determine the absolute structure and importance of the methylbutyrate side chain an analog series was synthesized with varying chain lengths and stereoformations. Truncated aliphatic tails were found to be devoid of activity and stereo isomerism in the methyl butyrate did not change the MIC. Elongation of the chain to hexanoate resulted in a 2-fold increase in potency. Hydrolyzed KA yielded a linear peptide that was devoid of activity [19].

A study from 2010 indicated that KF may act as a siderophore by complexing its numerous oxygen and nitrogen atoms to hexavalent octahedral iron. This may create a stabilizing system and as a switch. Through its stabilizing action, the effects of iron on KF could lead to increased structural stability and serve as a more suitable key in the lock and key model. Shifting polarity could also play a role as the complex dynamically transitions from solvents in different physiological environments [20].

A solid lipid nanoparticles (SLN) synthesis has been complete to improve bioavailability. In the study, the KF-derived compound PM02734 was entrained into cyclodextrin (CD) as well as the molten lipid Precirol®. Studies determined the zeta potential as well as the drug loading capacity within the particles. Canine in vivo studies determined increases in bioavailability plasma concentration. The Pricerol® SLN's showed higher capacity for drug loading than the cyclodextrins. The SLN's fared better than cyclodextrin (HPβCD) in terms of higher plasma concentration, but both proved efficacious. The CD's reached a higher plasma concentration quicker, but no drug could be detected after 48hrs indicating a faster clearance. SLN's maintained a higher plasma concentration for almost twice as long with concentrations of 1 ng/ml still being detected up to 96hrs later. The SLN's had aggregation problems, swelled, and leaked their drug content over time. It was hypothesized that the SLN's may have had issues with aggregation which may have caused a faster clearance. Reformulation was proposed as a solution to this problem. SLNs can be delivered orally, however, the bioavailability by this route was only about 1% (see Fig. 2) [21].

Previous work with binding KF to gold nanoparticles has shown a degree of success. Two cysteine analogs of KF were created to facilitate conjugation to gold nanoparticles. One analog replaced the D-13Val with D-Cys. Prior studies indicated that the D-13Val was not crucial for bioactivity [22]. The other analog replaced the D-Val with L-Cys. When conjugated to gold nanoparticles, both failed in clinical trials because of low activity. Studies in HeLa cells aimed at demonstrating high intracellular payload delivery failed to attain levels as high as the free amino peptide. 20 nm GNP conjugated KF particles showed little activity and failed to access the cell. After increasing the size by 20 nm, the particles failed to show any improvement in activity, although the larger 40 nm particles did show higher rates of particle take up [23].

Because KF showed activity against several microbes it was considered as a possible treatment for severe psoriasis unresponsive to methotrexate. The anti-psoriatic effects were originally noted upon infusion of 1 mg of the drug over an hour once a week for eight weeks. However, the side effects of systemic delivery outweighed the benefits. To more effectively guide the drug to the diseased site, a combination of KF and four experimental transdermal formulations were developed and tested. Four formulations composed of KF, ethanol, PEG400, Transcutol® (diethylene glycol monoethyl ether), N-methyl-2-pyrolidine (NMP),

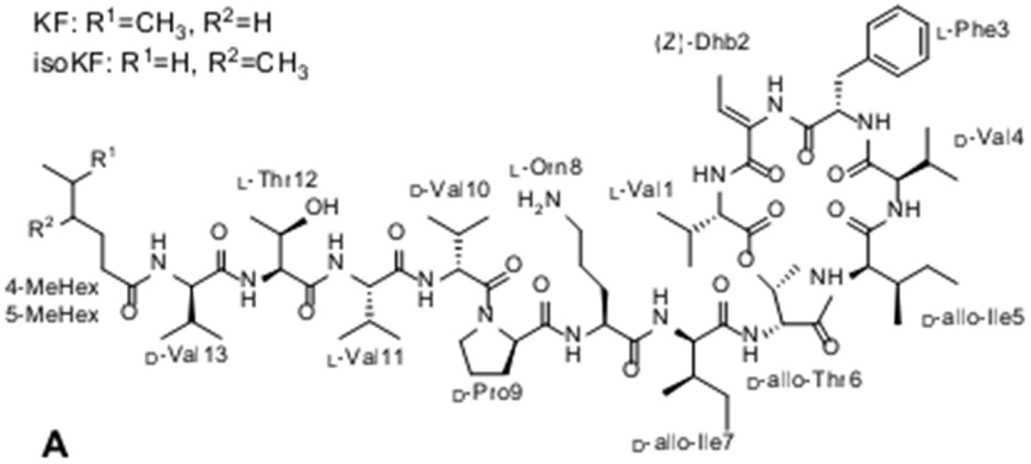

Fig. 2. The structure of KF and isoKF complete with the assigned stereochemistry at each of the amino acids. The presence of numerous atypical amino acids including D, D-*allo,* ornithine as well as other modifications greatly increase the complexity and cost for a traditional peptide synthesis approach to generate and optimize the drug.

ethyl oleate, Span 80, propylene glycol, labrasol, and isopropyl myristate were developed along with DMSO. [24]

The transdermal delivery of KF is not efficient owing to its cyclic nature as well its lipophilicity and large molecular weight. The stratum corneum is the primary barrier to absorption and several different methodologies were developed and investigated.

5. Mechanism of action

KF has a distinction of not causing apoptosis but kills cancer cells via necrosis or oncosis. This is characterized by swelling of the cell and eventual dissolution of the lipid bilayer. The mechanism of action is theorized to be split between aggregation of DNA, poration of the cell membrane in a mechanism similar to that of antibiotics, and deactivation of apoptosis control pathways such as AKT (protein kinase B) and receptor tyrosine kinase (ErbB/Her). The hallmark of KF's action on cells is blebbing, which involves a dramatic swelling and formation of large vacuoles as well as rapid disintegration of the cell membrane, which can result in detachment. Liposomal lysis causes a detectable increase in fatty acids within treated cells. Despite not being a caspase induced apoptosis, cells treated with Annexin V showed damage to the cell walls and increased permittivity [25].

The mechanism of action behind the suppression of ErbB has not been entirely elucidated. Upon exposure to KF a decrease in several ErbB receptors is observed, possibly because ErbB3 is a common binding agent. KF specifically targets ErbB3 [26], one of the four members of the EGFR (epidermal growth factor receptor) family of tyrosine kinase receptors. ErbB3 possesses no kinase activity of its own; however, it heterodimerizes with the other members of the EGFR family, particularly HER2 (ErbB2; *neu*), even in the absence of ligands, especially when ErbB2 is overexpressed [27].

This heterodimerization step is required for ErbB2 activation, resulting in the propagation of signals to pro-proliferative and pro-survival pathways in the cell, including MAPK (mitogen- activated protein kinase), PI3K (phosphoinositide 3- kinase)/Akt(protein kinase B, and JAK (janus kinase] /STAT (signal transducer and activator of transcription) [28,29,27,30]. ErbB2 overexpression is the main driver of 20–25% of breast cancer, whereas ErbB3 is also overexpressed in estrogen receptor positive and luminal breast tumors, where it is essential for their survival [31-39]. ErbB3 is also overexpressed in the colon and gastric cancers [27,40] and is the preferred heterodimeric partner for EGFR in melanoma and pancreatic carcinoma [41,42]. Notably, ErbB3 can also form heterotrimeric interactions with ErbB2 and IGF1R(insulin-like growth factor 1 receptor), which also acts as an oncogene in breast and colon tumors [43-49]. This ErbB3/ErbB2/IGF-1R heterotrimer is what confers resistance to Herceptin (Trastuzumab), posing a major challenge in HER2-targeted therapeutic management of breast cancer [50-53].

Overall, the contribution of ErbB3 to the resistance to hormonal (e.g. Tamoxifen) or targeted (e.g. Herceptin, Erlotinib) therapies against the EGFR family is well-documented [27]. KF results in downregulation of ErbB3 and negatively impacts viability specifically of ErbB3-overexpressing breast, colon, lung, vulval and hepatic cancer cell lines [26].

Beside caspase-mediated apoptosis, cells have been found to regulate apoptosis and necrosis through activation of calpains and cathepsins. However, coadministration of cathepsin or calpain inhibitors failed to prevent cell death. Importantly, ErbB3 ectopic overexpression in cells with low endogenous ErbB3 levels sensitizes these cells to KF-mediated cytotoxicity [26]. Although ErbB3 overexpression correlates with sensitivity to KF, this is not always the case. The non- small-cell lung carcinoma (NSCLC) cell line A549 had very low ErbB3 levels and displayed high sensitivity to KF. The survival of cell line A549 is dependent upon ErbB3 as indicated by experiments using RNA interference [22].

The mechanism behind ErbB3 downregulation is not clear. It is not caused by the downregulation of the receptor because pretreatment of the cell with the protein synthesis inhibitor cyclohexamide did not reduce ErbB3 expression, whereas other related proteins were downregulated. This indicates that downregulation may be caused by degradation. There are several ways to mark a protein for degradation by proteosomes, but pretreatment with the proteosome inhibitors MG-132 and PS-341 did not protect ErbB3 from KF induced depletion (Fig. 3). Data suggests that the ErbB3- Akt pathway contributes to KF response. Cells transfected with an Akt mutant were protected from KF sensitivity. However, no KF associated effect on Akt phosphorylation was observed [26].

The KF-derived compound, elisidepsin, interacts with glucosylceramides, as evident by the unresponsiveness of the cell subline HCT-116-Irv. This subline presents reduced levels of ceramide glucosyl transferase and thus lower levels of glucosylceramides. No accumulation of elisidepsin in the cell membrane was found and treatments did not result in necrotic death. A mutant cell line, GM95, derived from B16 mouse melanoma lacking ceramide glucosyl transferase activity, was also unaffected by KF. Over expressing ceramide glucosyl transferase rendered the same cells sensitive to elisidepsin. These results indicate that glycosyl ceramides may be membrane targets. Whereas KF reduces ErbB3 protein levels, elisidepsin disperses ErbB3 from the plasma membrane intracellularly and into the nucleus. Elisidepsin is also

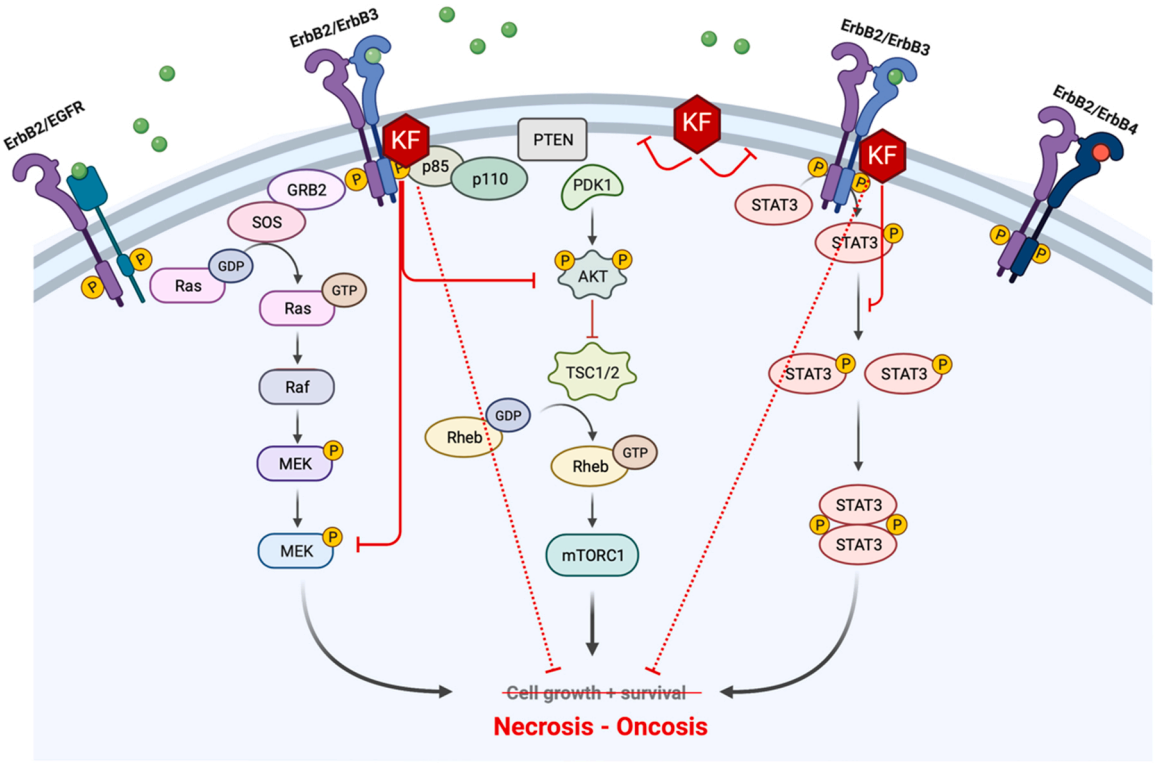

Fig. 3. A summary of the targets and pathways involved in KF's selective inhibition of tumor cells. (Created using BioRender.com).

dependent upon hydroxylase FA2H (fatty acid 2- hydroxylase). In another study, FA2H overexpression increased the sensitivity to elisidepsin, whereas a knockdown showed increased drug resistance [54].

The two common ErbB3 inhibition mechanisms are 1) antibody binding at the extracellular binding domain rendering ErbB3 inactive and/or preventing it from forming heterodimer with other receptor tyrosine kinase (RTK) such as ErbB2 and EGFR; and 2) small molecule targeting the pseudo kinase domain in the intracellular region [55]. The most current ErbB3 drugs under clinical trials are mono- or bi-antibody, such as patritumab, seribantumab, and istiratumab [55]. The inhibition mechanism of ErbB3 by KF remains unclear, however, it is most probable that KF bind at the pseudo kinase domain. Unpublished molecular docking data using previously described methods from our lab [56] has indicated that KF bind at the entrance of the ATP-binding pocket of the ErbB3 pseudo kinase domain, potentially interrupting phosphorylation activity and disturbing structure integrity (Figs. 4 and 5) [57]. KF presents an attractive drug-lead for the development of ErbB3 treatment, targeting the pseudo kinase domain.

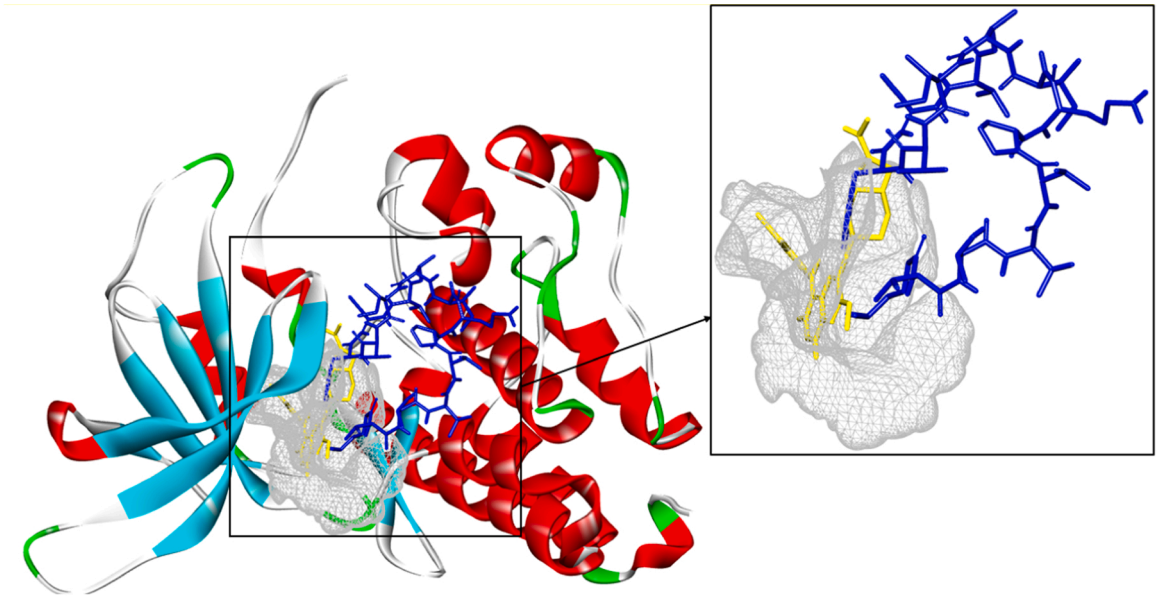

Fig. 4. KF (blue stick) and sapitinib (yellow stick) are shown docked to the ErbB3 pseudo kinase domain (PDB ID: 6OP9; binding coordinate: x-center = −51.522, y-center = −14.922, z-center = 14.951; grid box spacing = 1.0 Å; x-dimension = y-dimension = z-dimension = 30) at the entrance of the ATP-binding pocket.

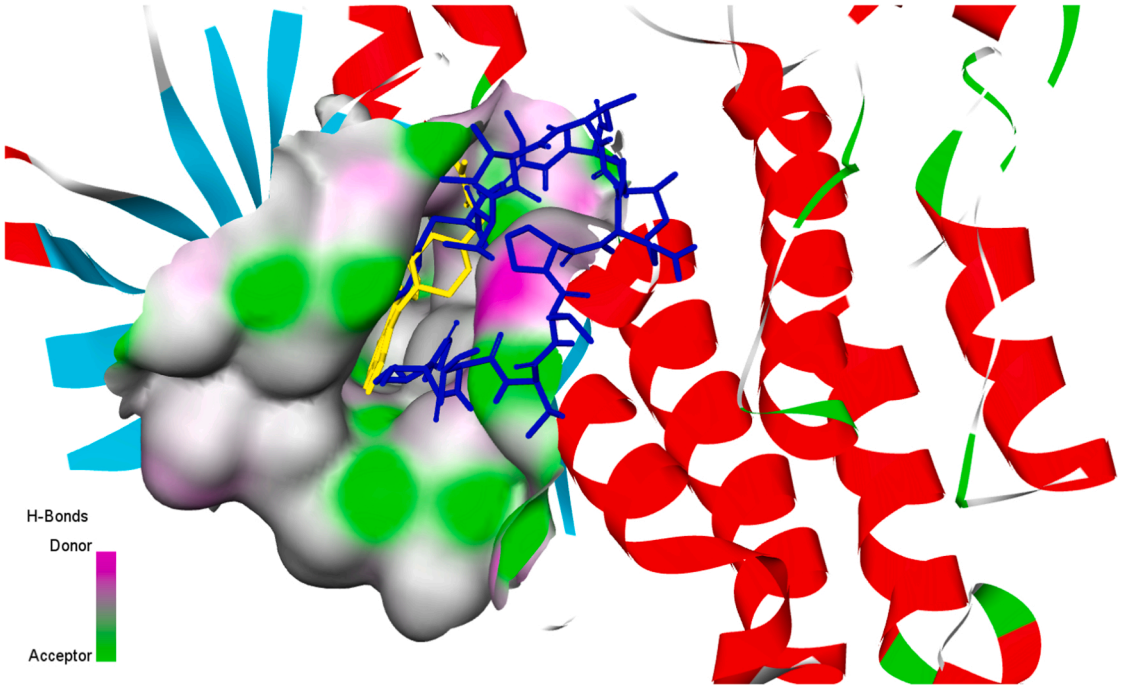

Fig. 5. An overlapped view of the H-bond surface for sapitinib (binding affinity: −9.9 kcal/mol; yellow stick) and kahalalide F (binding affinity: −7.4 kcal/mol; blue stick) ErbB3 (PBD ID: 6OP9).

Activity has been explained by inhibition of the PI3K-AKT pathway and down regulation of the ErbB3 protein making KF the first ErbB3 inhibiting anticancer drug derived from marine source. This is important because overexpression of ErbB3 is common in many types of cancers and to have a drug that targets this receptor could be a leap forward in treatment protocols.

In a recent metabolomics study using high resolution magic angle spinning (HRMAS) proton magnetic resonance spectroscopy (NMR), appropriate cell swelling, and fatty acid accumulation was observed in KF treated MCF7 cells. It is thought that the fatty acids were released from membranes in the phagosomes through lysosomal lipases. It has also been reported that KF can induce mitochondrial autophagy. In this study, membrane alterations might be linked to a decrease in N-acetyl aspartate, a precursor for membrane lipid synthesis through acetyl CoA [58].

6. Preclinical

IsoKF showed high stability in dog plasma for up to four hours while incubated at 37 °C. In animal trials at doses of up to 280 g/kg and a peak concentration of 1uM the half-life ($t_{1/2}$) peaked at 35 min. Intraperitoneally, the concentration peaked at 0.3 μM, approximately 1 h after dosing [7].

KF has been shown to be metabolically stable in vitro following incubation with microsomes, plasma and rat uridine 5′-diphosphoglucuronyl transferase [59]. Using the metabolite prediction software, GLORYx, (https://nerdd.univie.ac.at/gloryx/) the predicted metabolites of KF (pubchem ID: 9898671) are illustrated for both Phase I and II metabolism in Fig. 3 [60,61]. Phase II reactions show the highest priority score with N-acetylation of the aliphatic NH2 being the most likely. Further in vitro and in vivo studies are necessary to gain a better understanding of KFs metabolism and rapid elimination. Fig. 6.

7. Clinical trials

The maximum tolerated dose (MTD), profile of adverse events and dose-limiting toxicity of KF was first evaluated in a Phase I clinical and pharmacokinetic trial in patients with androgen refractory prostate cancer [62]. In humans, the active dose is usually 20–930 μg/m^2/ day delivered intravenously. Transient increases in blood transaminase levels have been observed, however fatigue, paresthesia, pruritus, nausea, vomiting, and rash were the most common KF-related adverse events [63,64]. KF has shown stability in a biomatrix for nine months at −20 °C and 24 h at room temperature. Degradation at 80 °C, the measured $t_{1/2}$ was 1.1 h at pH 0, 20 h at pH1, and 8 h at pH 7. At 26 °C the $t_{1/2}$ was 1.65 h at pH 11. KF is considered metabolically stable when tested against pooled human plasma, human microsomes, and uridine 5'- diphosphoglucuronyl transferase [7].

Population pharmacokinetic data for kahalalide F from six studies demonstrated linear plasma elimination as well as dose proportional exposure. Time independent pharmacokinetics has been consistently found and no clinically relevant co-variates have been identified. For solid tumors, intravenous dosages ranging from 266 to 6650 ug/m^2 administered in 1-, 3-, 6-, 12-, and 24-hour weekly infusions were well tolerated. In a Phase 2 trial for hepatic, melanoma, and non-small lung cancers, a dose of 650 ug/m^2 was tested and determined to be well tolerated but only had a negligeable effect on tumor growth. This study also indicated an in- vivo half-life of 0.52 h. Kahalalide G is the metabolic product and the lowest detection limit was 1 ng/ml [65].

A Phase I study involving 33 patients with androgen refractive pancreatic cancer using a dosage between 20 and 930 μg/day (MTD; 560 mg/m^2/ day). One patient displayed a decrease in PSA levels associated with improvement, along with a decrease in pain. Three patients showed stabilization of disease for two and seven months. A Phase I trial with an MTD of 1200 mg/m^2/ week with 25 patients showed clinical benefits in three of the patients with hepatocarcinoma, squamous carcinoma, and NSCLC, respectively. A Phase II trial with 60 patients showed a tendency to increase LDH(lactate dehydrogenase) and ALT:AP(alanine aminotransferase: alkaline phosphatase) > 5, which indicated reversible and dose dependent hepatocellular damage [66].

A Phase I trial in patients with advanced tumors such as colorectal, melanoma, pancreatic, and NSCLC were given weekly one-hour

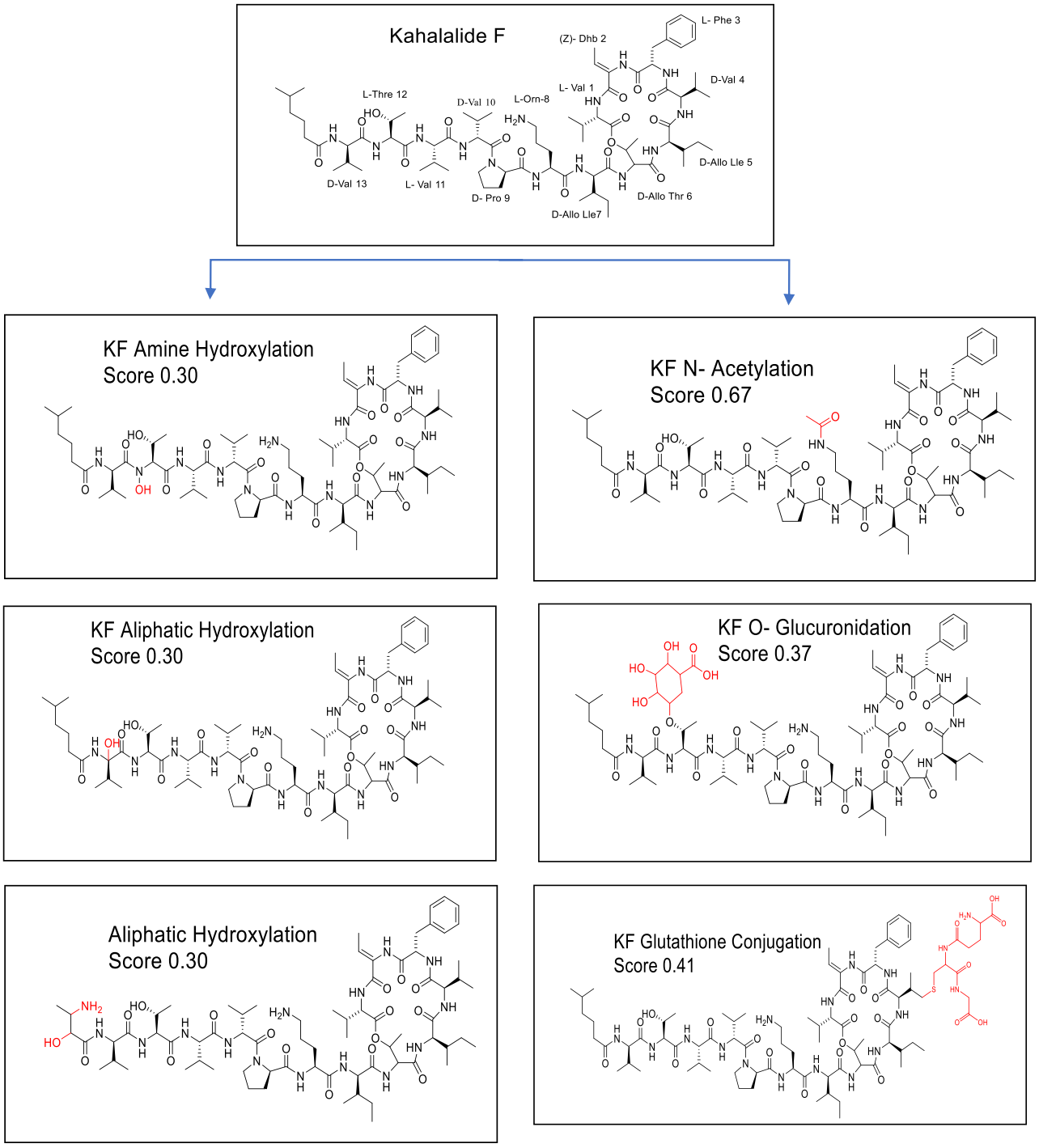

Fig. 6. Predicted Metabolites of Kahalalide F. KF is metabolically stable in vitro. Using the metabolite prediction software, GLORYx (https://nerdd.univie.ac.at/gloryx /) the predicted metabolites of KF (pubchem ID: 9898671) are illustrated for both Phase I and II metabolisms. The priority score for each proposed metabolite is listed below.

intravenous infusions starting at 266 μg/m^2/day and raised to 1200 μg/m^2/day. Toxicities included a 0.75% increase in transaminase blood levels and the maximum tolerance was 800 μg/m^2. No chronic toxicity was found, and a favorable safety profile was established with signs of antitumor activity such as tumor shrinkage and disease stabilization [63].

Another 2013 Phase I trial with 106 patients focused on three- and 24-hour infusions, with MTDs at 1200 μg/m^2 and 6650 μg/m^2, respectively. Both regimes were generally well tolerated and associated with manageable and predictable toxicities. One case of hypersensitivity was reported prior to implementation of specific guidelines for case management. Grade 3 lymphopenia occurred with some regularity in both schedules and anemia occurred in only 5% of treated patients in the 24-hour schedule. All grade 3 and 4 transaminase cases were transient and asymptomatic. No cases of liver dysfunction were discovered. The pharmacokinetic (PK) profiles for the 3- and 24-hour schedules were in agreement with other studies and characterized by linear kinetics, narrow volume of distribution, and $t_{1/2}$. The AUC (area under the curve)

was slightly lower than that of other studies and was attributed to the use of Cremophor® as a formulation vehicle. It is known that Cremophor® can cause micelles to form and sequester the drug, thereby decreasing available material and causing pseudo nonlinearity. The short half-life observed indicated that KF has limited extravascular binding and is rapidly eliminated, although the exact mechanism is unclear. It is thought that there is some limited conversion to kahalalide G, as this has been detected in incubated buffer solutions. Kahalalide G could not be detected in the human plasma because of signal interference. Major elimination routes have not been established yet and renal degradation is not expected due to low unchanged recuperation from urine. Prolonged disease stabilization occurred in eight heavily pretreated patients with non-small cell lung cancer, colorectal cancer, hepatocarcinoma, bladder cancer, and cholangiocarcinoma. Lung cancer, hepatocarcinoma, and colon cancer had already been indicated for sensitivity to KF in laboratory tests. A recommended dose (RD) was determined to be 1000 μg/m^2 for 3-hour infusions, but no RD was determined for 24-hour treatments. Studies have shown that the kidney, liver, and the central nervous system are the main organs affected by toxicity, with an additional symptom being pruritus paresthesia and fatigue [64].

Plitidepsin (aplidin) is a naturally occurring cyclic depsipeptide originally isolated from the Mediterranean tunicate *Aplidium albicans* but currently produced by chemical synthesis. This synthetic compound has also shown positive results in clinical trials. The pharmacokinetic studies of plitidepsin were evaluated in a Phase I trial in patients with solid tumors or non-Hodgkin's lymphoma. The PK parameters for plitidepsin showed a long plasma $t_{1/}$ (16.8 +/- 7.7 h) with a recommended 3.2 mg/m2/week dose. Muscle and liver toxicity were observed at 3.6 mg/m2/week [63].

8. Conclusion

Analogies can be drawn between the first in class antifungal caspofungin (Cancidas®) and kahalalides. While there are distinct similarities between the two compounds such as their cyclic peptidic structure and aliphatic tail, their chemical nature and development challenges are parallel. Caspofungin is derived from the natural product pneumocandin B0 found in the fungus *Glaria lozoyensis*. Many years of development eventually resulted in the successful marketing of caspofungin as a treatment for fungal infections caused by aspergillus and candida species. Many hurdles had to be crossed in regard to issues of SAR, solubility, stability, and bioavailability; issues that are also inherent to kahalalides. A lot can be learned by a comparative analysis of strategies for optimization utilized in the development of pneumocandin into caspofungin. The applicable methods range from mutagenesis to improve fermentation yields to chromatographic strategies developed to economically isolate precursors. Formulation schemes can also be applied due to similarities in structure and properties, this includes nanoscale liposomal preparations, aerosol delivery, and development of appropriate salts; as well as strategies in semisynthetic derivatization [67].

Advancements in the science of kahalalides have improved our understanding of the nature of their bioactivity, natural sources, and therapeutic protocols. Successes in translation between lab and clinic have been limited, but the science substantiates the activity and potential utility of kahalalides in the treatment of a wide range of cancers and psoriasis. Improved stability and PK could be the key to unlocking the potential of kahalalides, as well as the discovery of new kahalalides or new synthetic analogs. The recent discovery and genome sequencing of the kahalalide- producing symbiont of *Bryopsis* provides an alternative route for generating kahalalide derivatives, through the rational engineering and heterologous expression of their biosynthetic pathways. Deuterated versions have shown an increase in half-life due to the deuterium isotope effect.[14] Combination therapies of elisidepsin and 5-fluorouracil, gemcitabine, cisplatin, oxaplatin, and lapatinib have indicated improved success rate in the treatment of cancers in cell-based assays. This technique could translate into a real-world synergistic therapy where elisidepsin acts as a sensitizer and helps to unlock the clinical potential of KF [8].

CRediT authorship contribution statement

Study and design: Scott Wyer, Danyelle M. Townsend, Zhiwei Ye, Antonis Kourtidis, Yeun-Mun Choo, Luís Branco de Barros, Mohamed S. Donia, and Mark T. Hamann. Analysis and interpretation of results: Scott Wyer, Danyelle M. Townsend, Zhiwei Ye, Antonis Kourtidis, Yeun-Mun Choo, Luís. Branco de Barros, Mohamed S. Donia, and Mark T. Hamann. Draft Manuscript preparation: Scott Wyer, Danyelle M. Townsend, Zhiwei Ye, Antonis Kourtidis, Yeun-Mun Choo, Luís Branco de Barros, Mohamed S. Donia, and Mark T. Hamann, All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Data Availability

Data will be made available on request.

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