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Unraveling Anticancer Activity And Pharmacokinetics Of Dihydroartemisinin Dimer Oxime

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UNRAVELING ANTICANCER ACTIVITY AND PHARMACOKINETICS OF DIHYDROARTEMISININ OXIME

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ABSTRACT

Artemisinin is currently used as an antimalaria drug. Studies have shown its monomeric form exhibited promising anticancer effects by inhibiting the proliferation, inducing apoptosis, and increasing oxidative stress of tumor cells. Recently, several dimeric forms of artemisinin have been synthesized with more potent anticancer activity. In this study, nine dihydroartemisinin dimers with diversely functionalized linkers exhibited similar cytotoxicity against human breast adenocarcinoma MDA-MB-231 *in vitro*. Among these nine DHA dimers, DHA dimer oxime was selected for further anticancer activity and pharmacokinetic study. Our preliminary study showed that DHA dimer oxime displayed more than a 10-fold increase of antiproliferation effect over its monomeric forms. DHA dimer oxime inhibited the growth of eight tested cancer cell lines and reduced cell proliferation at submicromolar concentrations. It was also suggested DHA dimer oxime combination with gemcitabine, a standard treatment for advanced pancreatic cancer, had yielded a synergistic anticancer effect in selected cell lines compared to the mono-treatment. Furthermore, DHA dimer oxime displayed longer half-life to its monomeric form DHA in mice, remaining above the median IC_{50} for pancreatic cancer cells for 4h after oral administration. Together, this study demonstrated the potential use of DHA dimer oxime as an anticancer therapeutic candidate.

ACKNOWLEDGMENTS

I would like to express my appreciation to my advisor Dr. Chalet Tan for supporting my projects. During my two-years master study, she always patiently encouraged me and guided me to be a better researcher. She let me know how to be more accurate and efficient in doing experiments, as well as be humble and never stop learning. And thank Dr. Elsohly and Dr. Chambliss for being part of my committee members, their advice is so helpful and vital to me.

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I. INTRODUCTION

1. Introduction of artemisinin and its derivative dihydroartemisinin (DHA)

Artemisinin (Figure 1A), is a potent antimalarial drug isolated from *Artemisia annua L*. in 1972 [1]. Dihydroartemisinin (DHA) (Figure 1B), a semi-synthetic antimalarial compound, is a derivative of artemisinin. DHA has been reported as an active metabolite of artemisinin, and exhibited stronger effect against malaria [2, 3].

Increasing evidence revealed that artemisinin and its derivative DHA showed promising anticancer property which were related to their endoperoxide moiety. Endoperoxide bridge formed free radicals and reactive oxygen species (ROS) when it reacted with reduced heme and ferrous iron [4, 5]. Free radicals and ROS could cause DNA damages and activate mitochondrial-dependent apoptotic pathways in tumor cells [6]. Artemisinin and DHA displayed promising anticancer activity *in vitro* and *in vivo* with little toxicity to normal cells because their endoperoxide moiety will react with iron and heme, cancer cells have higher iron ion influx due to highly expressed transferrin receptors (TfR) on the cell surface [7]. For example, CCRF-CEM and U373 cells expressed transferrin receptor (TfR) in 95% and 48% of the cell population, respectively, whereas TfR expression in peripheral blood mononuclear cells of healthy individual accounted for 0.4–1.3%. Studies also showed that blocking the TfR by pretreatment with specific monoclonal antibodies abrogates artemisinin activity [8, 9]. The anticancer activity of artemisinin and DHA

significantly enhanced when iron complexes were added in the cell culture medium [10]. Moreover, the antineoplastic activity of artemisinin and DHA also were modulated by calcium metabolism and endoplasmic reticulum (ER) stress [11, 12]. In addition, artemisinin and DHA have been found to interfere with signal pathways involved in several hallmarks of malignancy. For instance, DHA can induce apoptosis in rat glioma (C6) cells by reducing the expression of hypoxia-inducible vascular endothelial growth factor (VEGF) and factor-1 (HIF-1α) [13]. Current data indicated that DHA inhibits the viability of pancreatic cancer cells by decreasing the levels of nuclear factor- kappa B (NF-kB) activity, which is inducible transcription factors [14]. Another study showed that DHA treatment in pancreatic cells (AsPC-1) inhibited cell viability by downregulating levels of proliferating cell nuclear antigen (PCNA) and cyclin D with updating p21 [15].

2. Dimeric dihydroartemisinin

Considerable progress has been made in the design of novel compounds with enhanced potency. The dimerization approach has been developed to join two DHA molecules without destroying their endoperoxide bridge[16]. Semi-synthetic artemisinin-derived dimers exhibited advanced antiproliferative properties and circumvented multidrug resistance of cancer cells [17]. The nature of the linker in dimers played a crucial role in imparting potent anticancer activity. As an example, novel C-10 non-acetal dimers with longer linker results in poor anticancer [18, 19]. C-10 carba artemisinin dimers with more carbon atoms in their linkers displayed a more powerful anticancer effect [20]. In this study, nine series of C-10 acetal dimers (Figure 1C-F) were synthesized and assessed for their anticancer activity. DHA dimer oxime (Figure 1J) was selected to elucidate mechanisms of anticancer action as a represent of those compounds.

3. The biological mechanism of DHA dimer oxime

It has been reported that the anticancer activity of DHA dimer oxime is associated with the generation of reactive oxidative species (ROS). ROS is a consequence of heme-mediated endoperoxide cleavage, leading to DNA damage and apoptosis [21]. Pretreatment with antioxidants could significantly impair anticancer effect of dimer. In the previous study, gene expression analysis identified more transcriptional DNA damage/stress response in dimer treatment. This response is possibly resulted from non-specific ROS production. RT-PCR data showed an increased expression of ER stress sensor and unfolded protein response (UPR) related mRNA following DHA dimer oxime treatment [22]. In addition, DHA dimer oxime is reported to be water-soluble and stable at room temperature [18].

4. The advantage of combination treatment

Nowadays, combination treatment has been explored more in the cancer research field because its improvement on anticancer treatment with potentiation and synergistic effect. It can also reduce drug- resistance and toxicity effect [23]. Cancer cells are characterized by multiple genetic defects, which may reduce the efficacy of single chemotherapeutic. Trying to stop them from one type of treatment just encourages them to find a new way to survive because cancer cells are always on the way to divide and mutate. Therefore, by using combination treatment to target either multiple pathways or, it is possible to reduce the chance of cancer cells evading treatment [24]. Combinations involving artemisinin and its derivatives have been studied *in vitro* and *in vivo* in various cancer types.

However, it is essential to know that not all the combination therapy facilitate the therapeutic purpose. For instance, Riganti et al. showed that artemisinin impaired antiproliferation of doxorubicin in colon cancer cells (HT-29) [25]. The intracellular accumulation of doxorubicin was decreased due to artemisinin, which could lead to activation of NF-κB/overexpression of P-glycoprotein (P-gp), which is one of the most recognized mechanisms of multidrug resistance (MDR). Therefore, it requires cautiousness for the developing of a successful combination therapy.

5. Introduction of conventional therapeutics and their combination with artemisinin or DHA

In this study, three conventional chemotherapeutics were selected for combinational treatment. Doxorubicin (DOX), an anthracycline drug, bind to DNA-associated enzyme. It intercalates the base pairs of DNA's double helix and inhibits the progression of topoisomerase II [26]. Topoisomerase II is an enzyme responsible for separating the double stranded DNA and is essential for the proliferation of cancer cells. It has been reported that dihydroartemisinin (DHA) can resensitize human colon tumor HCT/ADR cells to doxorubicin since the drug combination resulted in the downregulation of Bcl-xl [27]. Bcl-xl is an anti-apoptotic protein, which causes acquired chemoresistance due to overexpression in cancer [28]. Besides, a synergistic anti-proliferative effect was found between DHA and doxorubicin on breast cancer cells, and the combination treatment remarkably decreased the mitochondrial membrane potential and augmented the activation of caspase cascades. The combination indices (CIs) of doxorubicin combined with DHA varied from 0.5 to 1, which indicated a synergistic effect [29].

Paclitaxel, a mitosis inhibitor, is one of the most well-known natural products that have

antineoplastic effects. It can disrupt the microtubule polymer from disassembly, stabilizing the microtubule cytoskeleton [30]. Chromosomes, therefore, will not be able to develop the metaphase spindle configuration, leading to the termination of the mitosis process [31]. Currently, there is no literature reported the combination of paclitaxel with artemisinin or DHA, however, it showed a moderate synergistic effect when combined with DHA dimer oxime in selected cell lines.

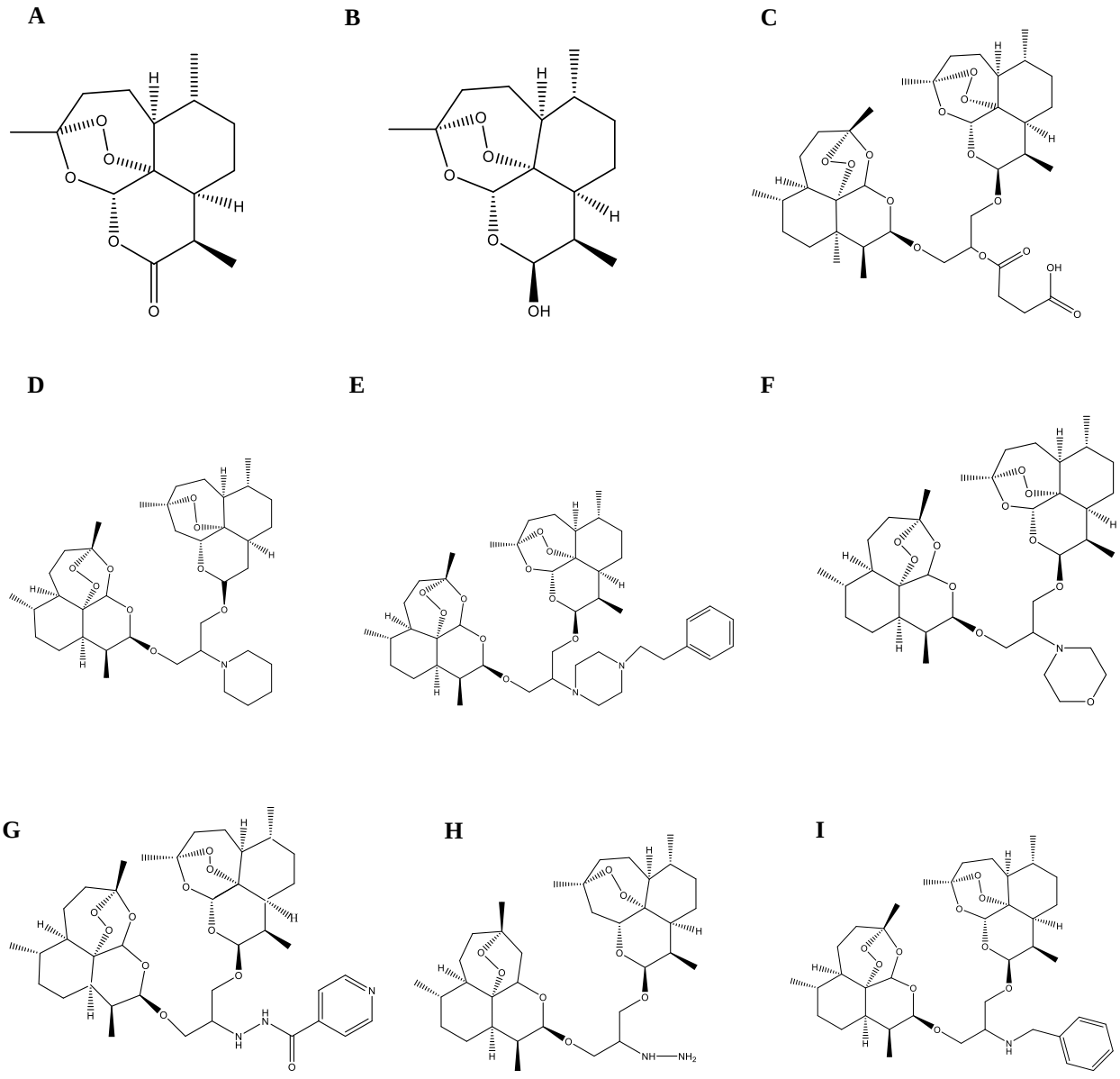
Gemcitabine (2', 2'-difluorodeoxycytidine, dFdC) is an S-phase-specific, fluorine-substituted pyrimidine. It is an FDA-approved first-line therapy for advanced or metastatic pancreatic cancer as a single agent. The parental form of gemcitabine is inactive, and gemcitabine will be phosphorylated intracellularly to the active gemcitabine diphosphate and gemcitabine triphosphate forms. Gemcitabine diphosphate inhibits ribonucleotide reductase, and the triphosphate can be incorporated into RNA, thus potentially inhibiting or altering RNA synthesis [32]. It has been reported that DHA improved the anticancer effect of gemcitabine. DHA potentiates the anti-tumor effect of gemcitabine on pancreatic cancer by inhibiting NF-κB signaling and regulating apoptosis regulator Bax and caspase-8 [33, 34].

DHA dimer oxime showed promising anticancer effect. However, cancer cells are always mutated and divided. Combinational treatment will improve anticancer effect, reduce drug-resistance and toxicity effect. In this study, combination study will be performed *in vitro.*

6. The purposes of this study

In present study, we aimed to evaluate anticancer activity and pharmacokinetics of a novel dimeric dihydroartemisinin. We firstly evaluated the cytotoxicity of nine dihydroartemisinin acetal dimers with diversely functionalized linker units on MDA-MB-231 cells. Next, we focused on

DHA dimer oxime and evaluated the anticancer effect against several different types of cancer cells. The cell cycle analysis was performed to observe the phase-specific cell cycle arrest. Paclitaxel, doxorubicin, and gemcitabine were used as combination therapeutics with DHA dimer oxime and the combination index (CI) was used as synergistic effect indicator. To further explore DHA dimer oxime as a potential anticancer agent, a battery of *in vitro* and *in vivo* pharmacokinetic experiments was performed to unravel the fate of DHA dimer oxime.



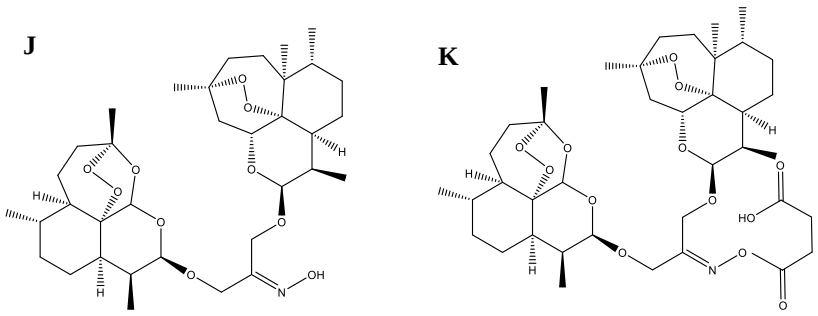


Figure 1. Chemical structures of artemisinin and its derivatives. Artemisinin (A), dihydroartemisinin (DHA) (B), Dimer HS (C), Dimer Piperidine (D), Dimer-2-Phenyleyl-Piperazine (E), Dimer Morpholine (F), Dimer Isoniazide (G), Dimer Amine (H), Dimer Benzyl Amine (I), Dimer oxime (J), Dimer HS (K)

II. MATERIAL AND METHOD

Chemicals and instrumentation

DHA dimers were kindly provided by Dr. Mahmoud A. Elsohly at the University of Mississippi. Paclitaxel, doxorubicin, and gemcitabine (free base) were purchased from LC Laboratories (Woburn, MA). B16-F10, SK-MEL-28, SKOV-3, and MIA PaCa-2 were grown in DMEM (Invitrogen, Carlsbad, CA) with 4 mM L-glutamine. MDA-MB-231, A549, and Panc-1 were grown in DMEM with 2mM L-glutamine. BxPC-3 and AsPC-1 were grown in RPMI 1640 medium, which contains 2.05mM L-glutamine. All these media were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Life Technologies). The cells were maintained at 37 ℃ with 5% CO_2 in a humidified incubator (Nuaire)

Cell proliferation assay

Cells were seeded at a density of 5,000–8,000 cells/well in 96-well plates and treated with DHA dimer oxime (1–50 μM), and their viability was determined after 72-h treatment. Subsequently, cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet, and the crystals dissolved in 10% acetic acid. The absorbance was quantified at 595 nm on a microplate reader (Bio-Tek, Winooski, VT). The relative cell viability was calculated as the percentage of absorbance of the drug-treated vs. the untreated controls. IC_{50} of DHA dimer oxime was calculated by Graphpad Prism 8.0.1 (GraphPad Software, La Jolla, CA）

Combination treatment assay

Cells were seeded at a density of 5,000–8,000 cells/well in 96-well plates, and were treated in triplicates with varying concentrations of paclitaxel (1–200 nM) or doxorubicin (0.025-5 μM) or gemcitabine (0.0125-10 μM) individually, or in combination with DHA dimer oxime (2.5 μM or 5 μM). The combination index (CI) was calculated by the ChouTalalay equation, which takes into account both potency (D_m or IC_{50}) and the shape of dose-effect curve. [23] The extent and direction of antitumor interactions were evaluated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA)[35].

The combination index (CI) between DHA dimer oxime and paclitaxel or doxorubicin or gemcitabine was calculated by CompuSyn software, where CI < 1, CI = 1and CI > 1 indicate synergistic, additive and antagonistic effects, respectively[36].

Cell cycle analysis

In 60 mm dishes (1.0×10^6 cells/dish), MDA-MB-231 and A549 were treated with DHA dimer oxime (5 μM) for 24 h and 48 h, Panc-1 was incubated in the presence of 0.25 μM DHA dimer oxime and/or gemcitabine (0.5 μM for Panc-1 cells and 0.25 μM for AsPC-1 cells) for 72 h. and then fixed in 70% ethanol at 4°C overnight, washed with cold PBS, re-suspended in a staining solution 50 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase A for 30 min at room temperature, and analyzed by a BD FACSCalibur Flow Cytometer System (San Jose, CA). The DNA content distribution was analyzed using the FlowJo 9.3.1 software (Tree Star, Ashland, OR).

Pharmacokinetic study of DHA dimer oxime in mice

Quantification of DHA dimer oxime in mice plasma was accomplished using AB Sciex QTrap 4500 (Manchester, UK) coupled with a Shimadzu Nexera dual pump UPLC (Waters, Milford, MA, USA). A UPLC-MS procedure was developed and validated for the quantification

of DHA dimer oxime using electrospray ionization in the positive mode (Table 1). The m/z 637, 624 were selected for analysis using selected ion recording (SIR) of DHA dimer oxime and IS, respectively. For method, Chromatographic separations were achieved on a column C18 (100Å 50 mm × 2 mm, 5 µm particle size: Phenomenex Luna, USA). The column temperature is 28° C. A gradient elution method of A: water with 0.1% formic acid and B acetonitrile with 0.1% formic acid (0 ~ 0.01 min 0 ~ 30 % B, 0.01 ~ 3.0 min 30 % B, 3.0 ~ 5.0 min 30 ~ 95 % B, 5.0 ~ 8.0 min 95 % B, 8.0 ~ 8.10 min 95-30 % B, 8.1 ~ 10.0 min 30 % B, 10.0 ~ 11.0 min 30-0%) was used for the analyses of the samples, and 5.0 µl sample was injected into the column each time with the mobile phase at a flow rate of 0.5 ml/min.

Table 1. MS Instrument and Parameters

Mass Spectrometer	AB Sciex QTRAP 3200
Scan Type	MRM
Polarity	Positive
Ion Source	Turbo Spray (ESI)
Curtain Gas	20 lpm (N_2)
Collision Gas	Low (N_2)
IonSpray Voltage	5500.0 V
Temperature	300.0° C
Ion Source Gas 1	60.0 lpm (Air)
Ion Source Gas 2	40.0 lpm (Air)

The plasma calibration curve was prepared by diluting DHA dimer oxime stock solution (1 mg/mL) with acetonitrile to varying the absolute concentration of DHA dimer oxime (10, 25, 50, 70 and 100 ng), each sample was spiked with 10 µL of IS solution (5 µg/mL Dimer glycerol). Plasma samples of DHA dimer oxime, including the blank, the blank with IS (internal standard), the calibration standards and test samples were extracted using a simple protein precipitation technique. For this technique, the plasma samples were spiked with 10 µL of IS solution (5 µg/mL Dimer glycerol). The extraction solvent, cold acetonitrile (500 µL), was added, and the samples were vortexed for few seconds and sonicate 30 seconds, Following centrifuge at 3100 rpm for 3mi

n, the supernatant was collected and evaporated at 37℃ until left 100 μL residue and vortex, then the residue was evaporated to completion. The residue was reconstituted with 100 μL acetonitrile and injected into the UPLC-MS for analysis.

Pharmacokinetic studies of DHA dimer oxime were performed in female BALB/c mice. The mice were randomly divided into two groups (n=3), one group was administered free DHA dimer oxime which dissolved in ethanol, Cremophor EL and phosphate buffer (pH 7.45) (1:1:8, v/v/v) (25 mg/kg) (dose concentration: 5 mg/mL) via the tail vein injection, and another group of mice was orally injected with DHA dimer oxime which was also dissolved in ethanol, Cremophor EL and phosphate buffer (1:1:8, v/v/v) mixed solution at a dosage of 100 mg/kg (dose concentration: 40 mg/mL). The time points of collecting the blood after intravenous injection were 2, 15, 30, 60 and 90 min, while oral gavage was 15, 30, 60, 120 and 480 min. The blood sample was centrifuged at 4 °C (12,000 g, 5 min), then the plasma was obtained by collecting the supernatant. The plasma samples were stored at -80 °C for further analysis. the plasma samples were spiked with 10 μL of internal standard solution (5 μg/mL Dimer glycerol), then the samples were analyzed using the same method described before.

III. RESULTS AND DISCUSSION

Cell proliferation assay

To determine the antiproliferation effect of DHA dimers with different linkers, they were screened for the antiproliferation against human breast adenocarcinoma cell MDA-MB-231. All of them exhibited a similar antiproliferative activity (Figure 2). DHA dimer oxime was selected for further investigation.

We first performed cell proliferation assay to study the effect of DHA dimer oxime in a variety of human and murine tumor cell lines harboring various oncogenic mutations (Figure 3). The IC_{50} of DHA dimer oxime was found to be in the low micromolar range in tumor cells originating from breast, lung, melanoma, and pancreas (Table 1). B16-F10, MDA-MB-231, Panc-1, MIA-PaCa2, AsPC-1, which contain the KRAS mutant gene, were more sensitive to DHA dimer oxime compared with SKOV-3, SK-MEL-28. It has been reported that RAS transformation renders cells sensitive to a ROS induced, iron-dependent mode of cell death [37, 38]. In addition, these results demonstrated that DHA dimer oxime is more active than artemisinin and DHA, although they were twice the concentration of DHA dimer oxime to maintain molar equivalency (Figure 3A

3B, and 3C)

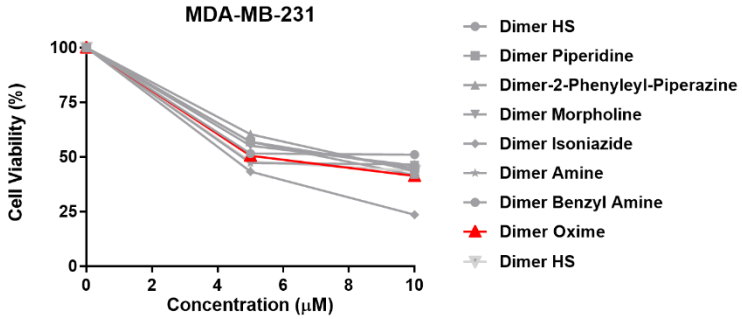


Figure 2. Antiproliferative activity of DHA dimers against MDA-MB-231 cells.

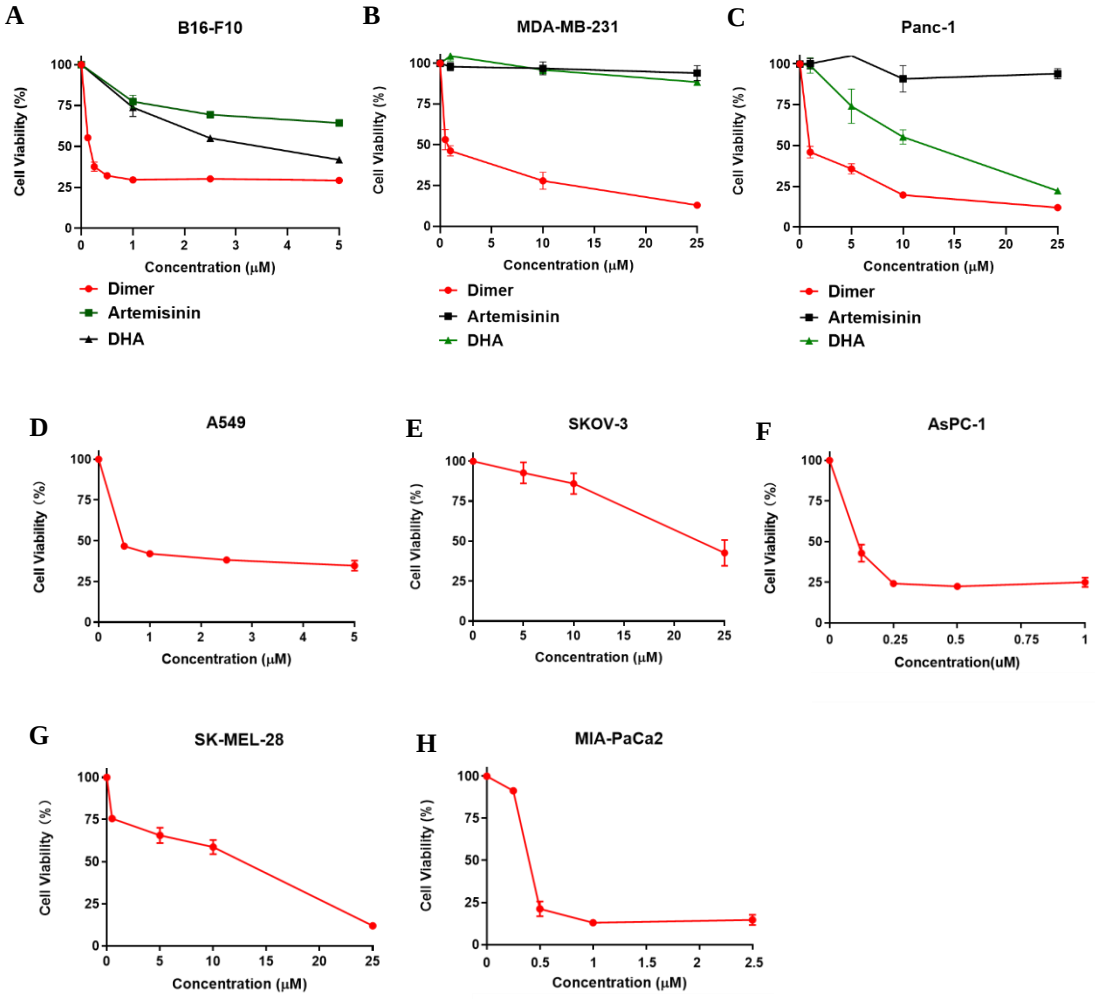


Figure 3. Antiproliferative activity of DHA dimer oxime, artemisinin and DHA on cancer cells. B16-F10 (A), MDA-MB-231(B), Panc-1 (C), A549 (D), SKOV-3 (E) AsPC-1 (F), SK-MEL-28 (G) and MIA-PaCa2 (H).

Table 2. Cytotoxicity of DHA dimer oxime in tumor cell lines with various tissue origins and oncogenic mutations.

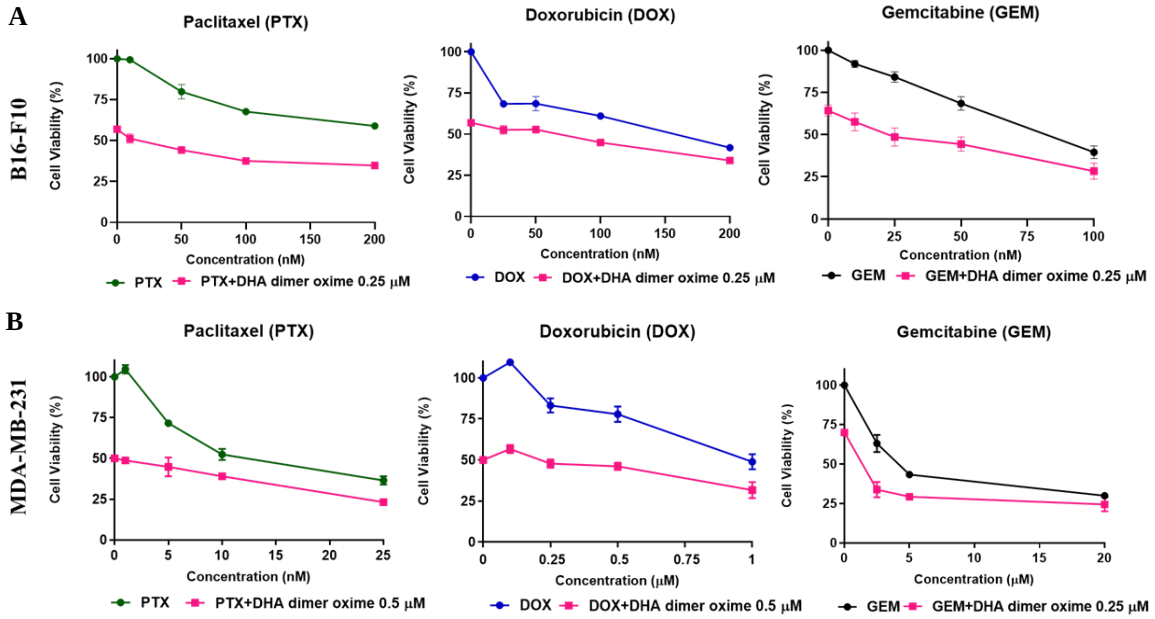
Cell lines	IC_{50} (μM)	Tissue	Mutated Genes
B16-F10	0.1	Melanoma(murine)	--
SK-MEL-28	8.7	Melanoma	BRAF, TP53, CDK4
MDA-MB-231	0.7	Breast cancer	KRAS, TP53, BRAF
A549	0.2	Lung cancer	KRAS
Panc-1	0.8	Pancreatic cancer	KRAS, TP53
AsPC-1	0.04	Pancreatic cancer	KRAS, TP53, CDKN2A
MIA-PaCa2	0.4	Pancreatic cancer	KRAS, TP53, ARID1A, KMT2C
SKOV-3	21.5	Ovarian cancer	TP53

IC_{50}: concentration causing 50% growth inhibition.

Combination treatment assay

Next, we sought to determine if combining DHA dimer oxime with conventional

chemotherapeutics could enhance the antiproliferation effect. DHA dimer oxime, together with paclitaxel, doxorubicin, or gemcitabine, was tested on B16-F10, MDA-MB-231 and Panc-1 cancer cell lines. The selected cell lines showed sensitive to DHA dimer oxime in previous cell proliferation assay. Combination indices (CIs) were obtained by using a constant dose of DHA dimer oxime in combination with varying doses of paclitaxel, doxorubicin or gemcitabine for 48 h. CIs were calculated by the ChouTalalay equation. The enhanced proliferation in the combined-treatment group could be observed between DHA dimer oxime with either paclitaxel, doxorubicin or gemcitabine compared to drug alone（Figure 4). The synergistic effect was determined based on the combination index (CI). The lower the value, the better synergistic effect it revealed. The result showed that the combination indices of DHA dimer oxime/gemcitabine scored well below 1.0 in selected cells, suggested a synergistic response in those cell lines. By contrast, combination indices of DHA dimer oxime/paclitaxel or DHA dimer oxime/doxorubicin were around 1.0, pointing towards an additive effect in those cell lines (Figure 5).



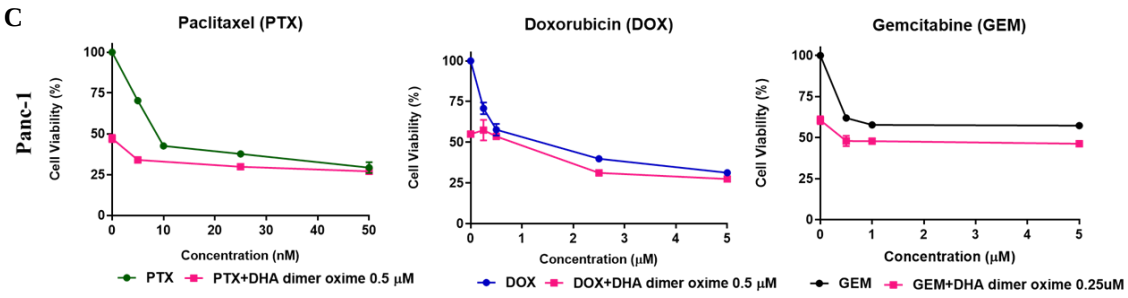


Figure 4. Cytotoxicity of combination treatments. B16-F10 (A), MDA-MB-231 (B) and Panc-1 (D) treated with varying concentrations of paclitaxel (PTX) or doxorubicin (DOX) or gemcitabine (GEM) alone or combined with DHA dimer oxime, treated for 48 hours.

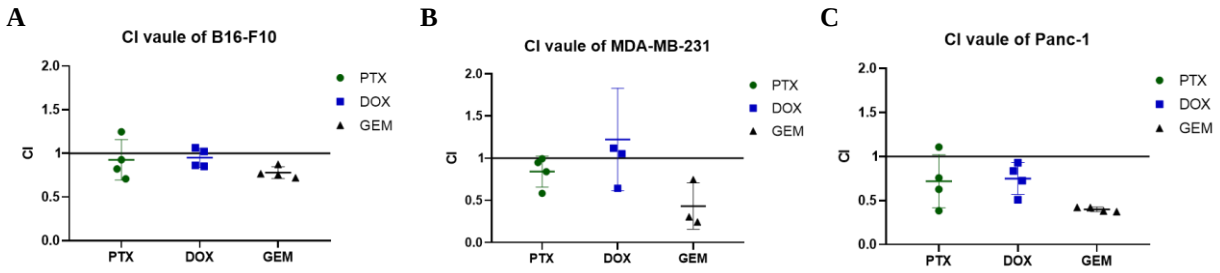


Figure 5. Comparison of synergistic effect in different combination treatments. Synergistic antitumor interactions of paclitaxel/DHA dimer oxime, doxorubicin/ DHA dimer oxime and gemcitabine/DHA dimer oxime in B16-F10 (A), MDA-MB-231 cells (B) and Panc-1 (C). Combination index (CI) values were calculated by CompuSyn software, where CI < 1, CI = 1and CI > 1 indicates synergistic, additive and antagonistic effects, respectively.

Since the combination of gemcitabine/ DHA dimer oxime exhibited an enhanced antiproliferative effect and gemcitabine is first-line therapy for advanced or metastatic pancreatic cancer, we next studied the effect of gemcitabine and DHA dimer oxime on different pancreatic cancer cell lines specifically. The combination of gemcitabine/DHA dimer oxime significantly inhibited the growth of Panc-1 and AsPC-1 to a greater extent compared with the same dose of each reagent individually

(Figure 6). The combination indices of DHA dimer oxime/gemcitabine were well under 1 in Panc-1 and AsPC-1 cells, showing a strong synergistic effect. (Figure 7).

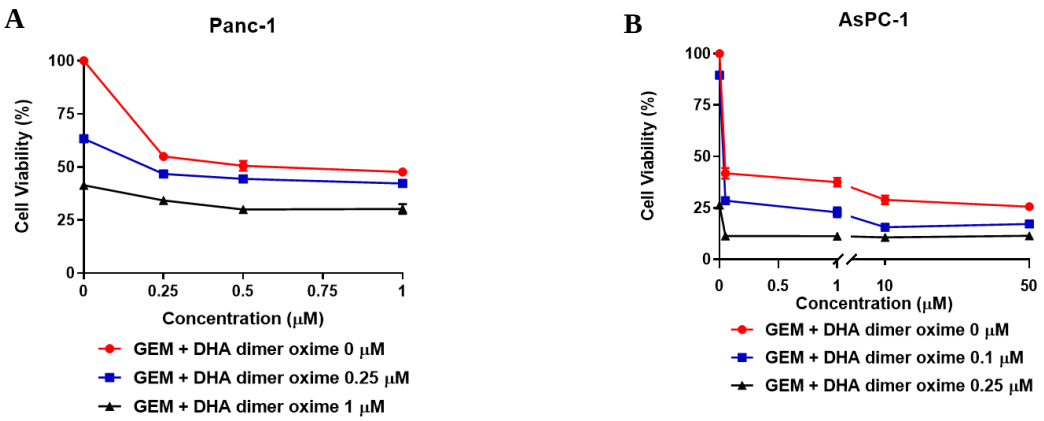


Figure 6. Cytotoxicity of combination treatments against pancreatic cancer cells. Cell viability of Panc-1 (A), AsPC-1(B) treated with varying concentrations of gemcitabine (GEM) in the absence or presence of DHA dimer oxime, administered for 48 hours.

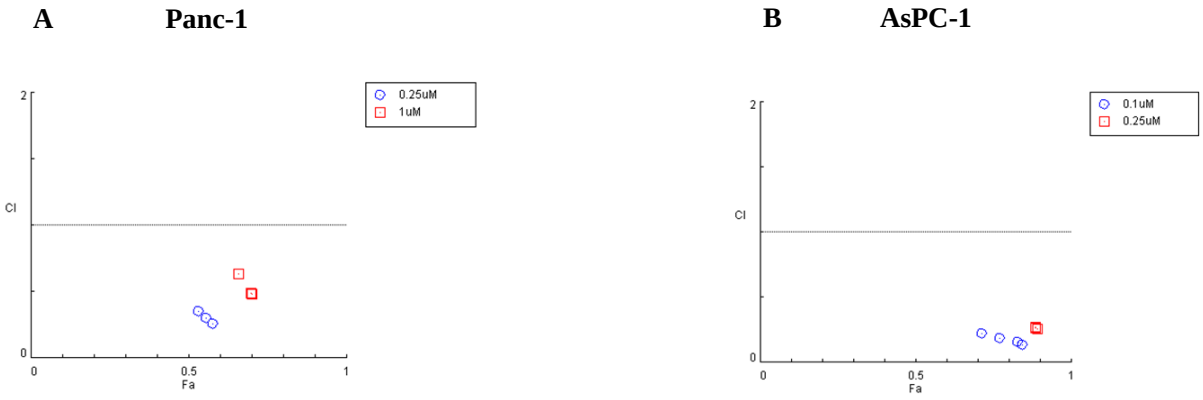


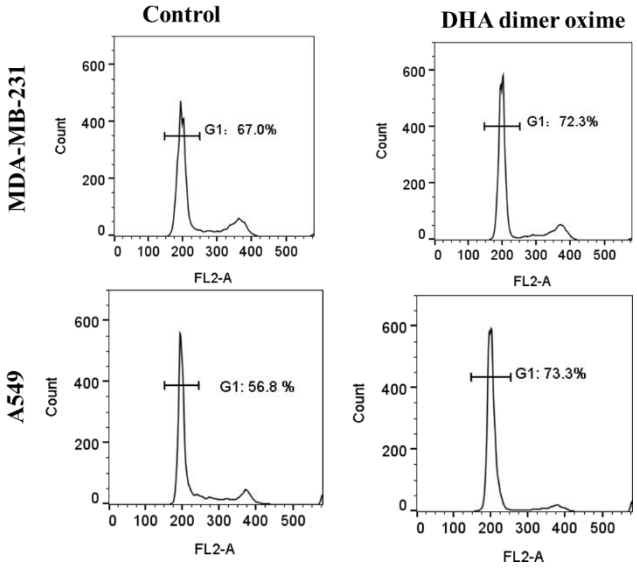
Figure 7. Synergistic effect of DHA dimer oxime and gemcitabine in pancreatic cancer cell lines. Panc-1(A), AsPC-1(B).

Cell cycle analysis

Proliferation in cancer cells is the result of mutation inducing amplification of growth factor, loss of sensitivity to growth inhibitors, dysregulation of checkpoints, and deregulation of apoptosis

[39]. Previous studies have shown that artemisinin and DHA could inhibit the proliferation of tumor cells by inducing the arrest of the cell cycle [40]. Here, we determined the cell cycle distribution of MDA-MB-231 and A549 cells after the treatment of DHA dimer oxime using flow cytometric analysis. MDA-MB-231 and A549 cells were treated with 5 μM DHA dimer oxime for 24 h. DHA dimer oxime treatment resulted in a significant G_1 phase arrest after 24 hours (Figure 8).

To further investigate the effect of gemcitabine and DHA dimer oxime combination on cell viability, a cell cycle analysis was performed in Panc-1 (Figure 9). In Panc-1 cells, the treatment



of DHA dimer oxime led to a pronounced shift of cell population from the G_2/M phase to the G_1 phase, whereas gemcitabine caused an accumulation of cells in S phase. The combination of DHA dimer oxime with gemcitabine resulted in cell cycle arrest in the G_1 phase. The number of cells undergoing apoptosis, as indicated by the sub-G_1 population, was slightly elevated after either DHA dimer oxime or DHA dimer oxime/gemcitabine treatment. These data suggest that the inhibitory effect of DHA dimer oxime and gemcitabine is primarily because of cell cycle arrest, and the enhanced antiproliferative effect is achieved by blockade of cell cycle progression.

Figure 8. DHA dimer oxime blocks cell cycle progression of MDA-MB-231 and A549 cells.

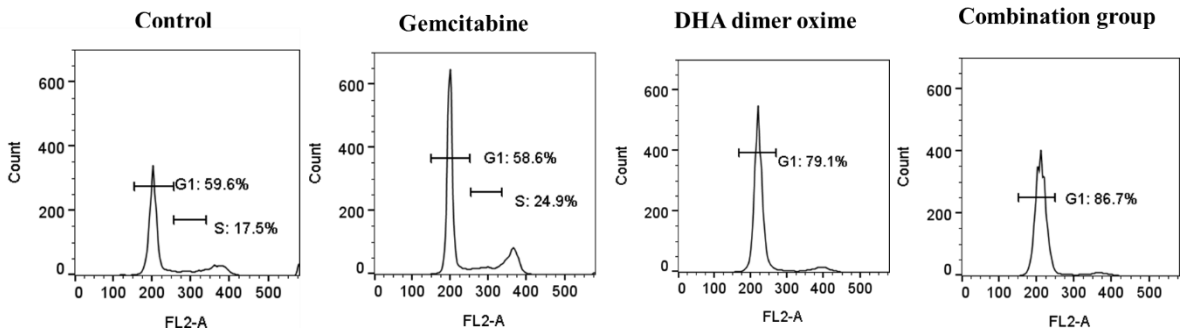


Figure 9. DHA dimer oxime/gemcitabine combination blocks cell cycle progression of Panc-1 cells.

Pharmacokinetics of DHA dimer oxime in mice

In the next study, we focused on exploring the pharmacokinetics of DHA dimer oxime in mice. First, we examined the stability of DHA dimer oxime in plasma, and the result indicated DHA dimer oxime was stable in plasma within 2 hours at 37 °C. Previous studies have shown that its monomeric form DHA was not stable at room temperature, and plasma samples were processed on ice.

The plasma concentration-time profile following a single oral or intravenous dose of DHA dimer oxime was shown in Figure 10, and the pharmacokinetic parameters were shown in Table 5. Detailed pharmacokinetic data for the artemisinin drugs in mice have not been reported. However, several studies have been conducted in healthy and malaria-infected rats, suggesting that the oral bioavailability of DHA was 19.3 ± 1.8% [41]. In this study, the absolute oral bioavailability of DHA dimer oxime was 18.62 ± 4.67 %. Following intravenous and oral administration, the V_d (2.60 ± 0.63 and 2.92 ± 0.19 L/kg) of DHA dimer oxime is larger than the total blood volume (0.058 L/kg) of the mice indicating the extra-vascular distribution [42].

This indicated that more of the compound was present in the central compartment (blood) than in the peripheral compartment (tissues). Following i.v. injection, DHA dimer oxime displayed a lower CL (1.85 ± 0.44 L/h kg) and longer $t_{1/2}$ (0.98 ± 0.05 h) when compared to DHA (CL, 50.9 L/h kg; $t_{1/2}$, 0.31h) in mice [43]. The plasma concentration of DHA dimer oxime remained above 1 μM for 4h, and DHA dimer oxime at 1 μM inhibited around 75% pancreatic cancer cells in previous proliferation study.

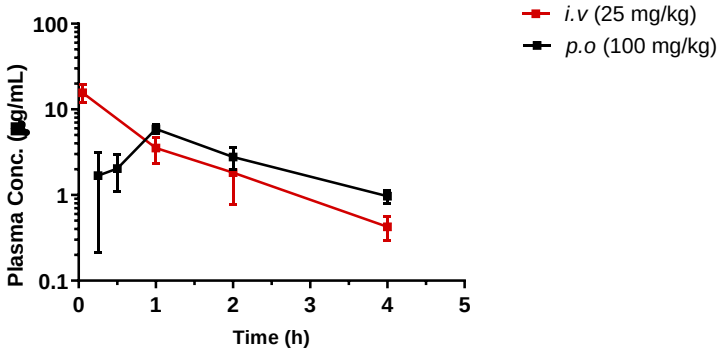


Figure 10. The pharmacokinetic profiles of DHA dimer oxime in mice. Results are presented as the mean ± SD of three mice per time point.

Table 3 Pharmacokinetic parameters of DHA dimer oxime after 25 mg/kg intravenous and 100 mg/kg oral routes of administration in female C57BL/6 mice.

Administration Routes	$t_{1/2}$ （h）	AUC_{INF_obs} (μg*h/mL)	K_{el} (h^{-1})	CL (L/h kg)	V_d (L/kg)	F (%)
i.v. 25 mg/kg	0.98 ± 0.05	14.44 ± 2.28	0.71± 0.04	1.85 ± 0.44	2.60 ± 0.63	100
p.o. 100 mg/kg	1.15 ± 0.09	10.76 ± 1.06	0.60 ± 0.04	1.75 ± 0.11	2.92 ± 0.19	18.62 ± 4.67

Each value represents N=3. mean ± SD

$t_{1/2}$, Elimination half-life; AUC_{INF_obs}, Area under the plasma concentration-time curve; F, Oral bioavailability; CL_T: Total clearance. V_d: Apparent volume of distribution

IV. CONCLUSION

In the current study, we have demonstrated the anticancer effect of DHA dimer oxime *in vitro* and explored the pharmacokinetic study *in vivo*. Firstly, we evaluated the cytotoxicity of nine DHA dimers against MDA-MB-231 cells. DHA dimer oxime was selected for further study. In cell proliferation assay, DHA dimer oxime is more active than its monomeric form artemisinin and DHA, although they were twice the concentration of DHA dimer oxime to maintain molar equivalency. Next, we examined the combination effect of DHA dimer oxime combined with paclitaxel, doxorubicin, and gemcitabine against selected cancer cells. The combination of DHA dimer oxime and gemcitabine exerts an enhanced antiproliferative impact in selected cancer cells, showing synergistic effect with CIs less than 1. Since gemcitabine considered as a first-line therapy for pancreatic cancer, it is essential to determine the effect of DHA dimer oxime/ gemcitabine on pancreatic cancer cells. The result suggested that DHA dimer oxime has the potential to be used in combination with gemcitabine to reduce the resistance of pancreatic cancer cells. We further evaluated the effect of the DHA dimer oxime/ gemcitabine treatment on cell cycle arrest and found that compared with single drug, the combination treatment enhanced gemcitabine-induced growth inhibition for Panc-1 cells. The pharmacokinetic studies demonstrated that DHA dimer oxime might be a better candidate for oral therapy than its monomeric form DHA, because it exhibited superior stability in plasma, longer elimination half-life than DHA. DHA dimer oxime showed acceptable oral bioavailability.

In summary, the present study demonstrated that DHA dimer oxime exhibited promising

anticancer properties. Furthermore, it enhanced the anticancer effect when combined with gemcitabine suggested that DHA dimer oxime could be potentially used in anticancer combination therapy. In the future, In the future, it is necessary to further develop formulations that can improve oral bioavailability of DHA dimer oxime and evaluate the therapeutic efficacy of DHA dimer oxime/gemcitabine combination in murine xenograft model.

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