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SYNTHESIS OF IR780-CHLORAMBUCIL AS A PHOTOSENSITISER FOR TREATING MCF-7 AND HEPG2 CANCER CELLS

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SUMMARY

Phototherapy is a targeted method for eradicating cancer cells with minimum collateral harm to healthy tissues. The method requires a photosensitiser for cancer real-time monitoring and treatment. Photosensitiser which composes from fluorophore and chemodrug would be an advantage compares to another since it could play many roles such as giving the image of cancer, generating the heat and releasing the drug to kill the cancer cells. IR-780 iodide, Heptamethine Cyanine dye, has been used as an ideal platform to construct theranostic agents for cancer imaging and therapy. Chlorambucil (CHL) is an effective chemodrug for cancer therapy which have been utilized for conjugation to synthesize new drug. Thus, in this study we attempted to combines the IR780 and CHL compounds for phototherapy purpose. The new synthesis of IR780-CHL was confirmed the structure by liquid chromatography – mass spectrum (LC-MS) and ^{1}H nuclear magnetic resonance (^{1}H NMR). The LC-MS findings indicated a mass of 616.35 MW, which corresponded to our predicted fraction. While the ^{1}H NMR demonstrated the presence of resonant peaks in IR780-CHL that are exclusive to CHL and IR780. UV-Vis measurements have revealed that the absorbance of IR780-CHL has moved to 756 nm instead of 780 nm. Before and after laser activation, the toxicity of IR780-CHL was comparable to that of CHL, indicating that the synthesis did not influence the *in vitro* effectiveness of the drug treatment. The development of IR780-CHL has considerable potential for cancer detection and treatment.

Keywords: Cancer, chlorambucil, drug release, IR780, phototherapy

INTRODUCTION

In cancer research, smart drug delivery is gaining popularity because it not only increases therapy success but also lessens side effects. The majority of methods developed to control drug release can be categorized into two groups: release by the microenvironments surrounding the tumor, such as low pH and higher body temperature, and release using external forces, such as the application of magnetic force, light, and ultrasound (Hayashi *et al.*, 2014; Wang *et al.*, 2022).

Phototherapy is the use of light to directly treat diseases such as skin-related conditions, wound healing, and sleep disorders, or indirectly to induce drug release at a specific spot. Typically, photosensitive links are responsible for light-induced drug release. For example, 1,2-bis (tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholin, one of the photosensitive phospholipids, under the irradiation of UV light at 254 nm, self-polymerizes and creates a pore on the liposome, allowing medicines to exit the liposome (Sine *et al.*, 2014). Another example is the release of a medication from a liposome in response to heat created by an overexposed fluorophore. For instant, IR780 generates heat when activated at 780 nm wavelength. This heat causes the thermally sensitive phospholipid (1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine) in the liposome (1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine) to melt, deforming the liposome and releasing the antibiotic (Ommen *et al.*, 2022). Besides, phototherapy use the light to trigger the heat of some photosensitiers and kill the cancer cells at the local (Jo *et al.*, 2022). In another example, the combretastatin, an anticancer drug, and IR783 have been conjugated via the cleavable linker. Then the drug was proven to release under near infrared light at 690 nm (Nani *et al.*, 2015a). Visible light and near-infrared light are advantageous in phototherapy because, unlike ultraviolet light, these wavelengths do not harm the cells. Near-infrared light is comparable to visible light since it may penetrate deeper tissues.

Near-infrared fluorophores (NIRF) have the ability to emit strong fluorescence between 700 and 1000 nm, making imaging diagnostics possible. They have several significant benefits for in vivo imaging, such as ligand labelling, higher tissue penetration, a broad range of material coupling, and reduced background fluorescence. Several NIRFs, including Cy5.5 and IRDye800-CW have been marketed with aptamers or antibodies as a targeted ligand for animal cancer imaging (Wang *et al.*, 2023; Yan *et al.*, 2016). The FDA has authorized indocyanine green, a heptamethine cyanine dye, as the only NIRF agent for medical diagnosis. However, the heptamethine cyanine dyes are prospective imaging and treatment agents for cancer. They have been conjugated to several cancer ligands, including metabolic substrates, cell surface peptides, growth factors, antibodies, and cancer biomarkers (Shao *et al.*, 2014). The heptamethine cyanine group, which consists of IR780, IR788, IR808, and MI-148, has been shown to accumulate in tumors and is nontoxic to healthy organs (Shi *et al.*, 2016). For example, IR780 have been conjugated with 17α-hydroxylase/C17,20-lyase for prostate cancer after androgen deprivation therapy (Yi *et al.*, 2016). Or IR780 have been conjugated to peptoids for chemophototherapy (Cai *et al.*, 2021). Various research could be found for the conjugation of heptamethine cyanine group

to the drugs in phototherapy, however, IR780 is one of the most chosen fluorophore for synthesis due to the appearance of chlorine on the its chemical structure which is easy to modify (Gorka *et al.*, 2014; Nani *et al.*, 2015a) (de Oliveira *et al.*, 2019).

Chlorambucil (CHL) is a synthetic alkylating agent and a type of chemotherapy medication used to treat various types of cancer, particularly certain forms of leukemia, lymphoma, and other malignancies (Gupta, Salvatore, 2014). It belongs to the class of nitrogen mustard compounds and works by interfering with the DNA replication process in rapidly dividing cells, thereby preventing the growth and spread of cancer cells (Gupta and Salvatore, 2014). CHL have been modified to link to various subjects in order to improve delivery and treatment efficacy (Fan *et al.*, 2015). For example, CHL have been linked to chitosan for improving delivery efficacy (Sang *et al.*, 2022). Or CHL have conjugated with folic acid and gold nanoparticle for cancer therapy (Vijayashree *et al.*, 2017). Or CHL conjugates with amino acid NGR a ligand for tumor targeting (Vats *et al.*, 2017).

Future estimates imply that the demand for smart drugs in cancer therapy, particularly phototherapy, will increase. Therefore, it is constantly required to discover the method for synthesizing novel light-activated drugs for cancer treatment, tracking and phototherapy. Based on the aforementioned benefits of both IR780 and CHL, therefore, in this study, we aim to synthesize the photosensitiser which comprise from IR780 and CHL for cancer therapy.

MATERIALS AND METHODS

Materials

All the materials, chlorambucil (C0253), IR780 (425311), (P2443), thionyl $SOCl_2$ (8.08154), N,N'-dimethylethylenediamine (D157805), dimethyl sulfoxide (DMSO) (472301), dichloromethane (DCM), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-2H-tetrazolium bromide (MTT) (M2128), used for the synthesis – with exceptions explicitly noted in the article – were purchased from Sigma Aldrich (St. Louis, MO, USA) and were used without any further purification. Materials used for animal cell culture were purchased from Gibco, Thermo Fisher Scientific, USA. All the solvents used in the study were reagent grade and HPLC grade.

The synthesis of IR780-CHL

The synthesis of IR780-CHL consisted of three main steps, as listed in Figure 1.

Synthesis of CHL- imidazole (1)

A three-necked flask was chilled in an ice bath and connected to the condenser system. CHL 1,216 g (4 mmol) was dissolved in benzene (25 mL). Next, $SOCl_2$ solution (30 mL; 48.44 g) was diluted in dry benzene (30 mL), then was added slowly to the reaction mixture over 30 minutes in the N_2 atmosphere while stirring. The mixture was allowed to reach room temperature (RT) and reflux for six hours. Until the reaction was completed, the product was checked frequently using thin layer chromatography (TCL), then the solvent was extracted at low pressure to get the crude product (Chlorambucil chloride), which was then refined by distillation at 240^{o}C/1 mmHg.

^{1}H-imidazole (0.34 g; 5 mmol) and trimethyl amine (0.885 g; 15 mmol) were added to a 25 mL round bottom flask with a magnetic stirrer bar and kept at 0^{o}C. Then chlorambucil chloride (1.615 g; 5 mmol) was added via dropping funnel. The

reaction was stirred at 0°C for 1 hour, and the resultant was warmed to room temperature for another hour. Water (10 mL) was added and stirred until the product precipitated. The precipitate was filtered under reduced pressure, and dried at 50°C in the oven for 5 hours to give compound CHL- imidazole (1).

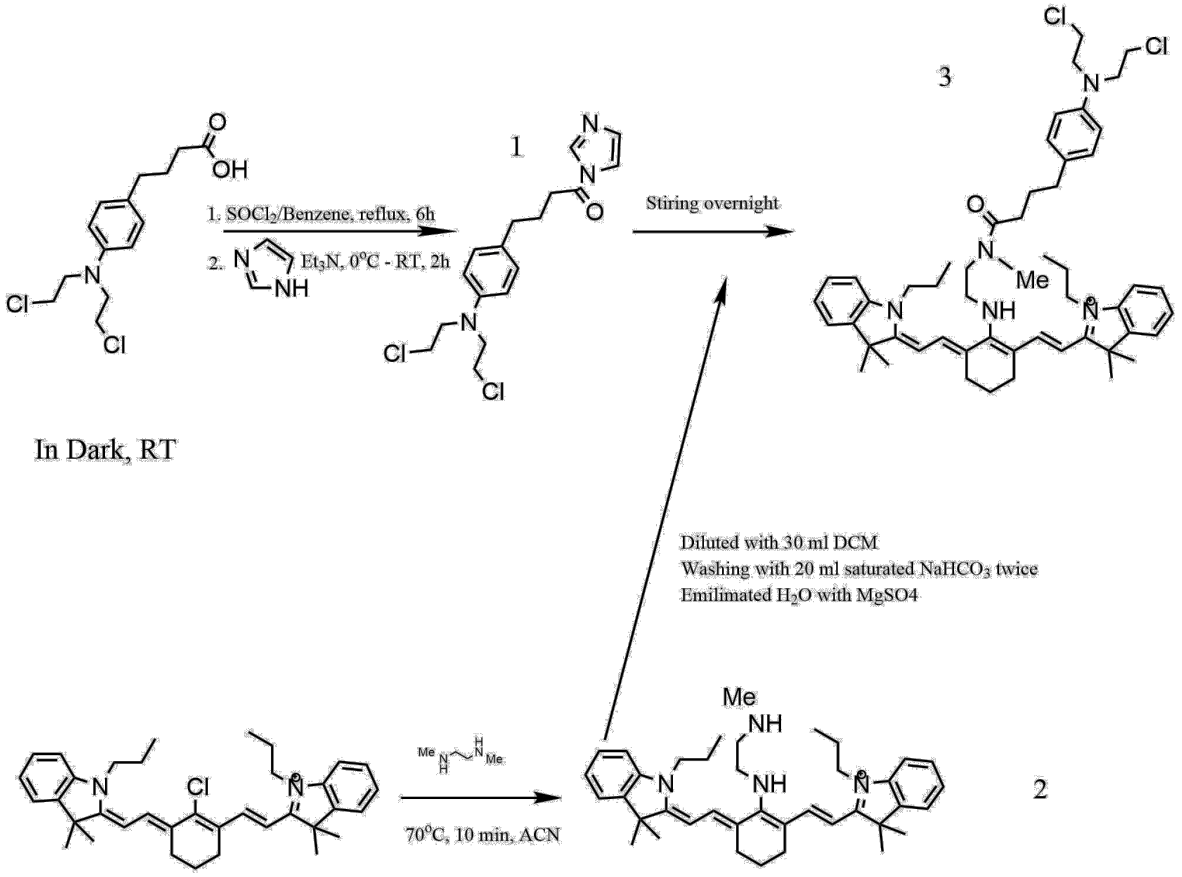

Figure 1. The synthesis of IR780-CHL. 1: The activation of CHL; 2: the synthesis of IR780 to DMEDA; 3: the synthesis of IR780-CHL.

Synthesis of IR780-DMADE (2)

The reaction was performed as described previously (Nani *et al.*, 2015a). Briefly, IR780 (0.15 mmol) and N,N'-dimethylethylenediamine (DMEDA) (0.75 mmol) were dissolved in dry acetonitrile (5 mL). Then, the bottle was closed tightly and warmed up to 70°C in oil for 5 minutes while stirring. At the time, the color of the reaction changed from dark green to dark blue. The reaction was then cooled to room temperature and diluted with 30 mL of dicloromethane. The organic layer was washed twice with 25 mL of saturated $NaHCO_3$. The tracer of water was removed by adding 200 mg of dry Na_2SO_4. The product (2) in figure 1 in DCM was collected by centrifugation.

Synthesis of IR780-CHL (3)

The product (1) was added to (2), and the reactions were stirred for 24 hours in the dark at RT. The DCM was removed by evaporation using a rotary evaporator at

20°C. Our final product (3) IR780-CHL was purified using column chromatography with silica gel (60 – 200 μm) in the dark. At first, the column was run with 100% ethyl acetate to remove the side product (red) until the filtration became clear. Then the mixture of ethyl acetate:methanol: dicloromethane (volume ratio 1:2:1) was added to collect the green/blue yield. The TCL was used to check the products at every step. Then, the final product (3) was collected by a rotary evaporator and kept at -20°C until use.

Liquid Chromatography – Mass spectrum

The product was sent to University of Science, Vietnam National University Ho Chi Minh city, Vietnam for LC-MS analysis (Agilent, Santa Clara, CA, USA).

^{1}H Nuclear Magnetic Resonance

The ^{1}H NMR spectrum of the product was checked in $CDCl_3$ Bruker 600 MHz (Germany), number of scans 16, acquisition time 2.75 seconds, at the Chemistry Department, Vietnam Academy of Science and Technology.

The UV-Vis of IR780-CHL

The absorbance of the IR780-CHL in acetonitrile was checked using NanoDrop OneC (ThermoScientific, USA).

Cell viability

The human breast cancer cell line MCF-7 (ATCC HTB-22) and the human hepatocellular carcinoma HepG2 (ATCC HB-8065) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (FBS), 1% mixture of antibiotics at 37°C, and 5% CO_2. Prior to the cell viability test, cells were seeded at 2000 cells per well in a 96-well plate. On the test day, the CHL and IR780-CHL were dissolving in DMSO at a concentration of 10 g/L. Cells were then treated with the positive control CHL concentrations of 0.2, 0.5, and 1 g/L, IR780-CHL at 0.2, 0.5, 1 and 1.5 g/L, and IR780-CHL at 0.2, 0.5, 1 and 1.5 g/L, after treatment with a near-infrared laser at wavelength 680 nm, 0.08 W/cm^2 for 5 minutes. After 48 hours of incubation, the cell viability was evaluated by the MTT assay. Briefly, 20 μL of MTT reagent (5 mg/mL) was added to the well and incubated for 3 hours. The culture medium was removed, and 100 μL of DMSO was added to dissolve the violet crystal. Then, the OD was measured at 570 nm using a BioTek ELX800.

Illustration and analysis

The Illustration and analysis in the study were designed and analysed by Chemdraw 18.1 and Sigmaplot 12.0.

RESULTS

Synthesis and characterization of IR780-CHL

The synthesis of IR780-CHL were done as described in Scheme 1 which comprised three main steps including: (1) The activation of CHL; (2) The synthesis of IR780-DMADE; (3) The reaction of (1) and (2).

The synthesis of IR780-DMADE was synthesized following the description of Gorka and colleagues (Gorka *et al.*, 2014). It could be noted that during the synthesis of IR780-DMEDA, the initial heat of IR780 at 70°C in 10 minutes partially decomposed the fluorophore into red products which could reduce the yield of final products. However, this red product could easily be seen and eliminated using ethyl acetate in column

chromatography. The synthesis of IR780-CHL was simply the mix reaction of product of (2) and (1) overnight. The final product, IR780-CHL, had a dark green-blue color compared to the green of IR780 (Figure 2A). The LC-MS showed that the main fragment mass spectrum was about 616.3549, which had the chemical formula $C_{34}H_{52}Cl_2N_5O$ (Figure 2B). This mass fracturing was somehow similar to the predicted fracturing structure and mass displayed in Figure 2C. Furthermore, the fracturing with a high molecular mass, like 763.6066 or event 815.2141, also appeared in the spectrum, which was higher than the mass of IR780 (539.32) and IR780-DMADE (577.43). The results have shown the success of the synthesis.

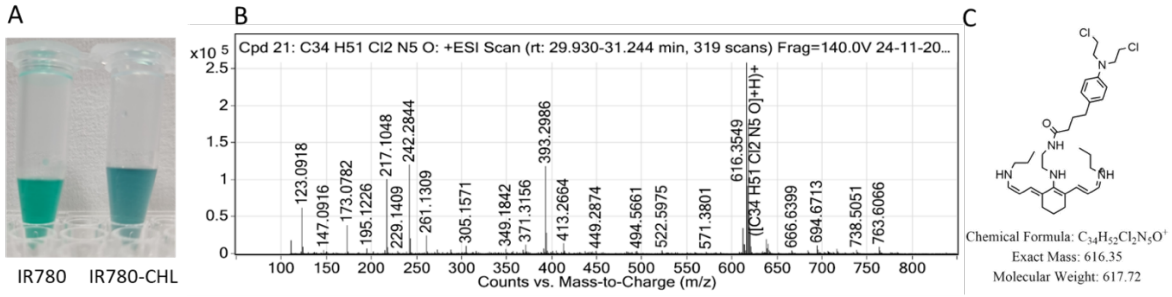

Figure 2. A. The LC-MS of the IR780-CHL. B. Structure and mass of predicted fragment. C. The photo indicated the color of IR780 and IR780-CHL.

NMR analysis

In the ^{1}H NMR spectrum (600 MH z), the IR780-CHL had some unique peaks that appear in CHL and IR780 (Figure 3). Its spectrum showed peaks characterized benzene protons appearing at 7.07 ppm (d, J 8.4, 2H) and 6.64 ppm (d, J 9.0 Hz, 2H) and multiple peaks in the region between 3.5 and 3.7 ppm were assigned to protons of the N, N-diethyl chloride, and the peaks belonging to protons of the butanoic acid in the region of 1.93–2.60 ppm (Prabhutendolkar *et al.*, 2006). In IR-780 spectrum, peaks between 7.1 and 7.4 ppm related to protons of double bonds (C=C) and peaks at region of 3.7 – 4.0 ppm assigned to the presence of protons in HC-N while peaks at 1.3 – 2.2 ppm corresponded to the protons of alkyl groups (Thavornpradit *et al.*, 2019). The spectrum of IR780-CHL showed the peaks related to its components. For instant, the peaks at 6.64 ppm represented for the presence of CHL component while the presence of peaks at 7.1 – 7.4 ppm and intensive peaks at 2.0 ppm assigned to IR780 component. The results confirmed the presence of IR780-CHL in the products.

The absorbance of IR780-CHL

Absorption reflected the spectrum of IR780-CHL's newly synthesized form (Figure 4). The spectra of the produced compound differed from that of IR780. The IR780-CHL featured a shoulder with wavelengths between 634 and 707 nm and a peak at 758 nm, whereas the IR780 had a minor shoulder at 720 nanometers and a shaped peak at 780 nanometers. The results demonstrated that IR780-CHL has shifted to the new wave length. The findings concurred with earlier research about how the adjustment of IR780 would affect its OD spectrum (Cai *et al.*, 2021; Gorka *et al.*, 2014; Sang *et al.*, 2022; Yi *et al.*, 2016). The shift of the spectrum would not affect the phototherapy since the excitation range

in the NIR regions. Also, the previous study showed that the drug was successfully released when excited at 690 nm (Gorka *et al.*, 2014). In this spectrum, the IR780-CHL was able to give the NIRF image as well as to generate the heat which caused it to have the same excitation wavelength as Cy5.5.

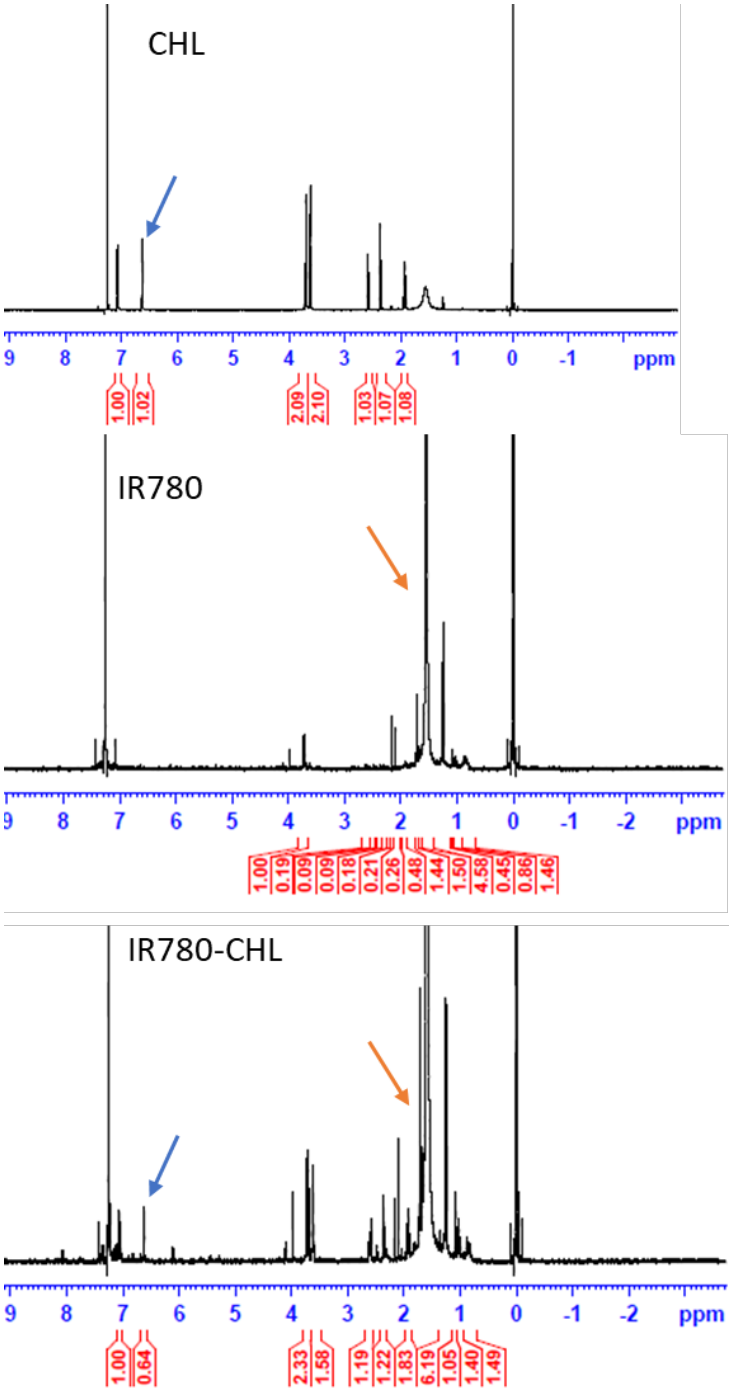

Figure 3. The 1H NMR spectrum of CHL, IR780 and IR780-CHL in $CDCl_3$.

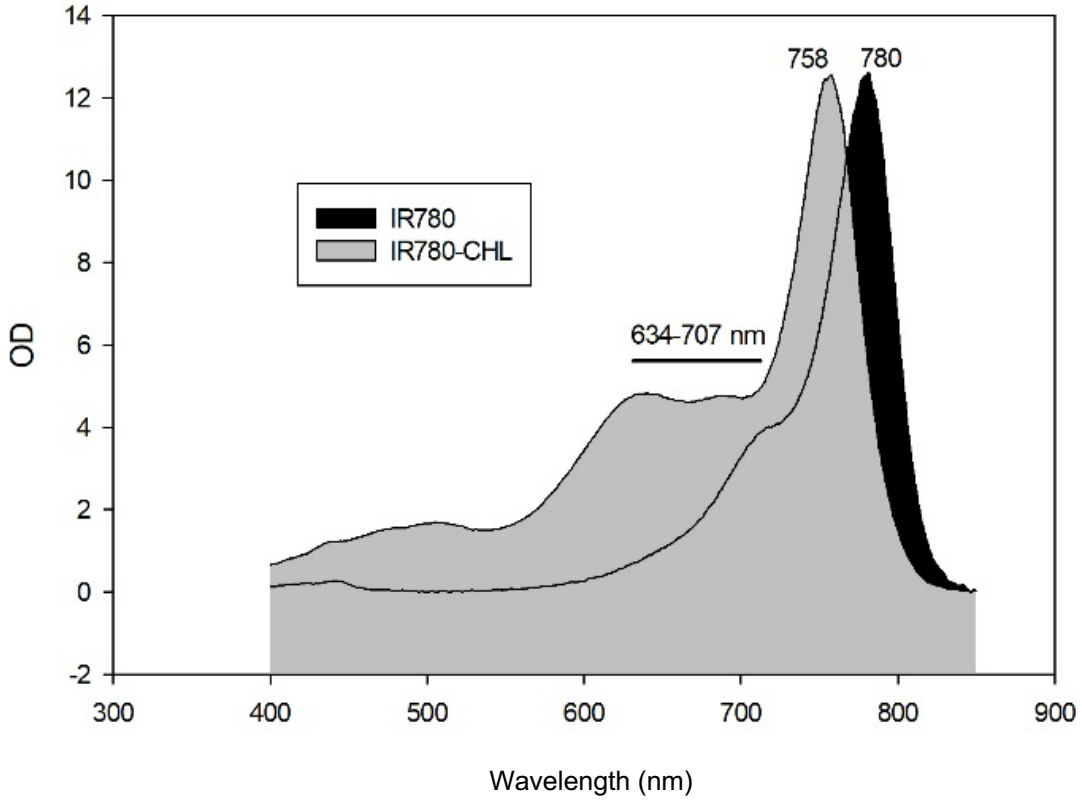

Figure 4. The absorbance of IR780-CHL and IR780 in acetonitrile

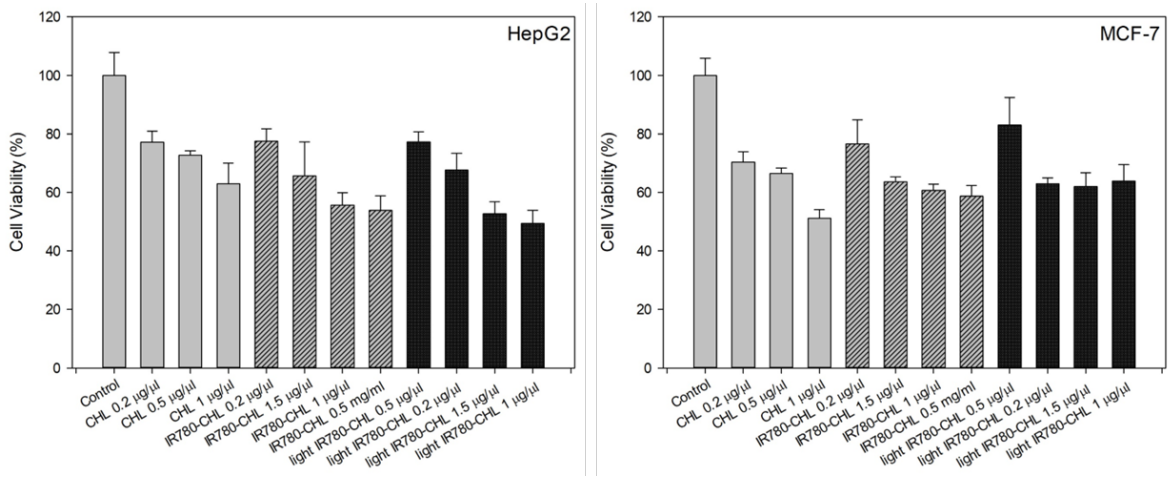

Figure 5. The cytotoxicity of CHL, IR780-CHL and IR780-CHL with laser light to HepG2 and MCF-7 at 48 hours of incubation. Cell: 2000 cells/well, n = 4.

The cytotoxicity of the IR780-CHL

Cytotoxicity of IR780-CHL was studied to determine the drug's efficacy against cancer cells. In this investigation, HepG2 and MCF-7 cancer cells were used (Figure

5). The cell viability of the positive control, CHL, IR780-CHL, and IR780-CHL following laser treatment were comparable at different concentrations. It was determined that the conjugation of CHL to IR780 did not reduce the cytotoxic effects of CHL to cancer cells. Besides, when comparing the toxicity between IR780-CHL and pre-treating the IR780-CHL with the NIR at 680 nm, there were not significant difference between them. It could imply that the drug after treating with laser as toxic to the cell as it before treatment.

DISCUSSION

Phototherapy has gained popularity and is utilized in several fields, including skin care, wound healing, and treatment of other illnesses such as cancer therapy. In cancer treatment, phototherapy required a photosensitizer for success. Photosensitizer might be an imaging agent that enables target monitoring. Or, when activated by light, it might either produce heat to burn tumors or release drugs to kill the cancer cells. In this study, we synthesized IR780-CHL, which can be used for multiple purposes, including as an imaging agent for locating the location of cancer, a photosensitizer for releasing CHL and creating heat when stimulated by NIR light for tumor treatment.

In our investigation, the IR780 and CHL were chosen due to their advantageous characteristics. First, IR780 is more stable than the FDA-approved medication ICG, which might be utilized for long-term tracking and treatment in the clinic (Wang *et al.*, 2016). Under the excitation of NIRF, IR780 also creates heat, and it is used to promote the drug release from thermally sensitive nanoparticles such as liposome (Ommen *et al.*, 2022) and the heat from it was also used for cancer treatment (Li *et al.*, 2019). Second, CHL is a chemotherapeutic agent that might suppress cancer development by inhibiting DNA replication in cancer cells (Gupta and Salvatore, 2014). In its chemical structure, CHL also possesses a carboxyl group that is amenable to modification for diverse applications (Jo *et al.*, 2022; Vats *et al.*, 2017; Vijayashree *et al.*, 2017).

Some research groups have coupled IR780 and 783 to different compounds, drugs, and targeted ligands for cancer treatment, as described in the literature (Gorka *et al.*, 2014; Nani *et al.*, 2015a; Nani *et al.*, 2015b). According to their method, the drug conjugated to IR780 through the linker DMADE is unstable and readily degraded by NIR light (Nani *et al.*, 2015a). Our IR780-CHL was synthesized using the same approach (Figure 1); hence, the CHL would be released by the same mechanism when triggered by NIR light. As with other heptamethine cyanine dyes, it is capable of generating heat upon light activation to destroy cancer cells.

CHL can be used directly to treat cancer or converted to form a prodrug. By regulating the release at the target region, the prodrug is advantageous for minimizing adverse effects. For example, chlorambucil-platinum(IV) is a conjugation of cisplatin and CHL for delivery to mitochondria; both drugs detected and impede DNA replication. (Aputen *et al.*, 2022; Pathak *et al.*, 2017). Or CHL were modified with choline and incorporated into sulfato—cyclodextrin as an enzyme-responsive supramolecular assembly for the treatment of cancer. Butyrylcholinesterase catalyzes the prodrug at the target site and releases CHL (Guan *et al.*, 2019). However, the conversion of prodrug will become difficult if the

conversion of the drug is not 100 percent successful, hence diminishing the treatment's efficacy. *In vitro*, our IR780-CHL demonstrated the same efficacy as CHL (Figure 5); consequently, it is likely to have the same impact in vivo. Our CHL-780 before and after treating with laser light the cytotoxicity stayed the same which show that laser triggering drug may not be necessary. However, IR780-CHL would be beneficial for real time tracing the distribution of drug in vivo as well as generating the heat at the local sites.

Our newly synthesized IR780-CHL have been demonstrated its great potential photosensitiser in phototherapy *in vitro*, however, further investigation of IR780-CHL is necessary. Some limitation of IR780-CHL should be improved like the targeting of the drug to cancer cells as well as reducing the toxicity to the normal cells. Also, in the near future, the IR780-CHL should be tested in vivo such as following the distribution and accumulation in tumor bearing mice, or monitoring tumor via NIRF image, or study the effects of drugs tumor with and without light triggering.

CONCLUSION

The novel IR780-CHL drug has been synthesized for several applications in monitoring and treating cancer. The results validated the structure of IR780-CHL and demonstrated its *in vitro* cytotoxicity. To validate its prospective applications, the IR780-drug might be studied further for NIR imaging, phototherapy *in vitro*, and phototherapy *in vivo*.

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