An-Najah National University

Faculty of Graduate Studies

SYNTHESIS OF DOXORUBICIN-URSODEOXYCHOLIC ACID CONJUGATES FOR TARGETED LIVER CANCER THERAPY

By

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Dedication

I dedicate this dissertation to Allah, the Almighty, who is my source of power, creativity, knowledge, and wisdom. I also dedicate my work to all the martyrs, captives, and injured.

I dedicate this piece to my loving father, who has always supported and inspired me in all aspects of my life. Also, thanks to the best mother who has supported me throughout my journey to become the person I am today by bearing my burdens, pushing me forward, and forgiving me for my errors. To my brothers, who have always encouraged and supported me while I was studying.

I also dedicate this thesis to all of my friends and everyone else who has helped me approach any work with enthusiasm and perseverance.

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In addition, most significantly, there are no words to adequately express how happy I am to have made my family proud. Throughout my years of study and research, I received moral and psychological support that I will never forget

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

SYNTHESIS OF DOXORUBICIN-URSODEOXYCHOLIC ACID CONJUGATES FOR TARGETED LIVER CANCER THERAPY

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: 05/09/2023

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SYNTHESIS OF DOXORUBICIN-URSODEOXYCHOLIC ACID CONJUGATES FOR TARGETED LIVER CANCER THERAPY

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Abstract

Liver cancer is a highly aggressive disease with high mortality rates. Doxorubicin has been utilized to treat hepatocellular carcinoma due to its broad-spectrum anticancer effect. However, due to the potential of cardiotoxicity and the development of multidrug resistance, it is typically dose-limited in therapeutic use, limiting its long-term usefulness. Ursodeoxycholic acid is a hydrophilic bile acid that binds to the bile acid receptors with higher affinity than any other type of bile acids.

The primary objective of this project is to develop a UDCA-DOX conjugate and evaluate its targeting capabilities *in vitro* to specifically deliver DOX to HCC cells. With these objectives, we aim to exploit the affinity of UDCA for HCC cell lines to minimize DOX toxicity on normal cells, while maintaining enough cytotoxicity against liver cancer cells.

UDCA-DOX conjugate was synthesized with an acid-labile linkage, then, its characteristics and release profile were studied by the HPLC. After that, the cytotoxicity of this conjugate was investigated by the MTS test *in vitro* in HepG2 ,Hep3B hepatic cancer cell lines, LX-2 cells the normal hepatic cells, and the none hepatic cells 3T3. The targeted cellular uptake of UDCA specifically by hepatic cancer cells was studied by fluorescence microscopy.

UDCA-DOX can spontaneously hydrolyze in acidic media. This conjugate showed significant cytotoxicity in hepatic cancer cell lines (HepG2 and, Hep3B). The successful delivery of DOX into the cancerous cells was confirmed by fluorescence microscopy. However, the cytotoxicity of UDCA-DOX was limited in non-cancerous cell lines

(LX-2 and 3T3), suggesting that the delivery of UDCA-DOX into the cells is dependent on specific features in the hepatic cancer cells. Moreover, the observed cytotoxicity of UDCA-DOX was less than that of free DOX, suggesting that the drug reached the cell via a rate-limited process, which endorses the hypothesis that the internalization of UDCA-DOX could be through bile acid receptor-mediated endocytosis.

This study demonstrated valuable insights into the potential of UDCA-DOX as a targeted therapeutic strategy for HCC to reduce the adverse effects associated with DOX therapy. Further investigations are required to test the in vivo efficacy and safety of this conjugate.

Keywords: Hepatocellular Carcinoma; Ursodeoxycholic acid; Doxorubicin; Cancer targeting; Acid-labile Linkage; Fluorescence.

Chapter One

Introduction and Literature Review

1.1 Cancer

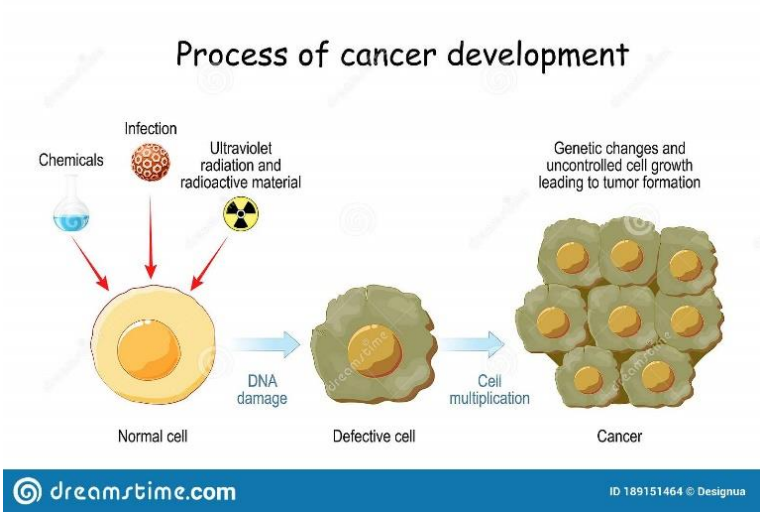
Cancer, also known as neoplasm or tumor, is defined by the American Cancer Society as an abnormal, uncontrollable overgrowth of cells and tissues of any organ with an invasion of adjacent parts and metastasis to other organs increasing the incidence of death (1). It's the second leading cause of death worldwide (2). According to a world cancer report, the incidence of neoplasms increasing globally by 50 % to 15 million cases in 2020. According to the last Word Health Organization (WHO) report in 2020, cancer is the highest disease increasing daily with 10 million deaths. However, the deadliest types of cancer are lung cancer, followed by colon and rectum, and liver cancer at the third leading cause of death with 830 000 deaths globally (3) .

It's still unknown what causes cancer, but there are many hypotheses discussing the mechanism of cancer i.e. viral-induced cancer, somatic mutation, multiple accumulated mutations, chromosomal-induced mutation, immunologically induced mutations, etc... (4). Risk factors of carcinogenesis are unlimited but, environment, diet, genetics, occupation, smoking, and infections are examples of common risk factors of cancer pathology (1).

However, cancer is classified according to the histology of origin, and according to the primary site. Histological classification is divided into Sarcoma, Myeloma, Leukemia, Lymphoma, Mixed, and Carcinoma. Carcinoma is defined as the most common type of cancer accounting for 80-90% and originated from epithelial tissue, for example, skin, the lining of the gastro-intestine (5). Scheme 1 shows the development process of cancer cells.

Scheme 1

Development process of Cancer cells



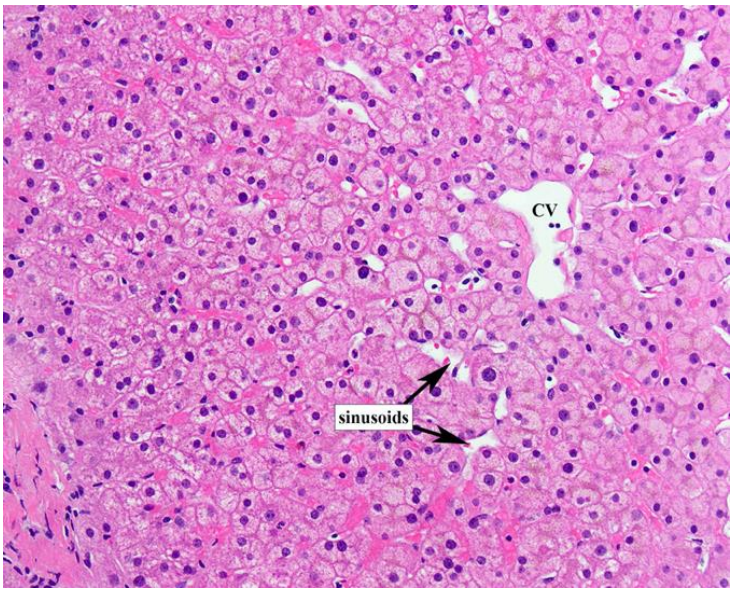
adopted from: dreamstime. Process of cancer cell development [cited 2023]. Available from: https://www.dreamstime.com/process-cancer-cell-development-process-cancer-development-cancer causing-agents-stages-transforming-normal-cell-image189151464

1.2 Hepatocellular Carcinoma (HCC)

The liver is the largest internal organ and consists of several cell types with a prominent type called hepatocytes cells (see Scheme 2), which is responsible for the metabolism of nutrients and drugs, production of clotting factors, and aid in the absorption of nutrients by delivering bile through the bile duct to the intestine in a process called enterohepatic circulation (6).

Scheme 2

Hepatocyte cells



adopted from: Bell P. [cited .[2023 Available from: https://www.aasld.org/liver-fellow-network/core-series/pathology-pearls/normal-liver-histology-101

However, liver cancer consists of a variety of heterogeneous malignancies with bad prognoses ranging from Hepatocellular carcinoma (the cancer of parenchymal cells of the liver), the most abundant type of primary liver cancer (80-90%), cholangiocarcinoma (the tumor of the lining of the bile duct) in a second manner, with less common fibrolamellar HCC, mixed HCC-CA, and pediatric hepatoblastoma (7-10).

The worthy prognosis of HCC increase with the presence of confounding factors, common and major risk factors include a chronic viral infection (Hepatitis B, Hepatitis C viruses) accounting for 80% of HCC cases globally, bad habits i.e. alcohol consumption, tobacco smoking, and aflatoxin consumption. Moreover, male gender, consumption of arsenic from drinking water, family history of HCC, iron overload, nonalcoholic liver fibrosis, androgens hormones, diabetes, and obesity (8, 9, 11).

Treatment of HCC is affected by the presence of other conditions like hepatitis infection, liver cirrhosis, and other risk factors (12). Generally, treatment option depends on whether primary HCC is metastatic or not (stage of malignancy), the presence of comorbidities, the overall function of the liver, the overall health of the patient, age, and the size of the tumor (13-15).

Patients with an early-stage disease with no metastatic regions, good liver function, good patient performance, and early discovery of disease may cure liver cancer with five years life expectancy.

However, early-stage management includes; surgical management by laparoscopy technique (partial liver resection), liver transplantation if surgical resection cannot be performed, and thermal ablation (destroying the tumor with very high and low temperatures) (15).

On the other hand, the goal of treatment in the advanced stages of HCC focuses on increasing the life expectancy of patients. Treatment options include; Embolization (using tumor-killing medications or radiation in targeting the beads of HCC) and chemotherapy either a single agent or combination regimen e.g. Sorafenib, Docetaxel, cisplatin, Gemcitabine, Oxaliplatin, 5-fluorouracil, GEMOX (gemcitabine plus oxaliplatin), FOLFOX (5-FU, Oxaliplatin, and leucovorin) and doxorubicin (15, 16).

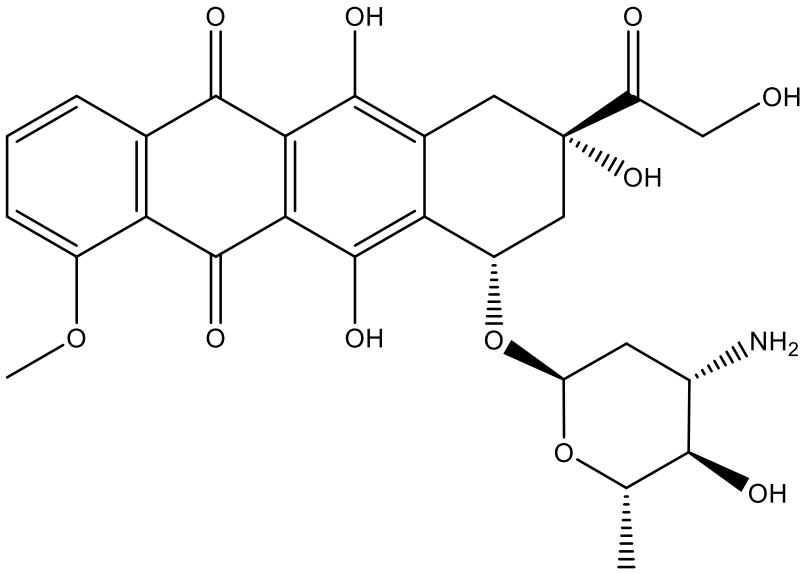
However, chemotherapy drugs can be given as systemic therapy (IV, oral) for metastatic tumors, and Regional therapy by administering the antitumor medication into the hepatic artery, so decreasing the systemic side effects of chemotherapeutic medications. On the other hand, the adverse effect profile of chemotherapy is widely depending on the dose, the type of drug, and the length of treatment. However, loss of appetite, hair loss, diarrhea, nausea, vomiting, immune system suppression, anemia, and mouth sores are common symptoms among patients on chemotherapy treatment (17).

1.3 Doxorubicin (DOX)

DOX is a chemotherapeutic glycoside anthracycline drug segregated from *Streptomyces paucities*. Scheme 3 shows the chemical structure of DOX.

Scheme 3

Chemical structure of DOX



DOX is a broad-spectrum potent anti-tumor drug clinically effective against solid and hematological cancer e.g. breast, prostate, uterine cancer, bile duct cancer, osteosarcoma, leukemia, esophageal tumor, and liver cancer. Additionally, DOX is a topoisomerase II inhibitor and covalently interacted with proteins responsible for nuclear and mitochondrial DNA replication and transcription leading to enhance apoptosis (18).

On another hand, the doxorubicin toxicity profile varied from nausea & vomiting, alopecia, hallucinations, baldness, extravasation, stomatitis, bone marrow depression, dose-dependent myelosuppression, and serious dose-dependent cardiotoxicity (acute and chronic with 50% mortality rate of irreversible cardiotoxicity) (18). *Sun, X et al* explain the mechanism of chronic cardiotoxicity by morphological changes of cardio myocytes leading to increase expression of ANP (Atrial natriuretic peptide), cytochrome P (CYP), and BNP (Brain natriuretic peptide) genes resulting in hypertrophy and congestive heart failure (19). Although, doxorubicin has the reversible chemo-brain effect (diminished cognitive abilities because of chemotherapy drug) related to the increased expression of TNF-α (18, 20). Doxorubicin negatively affects the liver, since it is metabolized in the liver. One of the liver toxicity hypotheses is the induction of the ROS system by doxorubicin leading to DNA damage and

hepatotoxicity. Doxorubicin decreases ATP, increasing AMP and ADP resulting in increased hepatocyte vacuolization (18).

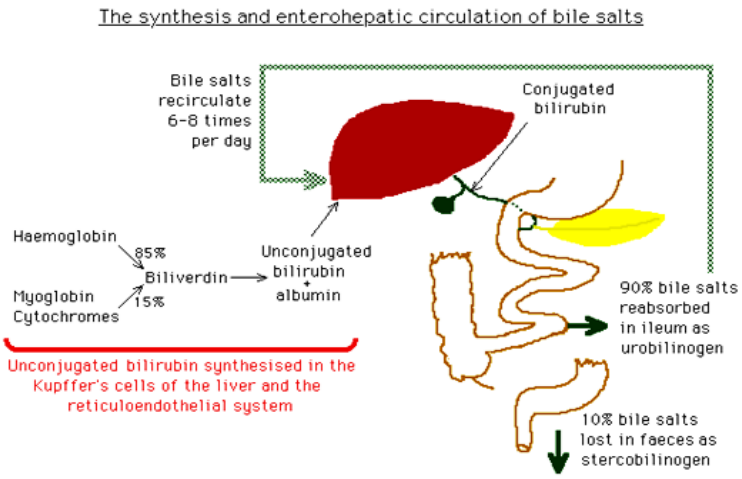
Doxorubicin shows a loss of sensitivity and cellular resistance. *Marine et al* explain the Low response, and loss of sensitivity in hepatocarcinoma due to the "resistome" complex and diverse mechanism of chemoresistance (MOC) by the involvement of more than (100) genes (21).

1.4 Bile Acid

Bile acid is an amphiphilic detergent sterol structured from cholesterol; conjugating with taurine or glycine catalyzing by cytochrome P450 (CYP) to improve their solubility. However, the chemical structure of bile acid is a steroidal tetracyclic nucleus with functional hydroxyl groups. Bile acids undergo an entero-hepatic circulation between the intestine and liver, see Scheme 4, which is key role in the intestinal digestion and absorption of fats, lipids, fat-soluble vitamins, triglycerides, cholesterol, and glucose metabolism. In addition, it's an important signaling molecule that regulates cellular process e.g. cell differentiation, proliferation, delivery of nutrients, and in gene expression since they have receptors at cells i.e. G-protein-coupled bile acid receptor-1 (GPBAR-1) and farnesoid X receptor (FXR) [28, 31].

Scheme 4

Synthesis and enterohepatic circulation of bile.

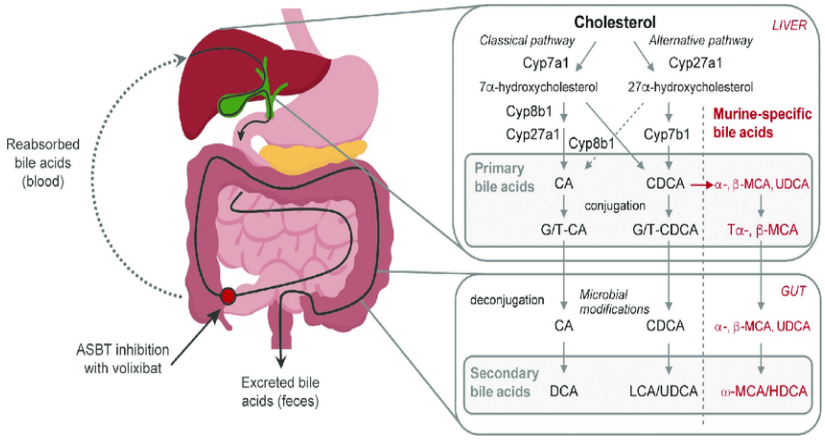


adopted from: GPnote. Synthesis and enterohepatic circulation of bile. 2023. Available from: https://gpnotebook.com/pages/cardiovascular-medicine/diagram-of-the-enterohepatic-circulation-of-bile-salts.

The synthesis of bile acids in the liver occurred through two pathways; the classical and the alternative (acidic) way. The acidic way accounts for 10% produce Chenodeoxycholic acid (CDCA) and is most regular in rodents. However, in humans, the most common one is the classical method accounts for 90% at which cholesterol in presence of CYP450 is converted to the primary bile acids; cholic acid (CA), and (CDCA) at equal ratios. Then with the aid of bacterial enzymes of the intestine i.e. bile acid-coA, bacterial 7α-dehydroxylase, and bile acyl-coA synthetase, covalently conjugate glycine or taurine to produce more soluble bile acids such as glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), UrsoDeoxycholic acid (UDCA) and taurocholic acid (TCA) (22-24) as in Scheme 5.

Scheme 5

Synthesis of Bile acids from Cholesterol



adopted from: Aguila MB, Salic K, Kleemann R, Wilkins-Port C, McNulty J, Verschuren L, et al. Apical sodium-dependent bile acid transporter inhibition with volixibat improves metabolic aspects and components of non-alcoholic steatohepatitis in Ldlr-/-.Leiden mice. Plos One. 2019;14(6):e0218459.

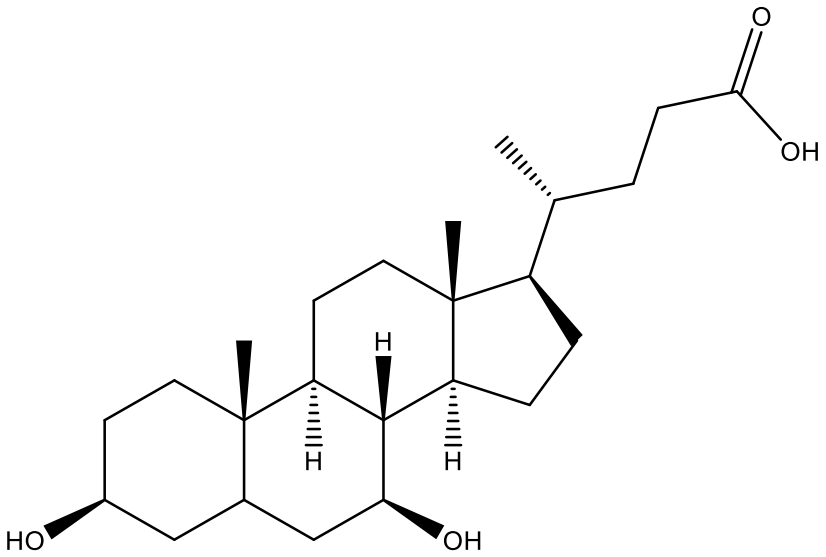
Previous studies showed a unique relationship between BA imbalances due to downregulated expression of BA transporters, which leads to the accumulation of harmful hydrophobic BA, and a bad prognosis of HCC (25).

Ursodeoxycholic acid (UDCA, 1-3% of total bile acids) is a secondary hydrophilic dihydroxy bile acid that was first discovered in the bile of the Chinese black bear known as genus Ursus. UDCA is structured from a cholesterol base as in Scheme 6.UDCA reduces the secretion of cholesterol, the intestinal absorption of bile, and enhances the dissolving of cholesterol gallstones. As a result, it's a hepatoprotective bile acid, which suppresses the tumergenesis process of HCC (26, 27). However, USDCA is transported

through multiple saturable and non-saturable Na+-TaurocholateCotransporting Polypeptide (NTCP) receptors (28, 29).

Scheme 6

structure of UrsoDeoxycholic acid



However, UDCA had a dual anti and pro-apoptotic action on HCC progression, since it is structured from cholesterol (has a conflicting role in cell death of HCC). As a result, recent researchers focusing on UDCA as a potential agent for chemoprevention and chemotherapy (29-31).

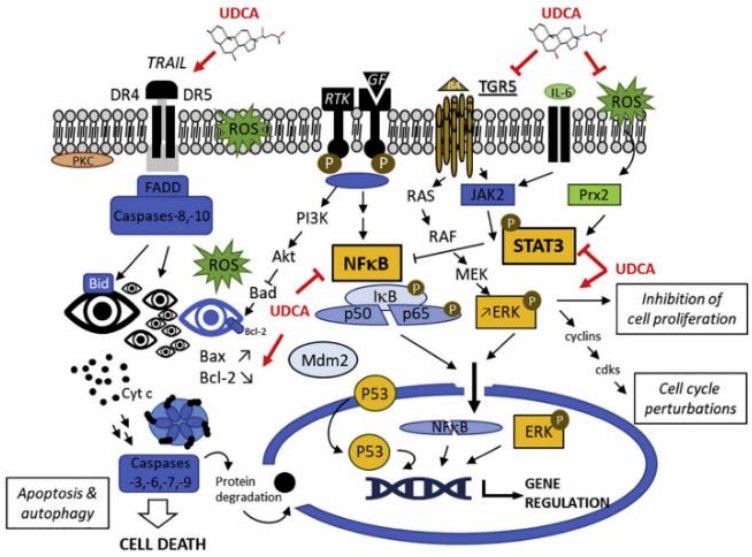
On one hand, UDCA as chemotherapy draw the attention of researchers. Until today, the exact mechanism is unclear but studies suggested some possible routes of chemotherapeutic activity of UDCA. *Zhu et al and Liu et al* suggested that UDCA induces apoptosis in HCC due to upregulated Livin and Bax, alternatively downregulated Smac and Bcl-2 proteins (27, 32). *Tsagarakis and collagenous* confirmed that Bax: Bcl-2 ratio is considered a critical point in the inhibition or activation of apoptosis of mitochondrial dysfunction. Additionally, they assist in the UDC activates the caspase pathway which may trigger apoptosis (33). *Goossens and others* study UDCA as chemotherapy in mice-model. They found that the effect of UDCA was a dose-dependent manner in reducing BEL7402 HCC growth and induced apoptosis (30). Moreover, *Yoon et al* showed that UDCA inhibits D1c1 protein in a ubiquitin-independent manner (26).

On another hand, many articles discussed the possible reasons for UDCA as a chemoprevention agent. *Cabrera and collagenous* summarized that UDCA promotes pre and anti-apoptotic effects on hepatocytes by different mechanisms. Firstly, it inhibits the classical pathway of apoptosis. Secondly, make representative morphological changes at chromatin fragmentation of apoptotic nuclei. Thirdly, inhibit the triggers apoptosis by FAS, TGF-β1, okadaic acid, and ethanol. And finally, reduces the release of cytochrome c to the cytoplasm from mitochondria (34).

Additionally, the interesting -a chemo-preventive feature of UDCA opens the door toward using it in protecting normal cells from chemotherapeutic medications damage. For instance, UDCA induces the release of intestinal anti-inflammatory cytokines and reduces gastrointestinal mucositis caused by 5-Flurorouracil. UDCA reduces the neurodegeneration effects of cisplatin by inhibiting the P35 pathway, also, converts the unpleased necrosis effects of oxaliplatin to apoptosis pathway by induction of P53-caspase 8 route in HepG2 cells, and reduces the cell injury of methotrexate in liver cells (30, 35). Scheme 7 summarized the mechanisms of UDCA anti-cancer.

Scheme 7

The possible mechanisms of UDCA anti-cancer probability



Very interesting research studied the possibility of the synergistic effect of UDCA and sorafenib, which are used in HCC management. The studies resulted that UDCA regulated OATP1B1 receptor, which is responsible for the release of sorafenib-β-D-

glucuronide metabolite. Additionally, co-treatment of sorafenib and UDCA improve the efficacy of sorafenib in inducing apoptosis through dephosphorylating of signal transducer and activator of transcription 3(STAT3) and activation of extracellular signal-regulated kinase (ERK) depending on (Reactive Oxygen Species) ROS system (36-38).

Dependently, UDCA is used as a delivery vector for anti-cancer medications in different types of cancer. Limited work is done on this approach especially in targeting liver cancer. *Zhang and others* increase in vivo half-life and in-vitro stability of cytarabine by conjugate cytarabine-UDCA (30, 39).

Tsagarakis and collagenous assessed the balance between cellular survival and receptor death in the HepG2 cell line. They summarized UDCA at higher concentrations inhibits HepG2 proliferation (33, 40). Moreover, Structure-activity relationship (SAR) studies showed that using bile acids as targeting agents could bring a better future for the management of liver carcinoma (41).

1.5 Literature Review

Traditional treatment of HCC with cytotoxic agents i.e. cytarabine, sorafenib, and doxorubicin had limited efficacy on carcinogenesis. Because of, systemic side effects, bad efficacy, and acquired resistance. However, doxorubicin chemoembolization was a good choice for liver carcinoma treatment with better efficacy and toxicity profile. Nevertheless, this strategy cannot overcome the acquired resistance of cancerous cells. The mechanism of the resistance is not clear yet, but it may be related to the alteration of the drug target, DNA modulation of cellular death, higher expression of drug efflux pump in HCC cells, and alteration of topoisomerase enzyme. Those changes are conducted from changes in mRNA and DNA expression, which make them prominent changes (42).

To overcome doxorubicin-induced resistance in HCC cells, researchers designed multiple forms of drug delivery for more selective and safer cytotoxicity. *Li al et al* designed a dual-ligand modified liposome-encapsulated doxorubicin (DOX-GA/PNA-lips). A black liposome was used and the addition of PNA and GA ligand on the surface of the liposome was then loaded with doxorubicin. Nevertheless, PNA and GA ligand

act as targeting agents of hepatoma cells; once they are attached they release doxorubicin to the cells in a frequent manner. This strategy showed better safety and efficacy profile (43).

However, bile acids act as solubilizing and permeation-modifying agents of poorly soluble and poorly permeable medications. Therefore, bile acid can be used as a drug delivery system either as a part of ligands in nanoparticles or as a conjugation subunit. The chemical structure of bile acids contains carboxyl and hydroxyl groups that can be covalently conjugated with low-permeable anti-cancer agents, therefore enhancing the efficacy and safety profile of these agents. However, previous studies showed that anticancer medications can be conjugated with bile acid at positions of C-24, C12, C-7, and C-3. Motherless, for medications that are recognized by the liver the best position of bile-drug conjugation, is at the C-3 position (48, 49).

Researchers studied the synergistic administration of anti-cancer agents with bile acids as a strategy for improving the cytotoxic effect on cancerous cells. *Zhang et al* showed ChenoDeoxycholic Acid bound to the extracellular structure of EGFR, therefore improving the inhibitory effect of sorafenib in the HepG2 cell line of liver hepatoma (44).

Bile acids are the attractive building block for designing a novel drug delivery system; Bile acid-drug conjugate (45). *Sreekanth and others* create lithocholic acid- Tamoxifen conjugate. Lithocholic acid phospholipid derivative (LCA-Tam-PC) is a stable bile acid at stomach PH, while Tamoxifen is a poorly soluble drug and precipitates at acidic PH. The LCA-Tam-PC-Tamoxifen conjugate had a prolongation life cycle, therefore, showed better solubility and stability. In addition, this conjugate resulted in improvement of cellular uptake, shrinkage of the tumor weight and volume, less drug hepatotoxicity, enhance the selectivity of Tamoxifen (higher accumulation of Tamoxifen at the site of cancer), and better anti-cancer effect in comparison with Tamoxifen alone (46).

Cunningham and collagenous formulated a new delivery technique depending on bile acid (Cholic acid) as a targeting agent for delivering doxorubicin by hydrophobic or electrostatic interactions by nanoparticles. However, nanomaterials showed better cellular internalization and lower IC_{50} (47).

UDCA-Cisplatin conjugate was formulated to overcome the chemotherapy resistance challenge and for attachment to colon adenocarcinoma and cholangiocarcinoma with high expression of ASBT receptors (48). Besides, Cytarabine is classified poorly soluble, poorly permeable agent. Efforts were conducted to conjugate cytarabine with different bile acids (CA-cytarabine, HDCA-cytarabine, CDCA-cytarabine, and UDCA-cytarabine conjugate).All of the conjugates were effective against HCC, HepG2 cell line, with better stability profile of UDCA-cytarabine conjugate (39).

Based on the previous review, our novel hypothesis will base on using UrsoDeoxycholic acid for targeting hepatoma cells to selective penetration of doxorubicin to improve therapeutic outcomes and reduce commonly associated toxicities.

1.6 Aim of the project

The project aims to develop a new approach to managing HCC using UDCA bile acid as a targeting agent. The new approach is based on the synthesis of doxorubicin-UDCA conjugates as a targeting agent to selectively release doxorubicin to hepatoma cells.

1.7 Objectives

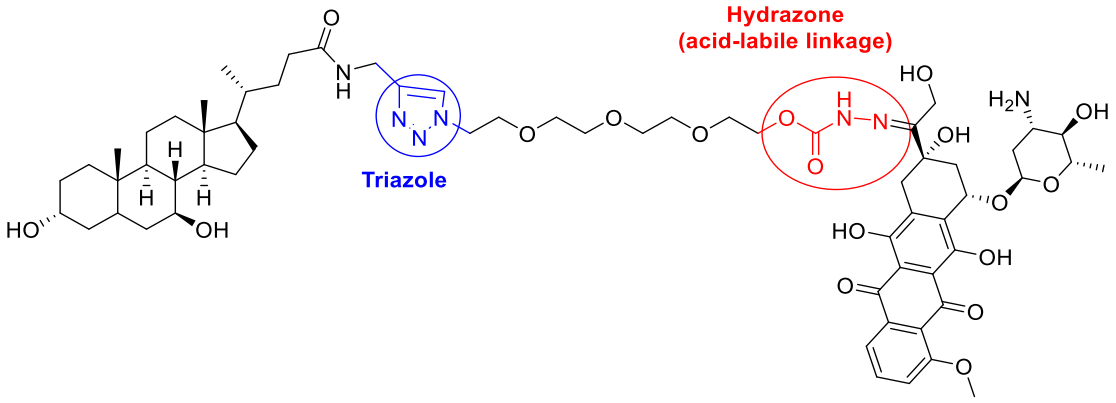
1. Synthesis of Doxorubicin-UDCA conjugates by acid-labile linkage.
2. Characterization of the synthesized conjugate.
3. The study of the release profile of the conjugates using the HPLC method.
4. Study anti-cancer activity of conjugates in the liver cancer cell line.
5. Study cell-overlay of UDCA-DOX conjugate in comparison with doxorubicin and UDCA alone.

1.8 A general method for synthesis of doxorubicin-UDCA conjugates

This thesis aimed to synthesize a conjugate of doxorubicin-UDCA using a hydrophilic linker that the ursodeoxycholic acid connected through triazole ring to the linker and on the another part the Doxorubicin connected through acid-labile linkage to the linker as shown in scheme 8.

Scheme 8

The chemical structure of the required UDCA-DOX conjugate



Chapter Two

Materials and Methods

2.1 Materials and reagents of synthesis part

All materials used in the experiments were of analytical grade. UrsoDeoxycholic acid (M.W:392.56, lot#:10231377), copper iodide (M.W:190.45), N, N-Diisopropylethylamine (DIPEA, M.W:129.25, lot#:STBF9602V), and Doxorubicin hydrochloride (M.W:579.98, lot#:LRAB3692) were purchased from Sigma-Aldrich company. 4-Nitrophenylchloroformate (M.W:201.57, lot#:10204524), TBTU (lot#: B23597), propargyl amine (M.W:55.08, lot#: H53495) and tetraethylene glycol (M.W:194.23, lot#:B23990) were purchased from Alfa-Aesar company, UK. Triethylamine (M.W: 101.19, lot#:40502L05), diethyl ether (Lot#:38132), and N, N-Dimethylformamide (DMF, M.W:73.095, lot#:55145) were purchased from SDFCL company, India.

Dichloromethane (DCM), Ethyl Acetate, sodium hydroxide, sodium chloridewere purchased from C.S. company, Haifa. Sodium azide (M.W:65.0099, lot#:0E30428) was purchased from RIEDEL-DEHAEN AG SEELZE-HANNOVER laboratories, Germany. Methanol and acetone were purchased from Sun Pharma drugstore. Trifluoroacetic acid (M.W:114.023, lot#: A12198) was purchased from Fisher Chemicals, UK.

2.2 Materials and reagents of the biological part

In the biological part, we used the following cell lines from ATCC. HepG2 (isolated from a white, male, 15-year-old with hepatocellular carcinoma), Hep3B (isolated from a Black young male, 8-year-old, with liver carcinoma with hepatitis B virus genome), LX-2 (human hepatic stellate cell line), and 3T3 (mouse isolated fibroblast cells).

Pen-Strep-Ampho. B Solution (lot #2144024, Penecillin10000 units/ml, streptomycin 10mg/ml, Amphotericin B 0.025mg/ml), RPMI (lot#05669), and DMEM (lot# 222201212) were purchased from Biological Industries, Jerusalem.

From sigma-Aldrich in the USA, we purchased the following items which are L-Glutamine solution (lot# RNBK9610), Trypsin-EDTA solution 1X (catalog # 59417C), fetal Bovine Serum (catalog # C8065). The MTS (catalog # G3581) was purchased from Promega (USA). From CellStar, USA we purchased a 96-well cell culture plate and a 12-well cell culture plate.

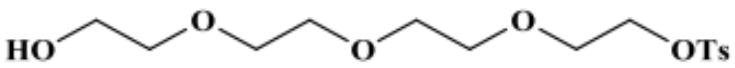
2.3 Techniques and instruments

Thin layer chromatography, DC-FertigfolienAlugeram® Sil G/UV 254 was purchased from Macherey Nagel Company, Germany. Column chromatography with silica gel, 230-400 mesh, was purchased from Sigma-Aldrich. Rotary Evaporator (MRC, ROVA-100, laboratory equipment manufacturer). High-performance liquid chromatography with C18 HPLC column (ODS-3V, 2.7μm100*4.6mm) purchased from RESTEK Company. NMR Bruker Avance 500 MHz was used for compound characterization.

For biological purposes, we used the following: Cell culture CO2 incubator (ESCO, serial#:2012-74317), Absorbance Microplate Reader (ELX 808, used at ʎ=490), and Olympus inverted microscope (6M45542, IX73PLF, OLYMPUS).

2.4 Synthesis Part

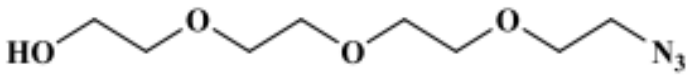
2.4.1 Synthesis of Tosyl-TEG-OH (1)



(1)

A solution of tetraethylene glycol (3 g, 15.45 mmol) and Et_3N (2.4 ml, 17.0 mmol, 1.1eq) in 20 ml DCM was stirred under ice conditions. Then tosyl chloride (3.24 g, 17.0 mmol, 1.1 eq) was added gradually drop by drop over 30 minutes. This reaction was left overnight under vigorous stirring at room temperature. The resultant product was extracted by 100 ml DCM, washed with 50 ml 1M HCl, then a drying agent was added and filtrated. The solvent was removed under vacuum. The crude product was purified by column chromatography in silica gel using DCM: MeOH (20:1) as a mobile phase, giving a pale yellow oil. The yield of pure product was 55.6% (2.2 g, 6.31 mmol). The NMR results were the same as we previously reported in our group (49).

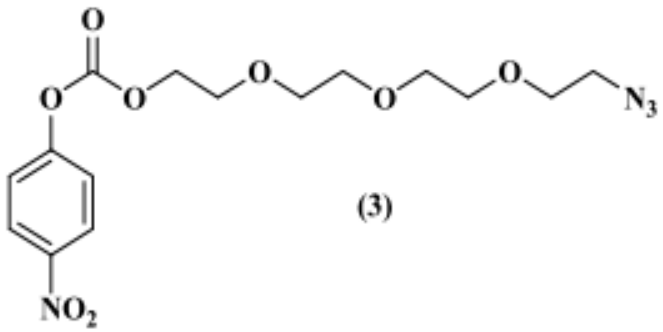
2.4.2 Synthesis of OH-TEG-N3 (2)



(2)

Dissolve compound (1) (0.96 g, 2.75 mmol, 1eq) and sodium azide (0.2 g, 3.025 mmol, 1.1eq) in 6 ml ethanol. The reaction was refluxed overnight at 70 ℃. After removing ethanol under vacuum. The reaction was diluted in 100 ml diethyl ether, and washed with 50 ml saturated NaCl. Removing solvents under vacuum gives a pure pale yellow oil compound. The yield of pure compound was 50.1% (0.4807 g, 2.19 mmol). The NMR results were the same as we previously reported in our group (49).

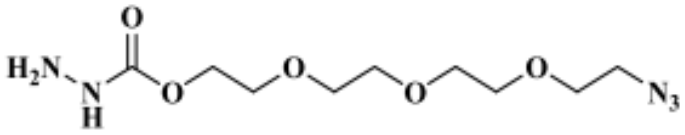
2.4.3 Synthesis of N3-TEG-nitrophenyl carbonate (3)



(3)

To a solution of compound (2) (0.25 g, 1.14 mmol, 1eq) and Et_3N (0.023 g, 0.23 mmol, 0.2 eq) under Argon add 6 ml of DCM at ice conditions for 2 hours. After that a solution of 4-NPC (0.023 g, 1.14 mmol, 1eq) in 7 ml DCM was added under argon. The reaction was left overnight at room temperature. The solvents were removed under vacuum and entered into the next step.

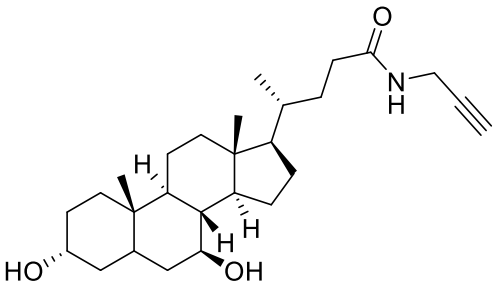
2.4.4 Synthesis of hydrazine-TEG-N3 (4)



(4)

A solution of compound (3) (0.47 g, 1.21 mmol, 1 eq.) and hydrazine hydrate (6.06 g, 121mmol, 100 eq) in 15 ml DCM refluxed overnight at 50 ˚C. The product was diluted with 40 ml DCM, and washed with 30 ml brine solution. The solvent was removed under vacuum. The crude product was purified by column chromatography in silica gel eluting with DCM: MeOH (20:1). The yield was 49.3% (0.1797g, 0.648 mmol). The NMR results were the same as we previously reported in our group (50).

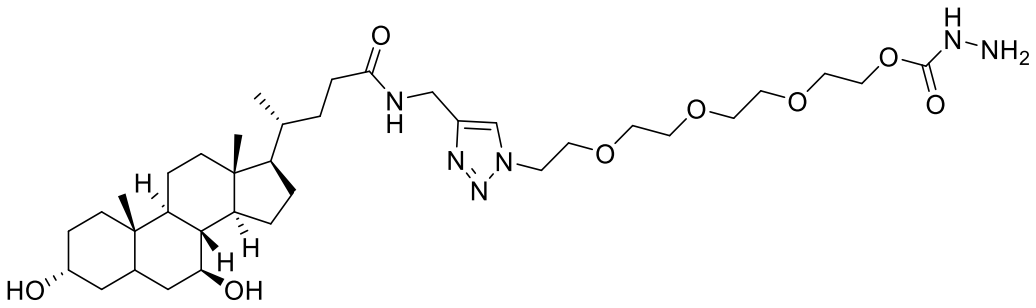
2.4.5 Synthesis of UDCA-alkyne (5)



A solution of UDCA (400 mg, 1.02 mmol, 1eq), propargyl amine (72.1 µl, 1.12 mmol, 1.1eq), TBTU (393 mg, 1.22 mmol, 1.2 eq), and DIPEA (533 µl, 3.06 mmol, 3eq) in 6 ml DMF was left overnight under argon. After that, DMF was removed under vacuum. The crude product was purified by column chromatography using the eluent DCM: MeOH (20:1). The yield was 51.5% (0.41 g, 0.952 mmol). R_f = 0.25, DCM: MeOH (20:1). ^{1}H NMR (500 MHz, DMSO): δ 8.20 (s, 1H, NH), 4.44 (s, 1H, OH), 3.87 (d, 1H, J = 5 Hz, OH), 3.82 (d, 2H, J = 3 Hz, $CH_2C{\equiv}C$), 3.63-3.60 (m, 1H, CHOH), 3.17-3.12 (m, 1H, CHOH), 3.06 (s, 1H, C≡CH), 2.12-1.10 (m, 26H, H rings, CH_2CO, CH_2CH_2CO, C<u>H2</u>C<u>H</u>(CH3)), 0.87 (s, 6H, $2CH_3$), 0.61 (s, 3H, CH_3).

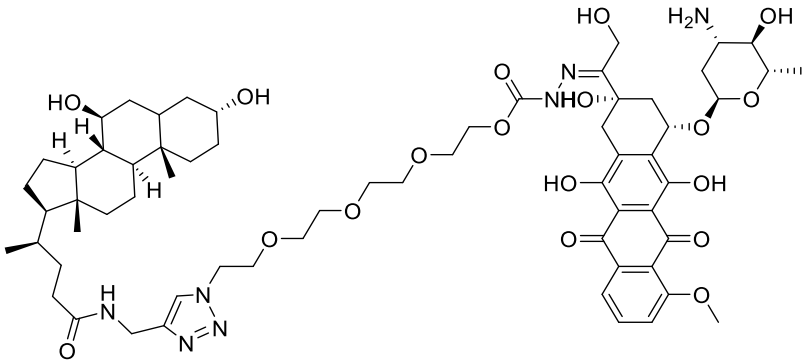
^{13}C NMR (125.7 MHz, DMSO): δ 172.8, 81.9, 73.2, 70.2, 69.9, 56.3, 55.2, 54.1, 43.6, 43.5, 42.7, 42.3, 38.2, 37.8, 35.4, 35.3, 34.2, 32.6, 32.0, 30.7, 28.7, 28.2, 27.2, 23.8, 21.3, 18.9, 13.0, 12.5.

2.4.6 Synthesis of UDCA-triazole-TEG-Hydrazine (6)



A solution of compound (5) (70 mg, 0.163 mmol, 1eq), compound (4) (50 mg, 0.18 mmol, 1.1 eq), copper iodide (6.2 mg, 0.0326 mmol, 0.2 eq), DIPEA (11.35 µl, 0.0651 mmol, 0.4 eq), and acetic acid (0.391 mg,10 µl, 0.006516 mmol) in 4 ml DCM was left overnight at room temperature under argon. The resulting product was extracted by 100 ml DCM, and washed with 50 ml 1M HCl. Solvents were removed under vacuum, and the crude product was directly entered to the next reaction.

2.4.7 Synthesis of UDCA-triazole-TEG-NHNH-DOX (7)



A solution of compound (6) (75 mg, 0.11 mmol, 1eq), doxorubicin (55.4 mg, 0.09554 mmol, 0.9eq), 3 drops of trifluoroacetic acid in 4 ml methanol was stirred for 48 hours at room temperature under argon. After that, the solvent was evaportated and the crude product was purified by column chromatography using the eluent DCM: MeOH (9:1). The yield was 46% (70 mg). R_f =0.45, DCM: MeOH (9:1). ^{1}H NMR (500 MHz, DMSO): δ 13.99 (s, 2H, 2OH Dox), 13.28 (s, 1H, NH hydrazone), 9.05 (bs, 1H, NH amide), 7.72-7.66 (m, 2H, Ar DOX), 7.13 (s, 1H, triazole), 6.99 (d, 1H, J = 5.5 Hz, Ar DOX), 6.02 (s, 1H, OH sugar DOX), 5.44 (d, 1H, J = 10 Hz, OH Dox), 5.32 (t, 1H, J = 5 Hz, CH_2O<u>H</u> DOX), 5.08 (bs, 1H, OH UDCA), 4.97 (dd, 2H, J =5.1 Hz, CH_2 sugar), 4.85 (t, 1H, J = 5.3 Hz, OH UDCA), 4.65 (bs, 1H, CH sugar), 4.59 (dd, 1H, J = 10 Hz,

CH sugar), 4.48 (bs, 1H, CH sugar), 4.39 (d, 1H, J = 5.3 Hz, CH-NH_2 sugar), 4.13 (t, 4H, J = 5.5 Hz, CH_2OCO, $C\underline{H_2}OH$), 4.00 (s, 2H, CH_2-triazole), 3.97 (t, 2H, J = 5.2 Hz, triazole-CH_2), 3.57 (s, 3H, OCH_3), 3.50-3.48 (m, 12H, $6CH_2O$), 3.06 (d, 2H, J = 15 Hz, CH_2 cyclohexyl DOX), 2.92 (d, 2H, J = 16Hz, CH2 cyclohexyl DOX), 2.27-2.25 (m, 2H, CH_2CO), 2.19 (d, 1H, J = 10Hz, $\underline{CH}NH_2$), 2.08-2.05 (m, 2H, $C\underline{H_2}CH$), 1.33-1.22 (m, 23H, H rings), 0.86 (t, J = 10 Hz, 12H, $4CH_3$).

^{13}C NMR (125.7 MHz, DMSO): δ 167.5, 161.3, 156.2, 153.8, 140.7, 134.6, 132.2, 132.1, 130.1, 129.1, 123.6, 123.2, 116.1, 111.3, 76.7, 70.3, 67.9, 64.4, 60.9, 57.1, 38.6, 36.9, 35.6, 34.9, 34.3, 33.1, 32.0, 31.8, 31.6, 30.3, 29.9, 29.5, 29.3, 29.2, 29.0, 28.8, 27.0, 23.7, 22.9, 22.6, 14.4, 11.3.

2.5 In vitro release of DOX

An HPLC method was developed to study the in vitro release profile of the DOX from the conjugate at acidic pH with time. The mobile phase of the HPLC was methanol:water (60:40) with a rate of 0.8 ml/ min using C18 column. Dectection UV was 233 nm.

2.5.1 Calibration curve of Doxorubicin HCl

A calibration curve of Doxorubicin HCl was built using the mentioned HPLC method at retention time of 3.261min using a serial dilution (0.8, 0.6, 0.4, and 0.2 mg/ml) of stock solution of Doxorubicin (1 mg/ml) in methanol. The calibration curve was constructed by plotting AUC vs. Concentration.

2.5.2 Calibration curve of UDCA-triazole-TEG-NHNH-DOX (7)

Same procedure as the prevcious section 2.5.1, a calibration curve of compound 7 was prepared at a retention time of 6.65 min using a serial dilution (0.8, 0.6, 0.4, and 0.2mg/ml) of stock solution of compound 7 (1 mg/ml) in methanol. The calibration curve was constructed by plotting AUC vs. Concentration.

2.5.3 The in vitro release study

The in vitro release of DOX was determined using phosphate buffer at pH 5.0. The concentration of the release DOX was measured upon time for 24 hours using the built calibration curve of DOX.

2.5.4 Stability study

The stability study was determined using phosphate buffer at pH 7.4 by measuring the concentration of compound 7 in HPLC upon time.

2.6 Anticancer

2.6.1 Cell Culture

Human Hepatoma cells Hep3B and HepG2 were implemented in RPMI (10% FBS, L-glutamine, and penicillin/streptomycin). LX-2 and 3T3 cells were maintained at DMEM (10% FBS, penicillin/streptomycin 100unit/ml, L-glutamate) in T-157 cell culture flask at 37c, 99% humidity˚, 5% CO_2 at CelCulture CO_2 incubator. After 27 hr, cell confluence was enough to make subculture, suctioned the media and wash cells with physiological phosphate Ca^{2+} free buffer (PBS). Flowingly, the cells trypsinized with 0.025% trypsin and left for 5 minutes in the incubator. After the detachment of cells, inhibit the trypsin activity using suitable media. Then, the cell suspension was collected and the trypan blue stain was used for cell counting and culturing.

2.6.2 Cell Viability

MTS was used for measuring the viability of cells at ʎ490 nm using Absorbance Microplate Reader (ELX 808). Each of HepG2, Hep3B, LX-2, and 3T3 cells were cultured at 96-well plates at concentrations $5*10^4$cell/ml (100µl/well, 5000cell/well) and incubated for 24 hr. Then, cells treated with doxorubicin, UDCA-DOX, and UDCA at concentrations (1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, and 16 µg/ml) for 48 hr under physiological PH. after 48 hrs of incubation 20µlMTS was added to each well of the plates (net volume 100µl/well) incubated for 2hr at the standard incubator and read by the microplate reader. Each trial was repeated three times.

One-way ANOVA using GraphPad Prism Software analyzed the difference between control cells and treated cells with statistical significance $p< 0.05$.

2.6.3 Evaluation of DOX uptake by fluorescence cell imaging

For studying the 3D cell model of HepG2, Hep3B, LX-2, and 3T3 cells. Cells were cultured in a 12-well plate at $10*10^4$ cell/ well for 24 hr, and then treated with doxorubicin, UDCA-DOX, and UDCA (16mcg/ml, 64mcg/ml) incubated for 2hr at a standard incubator. After that, the plates were washed with PBS Ca^{+2} free buffer and replaced with fresh media (1ml/well). then, capture two fields of cells (Bright field, and fluoresce field) using Olympus inverted microscope (6M45542, IX73PLF, OLYMPUS). The cultured cells were assessed and overlaid using the ImageJ software program.

Chapter Three

Results & Discussion

This thesis aims to synthesize a new-targeted chemotherapy in HCC management depending on using bile acid as a targeting agent and doxorubicin as a chemotherapeutic agent. Because of the limited number of research articles on using bile acid as a targeting agent in the management of liver carcinoma, in addition to, the cardiotoxicity side effect of doxorubicin. There is an emerging need for developing a more effective, selective, and safer method in the treatment of advanced liver cancer.

Moreover, SAR studies show that using bile acid as a targeting agent will enhance the selectivity, efficacy, and safety of the anti-cancer agents for liver carcinoma (51). This research is focusing on the synthesis of a new brand agent in the management of liver carcinoma by using UDCA as a targeting agent of doxorubicin.

Doxorubicin is an anthracycline anticancer that inhibits bimolecular synthesis through the inhibition of the topoisomerase II enzyme and stops cellular replication (52). Although its broad-spectrum activity, it has limited action in HCC even with systemic administration via the hepatic artery (53). Moreover, cardiotoxicity is a black-box warning of doxorubicin so, developing more selective, effective, and safer methods rather than systemic administration such as targeted chemotherapy.

In this research, we aim to target doxorubicin to HCC cells without affecting non-cancerous cells by attaching the bile acid derivative UDCA to enhance the delivery to the cancer cells

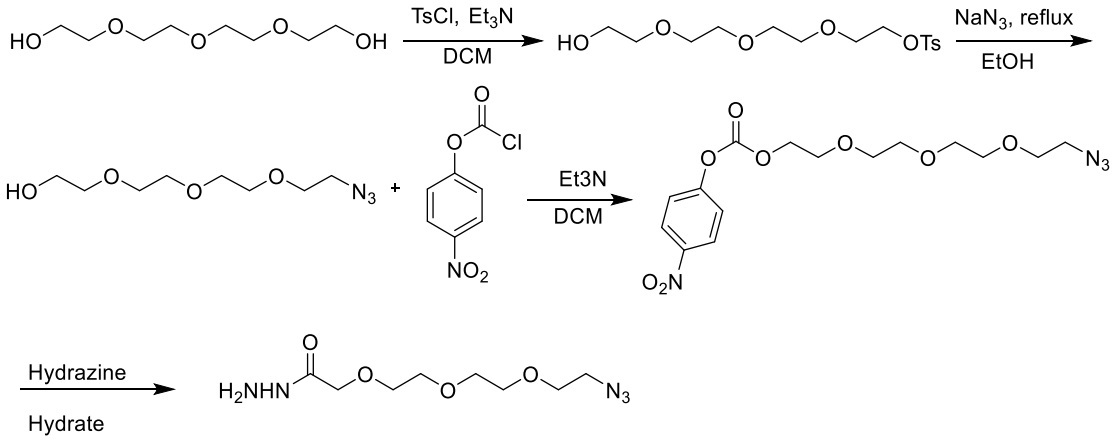
3.1 Synthesis of UDCA-DOX conjugate

To synthesize UDCA-DOX conjugate. The first step is the synthesis of an acid-labile linkage since the cancerous cells produce lactic acid that is responsible for the acidic environment (50). Followed by reacting UDCA with propargyl amine to have terminal alkyne that can react with the linker through click reaction to form a triazole ring. Then, a condensation reaction was followed with doxorubicin to obtain the final compound (7).

To synthesize the hydrophilic bifunctionalized linker, a tosylation reaction of tetramethylene glycol was proceeded in presence of trimethylamine in DCM. Then the monotosylated compound (1) was refluxed at 70 ˚C with sodium Azide to obtain alinker terminated with azide group (2). After that, compound (2) was reacted with 3-nitophenyl chloroformate to obtain compound (3). Finally, it was reacted with hydrazine hydrate in DCM to obtain NH_2-NH-TEG-N_3 (4) as shown in Scheme 9.

Scheme 9

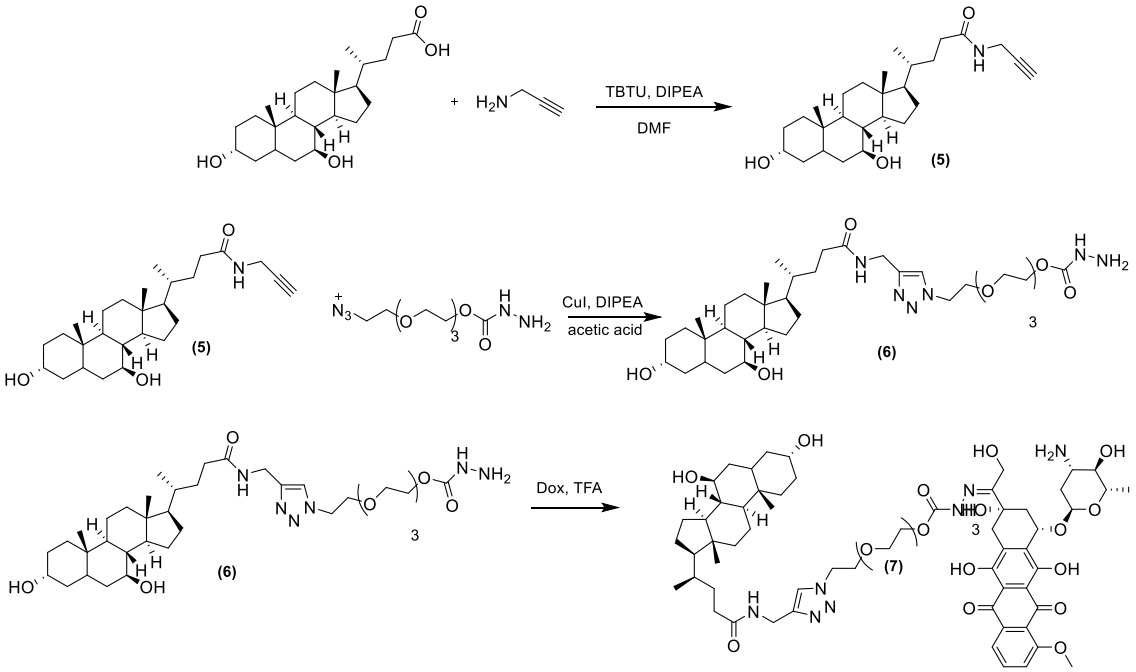
Synthesis of an acid-labile linkage



To conjugate UDCA with the synthesized linker thorugh click reactio. The UDCA was reacted with propargyl amine in the presence of TBTU and DIPEA as a base to obtain a termina alkyne as shown in scheme 10. After that a click reaction was performed using copper iodide, and DIPEA to conjugate the UDCA-alkyne with the linker azide-TEG-hydrazine to form the triazole ring has shown in scheme 10. In a final step, a condensation reaction was performed with doxorubicin in the presence of TFA to form the acid-labile linkage (hydrazine) with DOX as shown in scheme 10. The structures of all synthesized compounds have been confirmed by ^{1}H and ^{13}C NMR as shown in chapter two materials and methods.

Scheme 10

synthesis of UDCA-DOX conjugate



3.2 In vitro doxorubicin release study

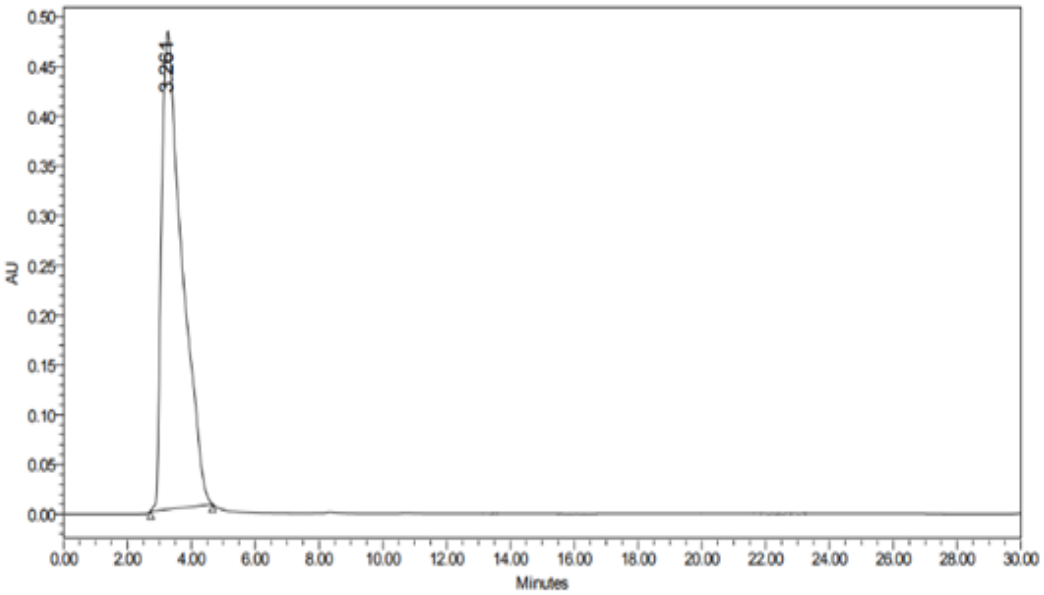
To study the release profile of Doxorubicin at acidic and neutral media, the HPLC method was developed using HPLC C18 column (ODS-3V, 2.7µm 100*4.6 mm). The elution was at 0.8 ml/min flow rate for 30 minutes run. The UV- detector was at wavelength 233nm for the detection of DOX and UDCA-DOX conjugate. The mobile phase consisted of 60% Methanol, and 40% Water.

3.2.1 Retention Time and Calibration Curve of Doxorubicin and UDCA-DOX

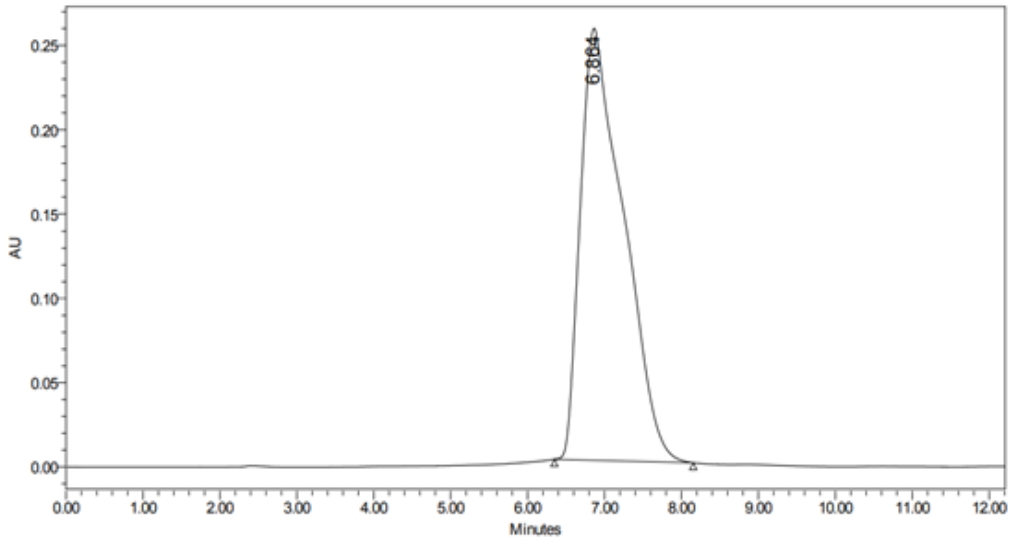
Doxorubicin eluted at 2.65 minutes, while UDCA-DOX conjugate eluted at 6.86 minutes as in shown in Figure 1.

Figure 1

Determination of the retention time of DOX and UDCA-DOX



A: *The retention time of DOX at 3.261min.*



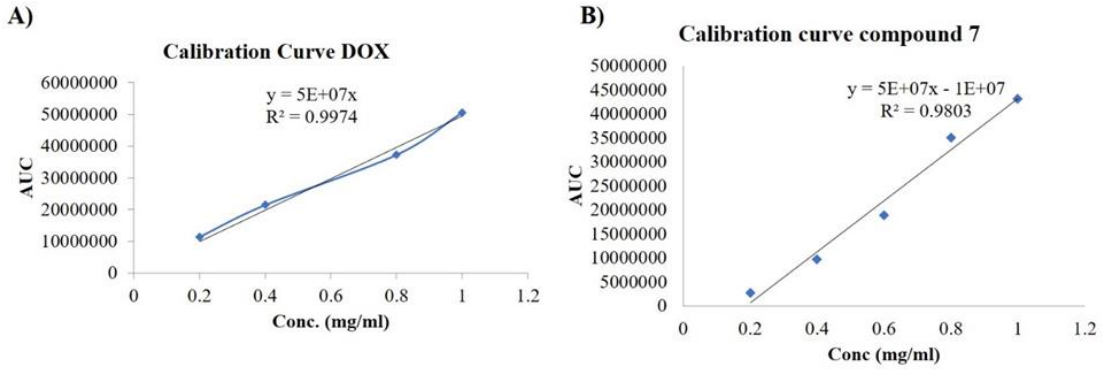
B: *The retention time of UDCA-DOX at 6.86min.*

After knowing the retention time of dox and the UDCA-Dox conjugate, a calibration curve of each compound was conducted based on HPLC analysis. As shown in Figure 2, the calibration curve of doxorubicin has been constructed at the range of concentrations (0.2-1 mg/ml), with an R^2=0.9974. Whereas, R^2 of UDCA-DOX at the

same concentration gradient was 0.9803. The two compounds show good linearity over the concentration gradients.

Figure 2

A. Calibration curve of DOX based on HPLC analysis B. calibration curve of UDCA-DOX based on HPLC analysis



3.2.2 In Vitro Drug Release

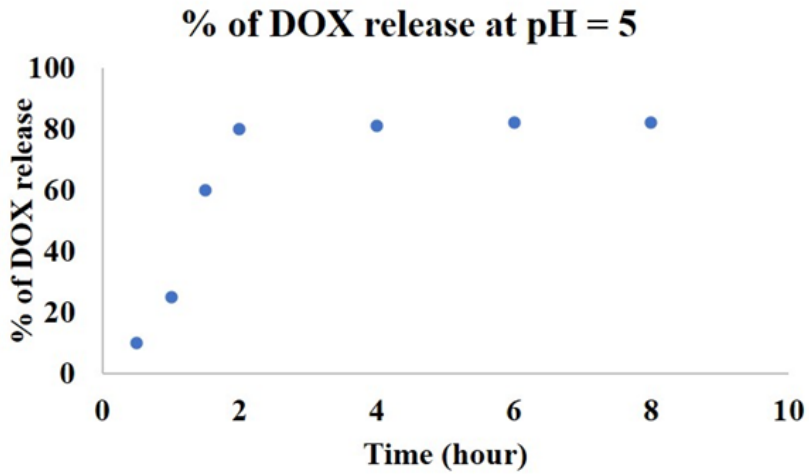
As we mentioned earlier, cancer cells are characterized by an acidic environment due to the higher production of lactic acid(50). As a result, we suggest that the synthesis of our conjugation by acid-labile linkage will confirm the release of DOX from our pro-drug at cancerous cells. Therefore, we took this advantage in studying the release profile of UDCA-DOX conjugate.

To conduct the release profile of dox at acidic media, a phosphate buffer with pH=5 was prepared. In addition, the stability of UDCA-DOX was also conducted at a neutral phosphate buffer (pH = 7.4). The UDCA-DOX conjugate showed no hydrolysis process at neutral pH, which means it is completely stable at a physiological buffer. Whereas, once the acidic phosphate buffer incubated UDCA-DOX gradually hydrolyzed and release doxorubicin at a time. This is shown by the decrease of UDCA-DOX peaks and the appearance of the dox peak. However, after 2 hrs of the acidic buffer incubation, the peak of dox was at its highest level with almost 85% degradation of the acid-labile linker of UDCA-DOX. As a consequence of the hydrazone linkage, our product shows a complete stability profile at physiological pH and starts the release of dox from our pro-drug at a slightly acidic pH. The release profile optimizes that our pro-drug achieves the

required physical and chemical properties and the acquired optimum release in a cancerous environment without affecting the surrounding environment.

Figure 2

C. *In vitro release of Doxorubicin at acidic media*



Therefore, it is confirmed that the synthesized UDCA-DOX prodrug is stable at physiological environment and it will be hydrolyzed at slightly acidic (pH = 5) that mimics the cancer environment thanks to the hydrazone acid-labile linkage.

3.3 Investigating the cytotoxity of UDCA-DOX in vitro

HepG2 and Hep3B hepatic cancer cell lines were used to investigate the anticancer of UDCA-DOX *in vitro*. The cytotoxicity was also investigated in the noncancerous hepatic LX2 cells and in the non-hepatic 3T3 cell line to investigate the potential targeting property. The unconjugated doxorubicin and UDCA were used as positive and negative controls respectively.

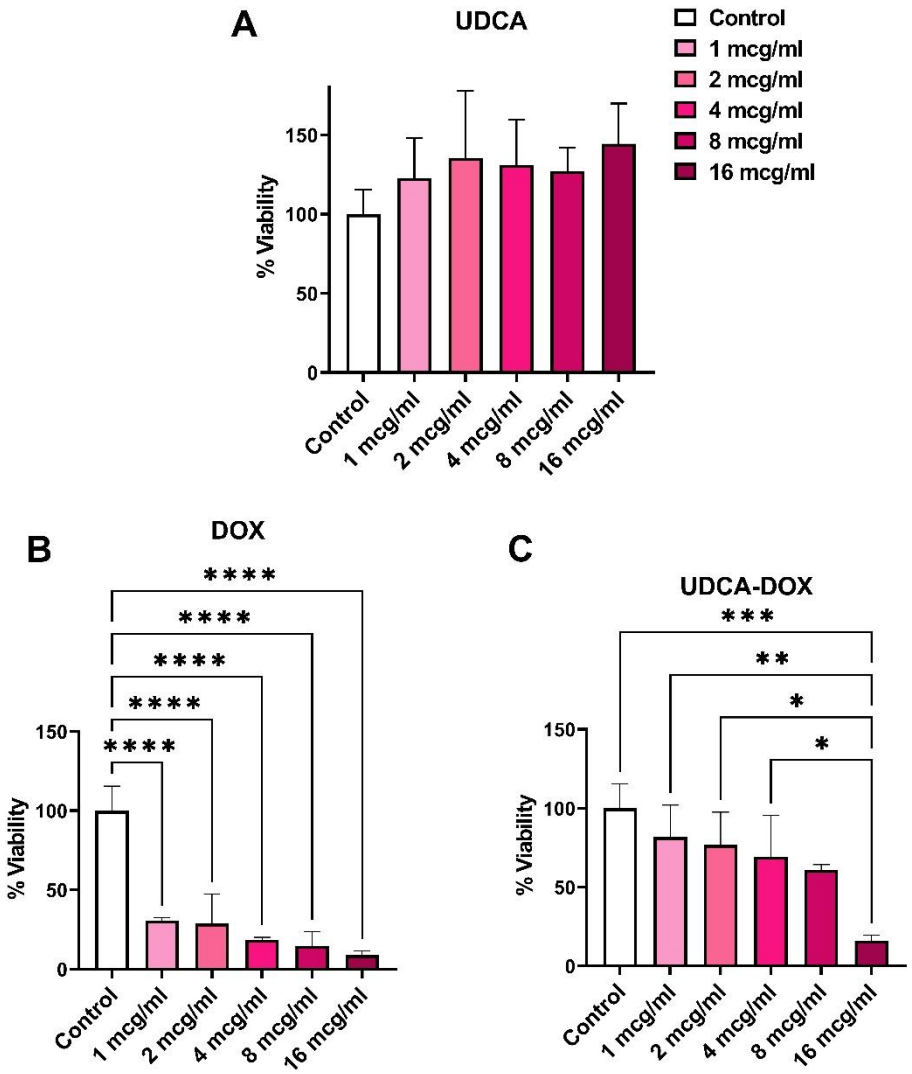
3.3.1 Investigation of the cytotoxicity in HepG2 and Hep3B cell line

The effect of UDCA-DOX on the viability of HepG2 cells was evaluated using the MTS assay. HepG2 cells were exposed to DOX, UDCA-DOX, and UDCA for a duration of 48 hours. The cytotoxicity of UDCA-DOX was compared to that of DOX alone. Figure 3 illustrates the concentration-dependent reduction in cellular proliferation of the HepG2 cell line by UDCA-DOX. The data clearly indicate that the cytotoxicity of free DOX was significantly higher than that of UDCA-DOX. This observation suggests the involvement of receptor-mediated endocytosis rather than simple diffusion, which

holds promise for targeted therapy against liver cancer cells. Interestingly, UDCA itself exhibited no cytotoxicity towards HepG2 cells, indicating that the observed cytotoxicity can be attributed solely to the intracellular release of DOX.

Figure 3

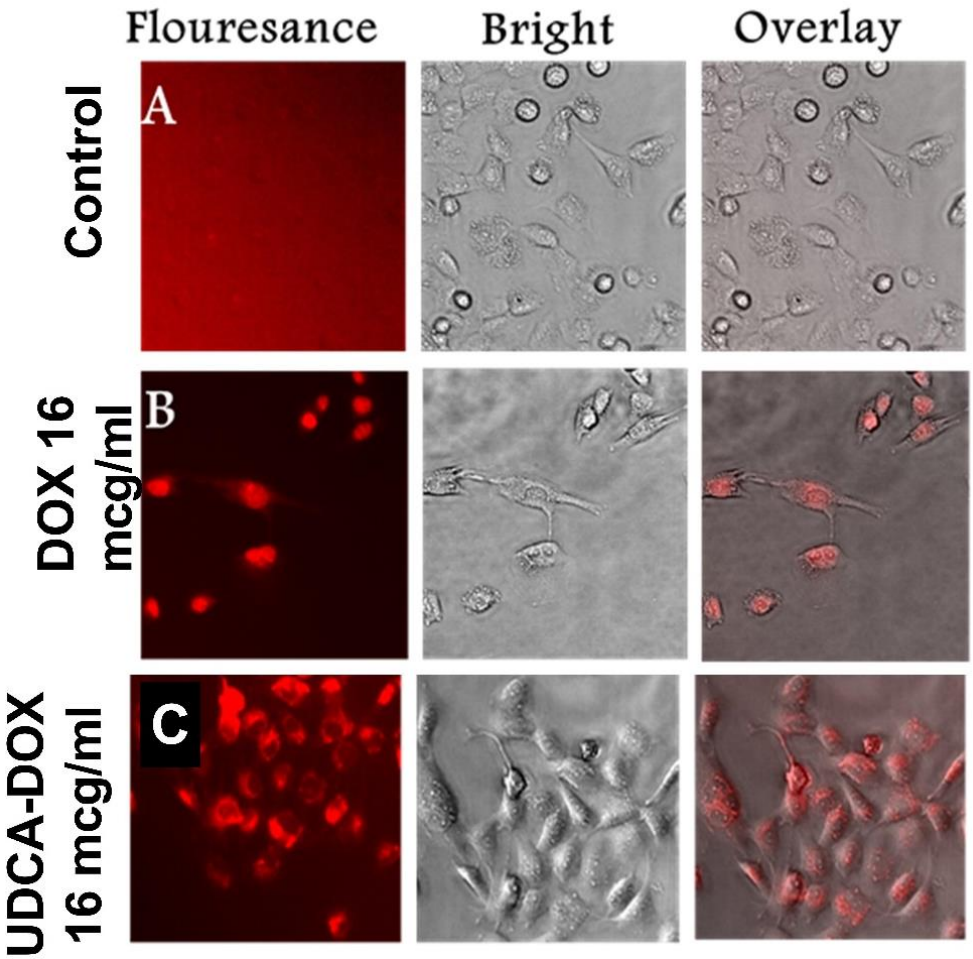
*MTS test for investigating the cellular viability of HepG2 cells treated with different concentrations of the test compounds for 48 hr. A: treatment with UDCA. B: treatment with DOX. C: treatment with UDCA-DOX. The data is presented as mean±SEM, * p-value ≤ 0.05, n= 3.*



In addition, fluorescence imaging of cells treated with UDCA-DOX, DOX, and UDCA was performed to investigate the uptake of DOX by HepG2 cells (figure 4). The fluorescence signal of DOX was observed in HepG2 cells treated with UDCA-DOX and free DOX. The images demonstrated similar subcellular distribution. No fluorescence signal was observed in UDCA, which validates that the observed signal is specific to DOX. Moreover, the morphology of the observed cells is consistent with the observed cytotoxicity in the MTS test.

Figure 4

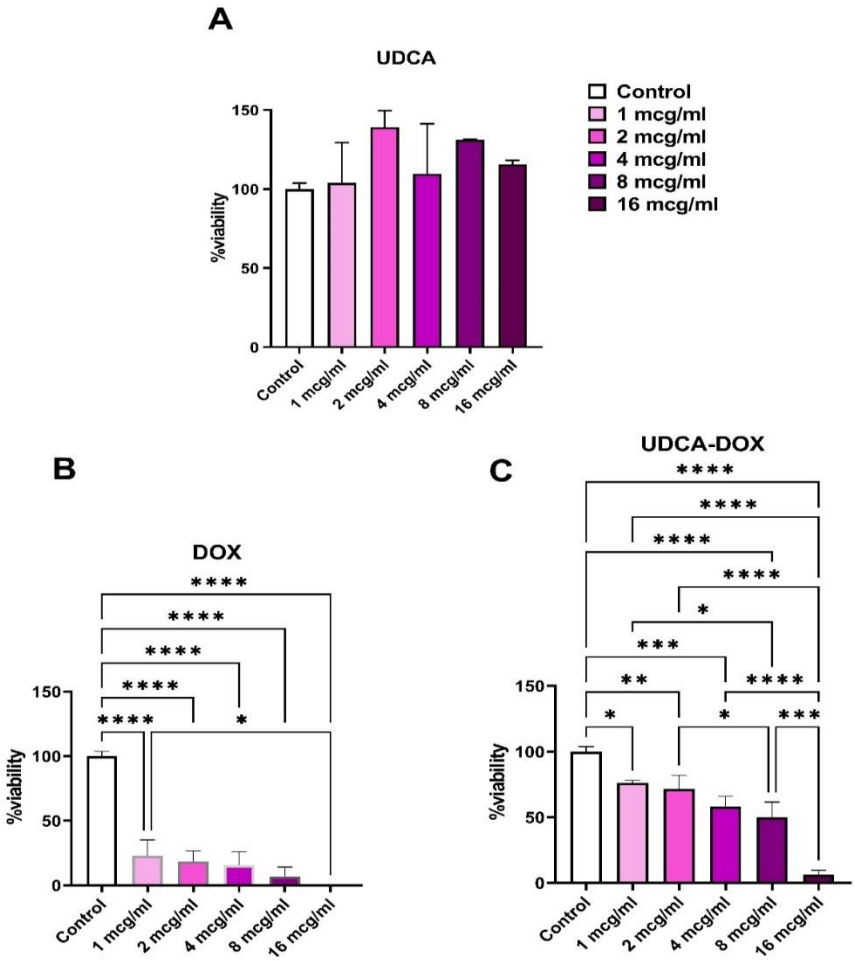
Investigating the uptake of DOX by HepG2 cells by fluorescence imaging. Left: Fluorescence field. Middle: Bright field. Right: Overlay. A: control cells. B: 16µg/ml DOX. C: 16µg/ml UDCA-DOX.



To assess the effectiveness of UDCA-DOX in targeting liver cancer cells, MTS cytotoxicity assays were conducted on another hepatic cancer cell line, Hep3B cells. Similar to the HepG2 experiments, Hep3B cells were exposed to doxorubicin, UDCA-DOX, and UDCA for a duration of 48 hours. Comparable to the previous cell line, free DOX exhibited significant efficacy against Hep3B cells. However, the cytotoxicity of an equivalent concentration of DOX in the form of UDCA-DOX was comparatively lower. This observation suggests that the complex's entry into the cells may not occur through passive diffusion, but potentially involves a bile acid receptor-mediated mechanism (Figure 5).

Figure 5

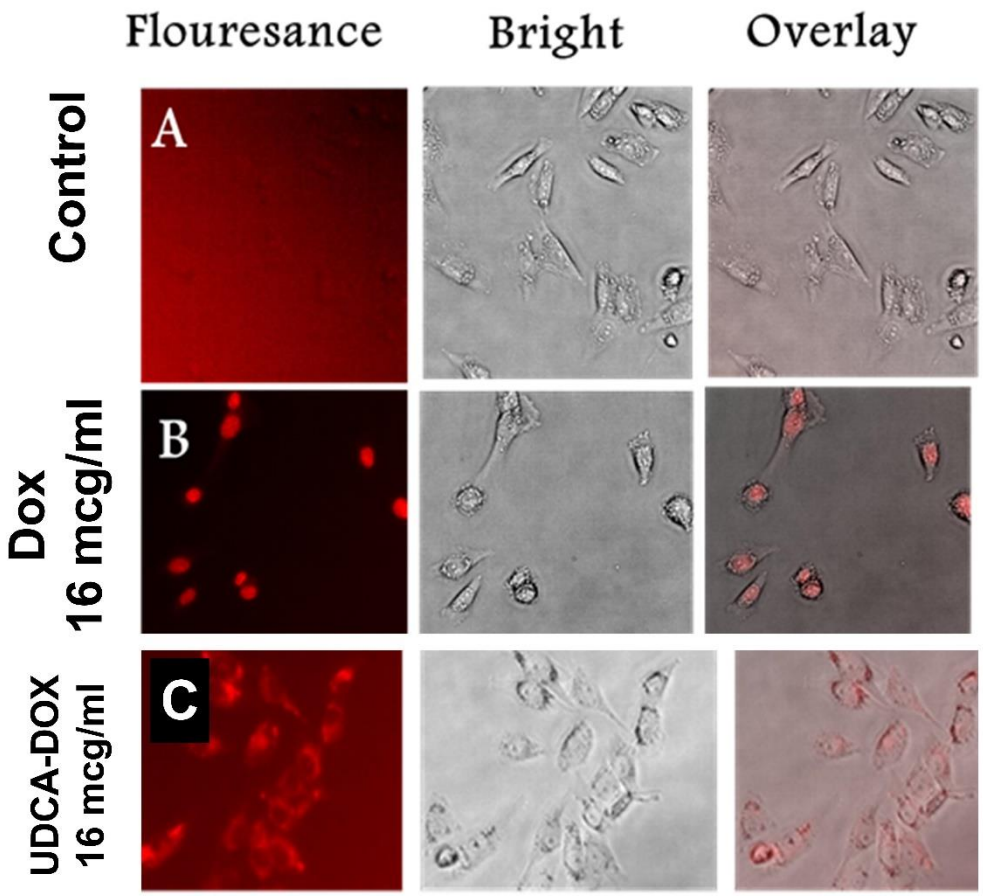
*MTS test for investigating the cellular viability of Hep3B cells treated with different concentrations of the test compounds for 48 hr. A: treatment with UDCA. B: treatment with DOX. C: treatment with UDCA-DOX. The data is presented as mean±SEM, * p-value ≤ 0.05, n= 3.*



Again similar to the HepG2 cells, the fluorescence signal of DOX was clearly observed in Hep3B cells treated for 2 hr. with either DOX or UDCA-DOX (figure 6).

Figure 6

Investigating the uptake of DOX by Hep3B cells by fluorescence imaging. Left: Fluorescence field. Middle: Bright field. Right: Overlay. A: control cells. B: 16µg/ml DOX. C: 16µg/ml UDCA-DOX



The concentration-dependent cytotoxicity of UDCA-DOX and the pH-dependent release of DOX from our novel acid-labile conjugate suggests that UDCA-DOX could be promising against HCC (50).

UDCA-DOX increases the cellular accumulation of DOX, however, the receptor recognition of UDCA and the slight hydrolysis of the acid-labile linkage into acidic media act as a targeting factor toward hepatic cancer cells.

The antiproliferative effect of the UDCA-DOX conjugate is comparable to that of the UDCA-cytarabine conjugate. The cytarabine conjugate is specifically designed to shield cytarabine from deamination after being taken up by receptors in HCC (hepatocellular carcinoma) cells. Among all the cytarabine conjugates, the UDCA-cytarabine conjugate demonstrates the highest degree of cytarabine release in its free form under acidic pH conditions (39).

Furthermore, there is a strong rationale for utilizing conjugated compounds derived from bile acids in the treatment of liver cancer. This strategy is based on three fundamental assumptions. Firstly, the synthetic bile acid conjugate should retain the ability to be taken up by bile acid transporters. Secondly, it is assumed that liver tumors still express certain bile acid transporters. And finally, the new complex should preserve some of the pharmacological effects of the parent drug. These assumptions provide a solid foundation for the development and application of bile acid conjugates in liver cancer therapy (54).

Moreover, the uptake of UDCA-DOX is influenced by the expression level of bile acid receptors, with one of the key receptors being the Farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (TGR5). The Farnesoid X receptor (FXR), belonging to the nuclear receptor superfamily, plays a crucial role in regulating various metabolic processes. Once activated by bile acids like CDCA (chenodeoxycholic acid) and CA (cholic acid), FXR controls bile acid production, conjugation, and transport, as well as several aspects of lipid and glucose metabolism. This indicates the involvement of FXR and related bile acid receptors in mediating the uptake and cellular effects of UDCA-DOX, contributing to its therapeutic potential in liver cancer treatment (55). However, FXR receptors not only regulate the uptake of bile acid but also have an impact effect in HCC (56).

The FXR plays a vital role in controlling a wide range of physiological processes including differentiation, metabolism, and homeostasis. FXR is prominently expressed in the liver, gut, kidney, and adrenal glands. The identification of bile acids as bona fide endogenous ligands for FXR has uncovered the pivotal role of FXR in regulating bile acid metabolism. Extensive research indicates that FXR primarily functions to maintain bile acid homeostasis and protect against bile acid-induced liver damage. Recent studies

have revealed that FXR mediates the effects of bile acid signaling on normal liver regeneration. Notably, the absence of FXR has been associated with a heightened incidence of hepatocellular carcinoma (HCC), underscoring the significance of FXR in liver health and the prevention of liver cancer (58).

FXR appears to have diverse effects on the carcinogenesis of other tissues, although it has anti-tumor effects on hepatic and intestinal malignancies. Depending on the type of tissue, FXR may have pleiotropic effects on carcinogenesis; while it may largely function as a tumor suppressor gene in enterohepatic tissues, it can also function as a proto-oncogene in other tissues, such as the breast and lung. In the absence of FXR, mice develop liver tumors on their own, along with pronounced hepatic damage and inflammation. Interferon-gamma (IFN) is upregulated in the liver of FXR-deficient mice and inhibits the development of cancer by promoting the production of p53 and preventing the activation of signal transducer and activator of transcription 3 (STAT3). Hepatoblastoma development is connected to the suppression of hepatic stem cell differentiation into hepatocytes. By inhibiting the ankyrin oncogene's ability to stimulate the development of hepatic stem cells, which was mediated by tumor suppressor proteins like p53, FXR prevented this from occurring (57).

The membrane BA receptor TGR5 is critical for mediating some many several BA signaling-related processes. TGR5 is crucial for controlling metabolism and the inflammatory response. TGR5 may function as a tumor suppressor in cases of liver cancer. Also, modify the activation of STAT3 receptors, which is critical for liver inflammation and liver cancer. However, STAT3 undergoes de-phosphorylation because of TGR5 activation, therefore inhibiting the DNA binding capacity of STAT3. Therefore, FXR and TGR5 are considered good choices for targeting therapy cancer (56).

3.3.2 Investigation of the cytotoxicity in LX2 cell line

To assess the cytotoxicity of UDCA-DOX on healthy hepatic cells, MTS experiments were conducted on the LX2 noncancerous hepatic cell line using the same experimental conditions described earlier for the cancer cell lines. As depicted in Figure 7, UDCA-DOX exhibited notable cytotoxicity at a concentration of 16 ug/ml. At this concentration, UDCA-DOX reduced the cellular viability of LX2 cells by 56.6%

compared to the 7.9% reduction observed with doxorubicin at the same concentration. Interestingly, no cytotoxicity was observed by the other tested concentrations. These findings highlight a level of selective cytotoxicity against liver cancer cells.

Figure 7

*MTS test for investigating the cellular viability of Lx2 cells treated with different concentrations of the test compounds for 48 hr. A: treatment with UDCA. B: treatment with DOX. C: treatment with UDCA-DOX. The data is presented as mean±SEM, * p-value ≤ 0.05, n= 3.*

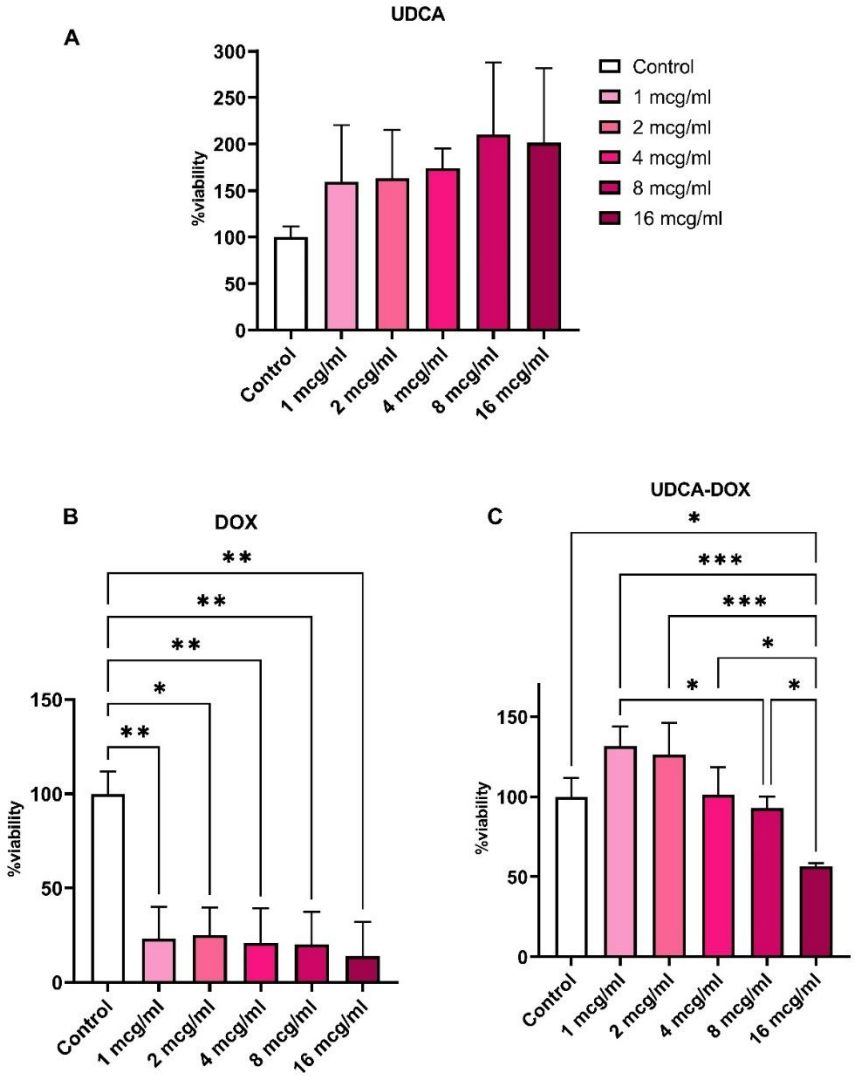
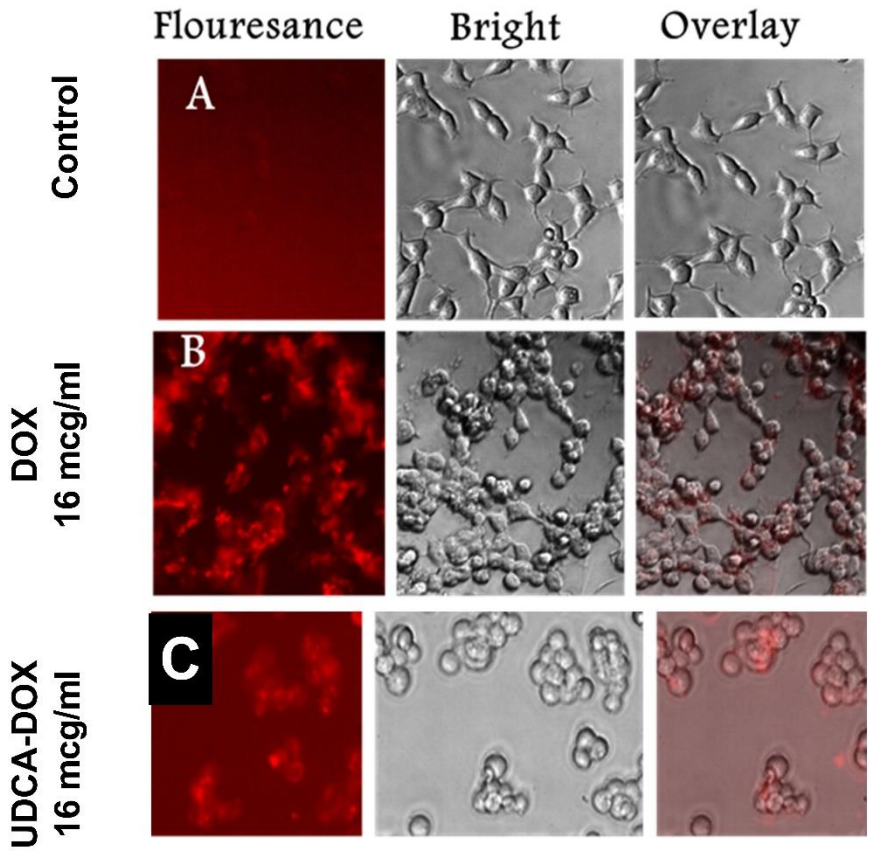


Figure 8

Investigating the uptake of DOX by LX2 cells by fluorescence imaging. Left: Fluorescence field. Middle: Bright field. Right: Overlay. A: control cells. B: 16µg/ml DOX. C: 16µg/ml UDCA-DOX.



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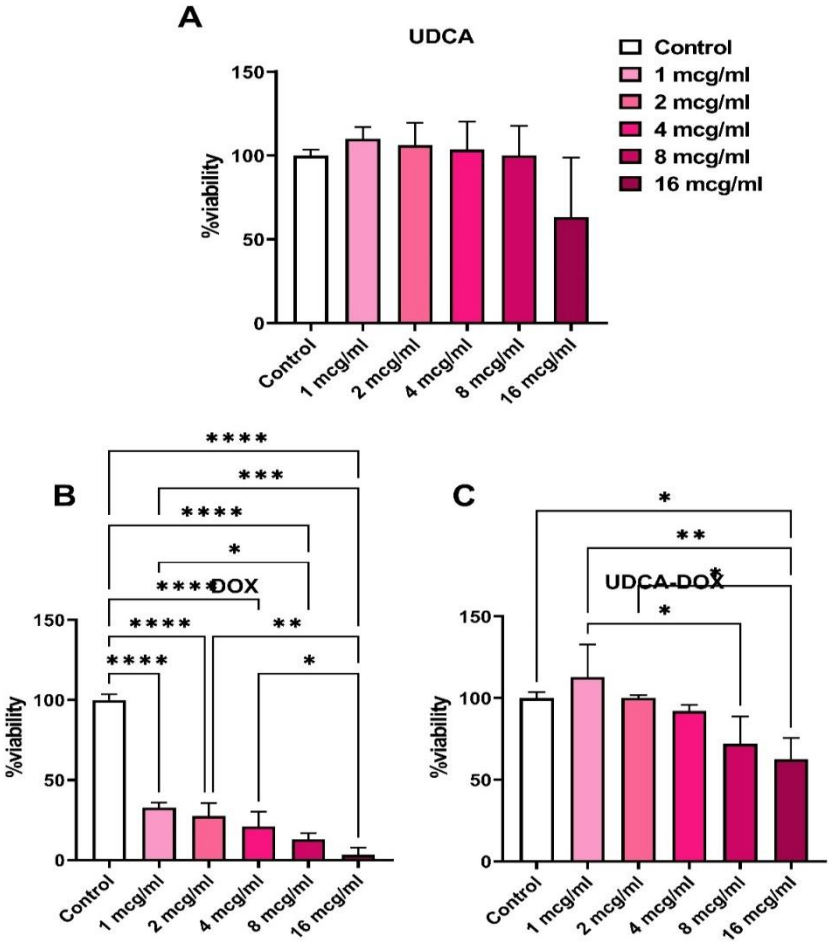
3.3.3 Investigation of the cytotoxicity in 3T3 cell line

To assess the specificity of UDCA-DOX cytotoxicity towards hepatic cancer cells, it was important to investigate whether this cytotoxicity was influenced by the specific uptake of bile acids by hepatic cells. To explore this aspect, 3T3 cells were chosen as a model, as they are known to lack bile acid receptors. Similar to the previous MTS experiments, 3T3 cells were cultured with DOX, UDCA-DOX, and UDCA for a duration of 48 hours.

Figure 9 clearly demonstrates that free DOX exhibited high effectiveness against 3T3 cells. This outcome aligns with the expected behavior of DOX as a cell cycle-nonspecific drug, capable of affecting various cell types, including both healthy and cancerous cells. However, when an equivalent concentration of DOX was delivered through UDCA-DOX, the resulting cytotoxicity was only mild to moderate. Notably, this level of cytotoxicity was significantly lower compared to the observations made in previous experiments involving hepatic cancer cells. This discrepancy can be attributed to the absence of bile acid receptors on 3T3 cells, which limits the entry of DOX and consequently reduces its cytotoxicity in these cells.

Figure 9

*MTS test for investigating the cellular viability of 3T3 cells treated with different concentrations of the test compounds for 48 hr. A: treatment with UDCA. B: treatment with DOX. C: treatment with UDCA-DOX. The data is presented as mean±SEM, * p-value ≤ 0.05, n= 3*

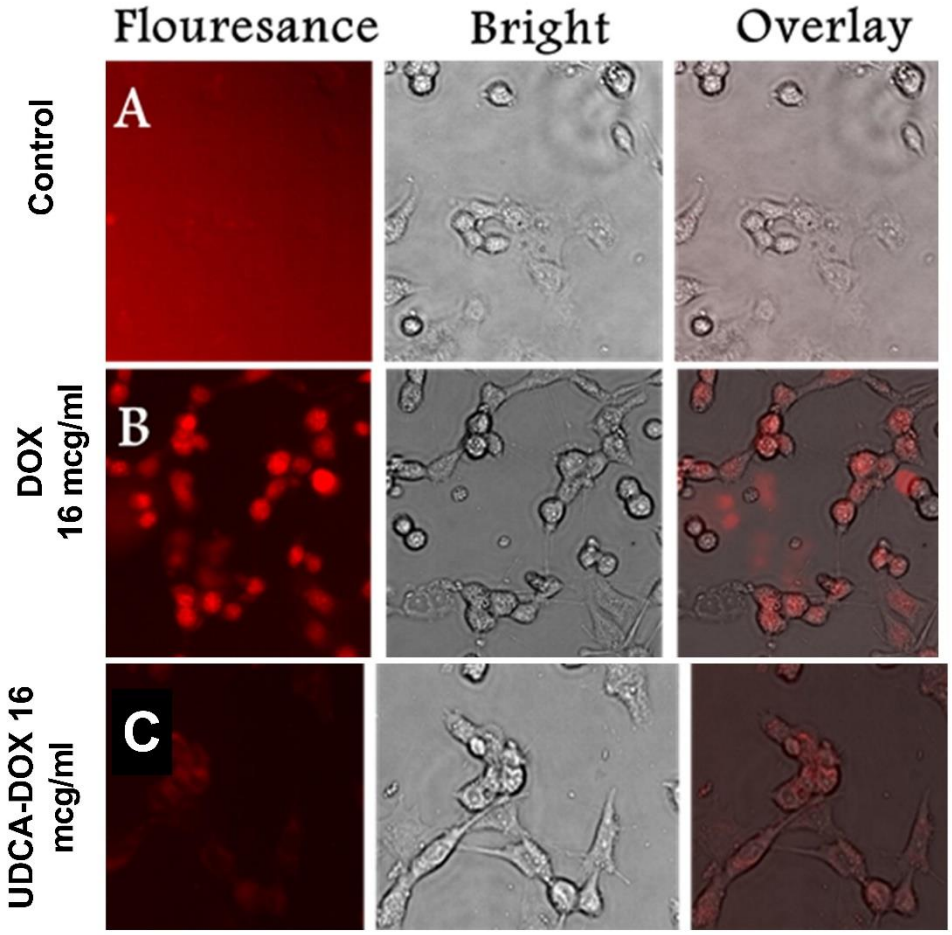


To gain more insight to the potential of UDCA-DOX uptake, fluorescence microscopy was performed in 3T3 cells incubated for 2 hr with UDCA-DOX and free DOX. As shown in figure 10, DOX signal could be detected within the cells, however, as this is only a qualitative test, it was not possible to perform a quatitiative comparison for DOX uptake between DOX and UDCA-DOX. Taken this observation together with the MTS data in figures 3, 5, and 7, we propose that UDCA-DOX might be uptaken by 3T3 cells in relatively lower amounts compared to free DOX, which may impart a level of selectivity to hepatic cancer cells. The exact mechanism by which UDCA-DOX could be uptaken requires further investigation.

The cells that were treated with UDCA alone showed enhancement of proliferation at the same concentration gradients. The cell growth of HepG2, Hep3B, and LX2 was 144%, 115%, and 200% at 16 µg/ml of UDCA, respectively. Whereas, the cell growth of 3T3 cells was 63% at the highest concentration in comparison with lower concentrations that showed an increase in cellular proliferation, as in Figure 10 below. Although UDCA has chemotherapeutic and chemoprevention actions, in our research, UDCA alone increased the cellular proliferation of hepatoma cells. Oppositely, UDCA decreased the cellular proliferation of 3T3 cells at 16 mcg/mL, and this effect disappear at other concentrations. The UDCA produced no cytotoxicity effect indicating its safety.

Figure 10

Investigating the uptake of DOX by 3T3 cells by fluorescence imaging. Left: Fluorescence field. Middle: Bright field. Right: Overlay. A: control cells. B: 16µg/ml DOX. C: 16µg/ml UDCA-DOX.



The results of the cell growth test show that UDCA-DOX achieves effective, specific, and selective inhibition of hepatoma cell proliferation in comparison with DOX alone. In addition, UDCA-DOX is relatively safer than free DOX in the LX2 normal hepatic cell line. Those results indicate that our novel conjugate constitutes a paradigm shift in the targeting therapy of hepatocellular carcinoma. UDCA-DOX demonstrated reduced toxicity against normal cells.

3.3.4 Limitations and Recommendations

Despite the demonstrated safety, efficacy, and selectivity of UDCA-DOX in the treatment of hepatocellular carcinoma (HCC), further research is still necessary to enhance our understanding of several key aspects. Firstly, more studies are needed to investigate the prodrug's pharmacokinetics, including its absorption, distribution, metabolism, and elimination patterns. These investigations will provide valuable insights into the prodrug's behavior within the body and its potential interactions with other drugs or physiological processes.

Secondly, additional research is required to evaluate the cytotoxicity of UDCA-DOX in various cellular contexts and to determine its potential side effects or adverse reactions. This will help assess the prodrug's safety profile and identify any potential limitations or concerns.

Moreover, animal models can provide crucial insights into the in vivo behavior and therapeutic potential of UDCA-DOX. Further research using animal models, such as mice or rats, can help validate the prodrug's effectiveness, assess its long-term impact, and provide valuable data for potential clinical translation.

Lastly, a comprehensive understanding of the targeting and uptake mechanisms of the UDCA-DOX conjugate is essential. Investigating the specific receptors or transporters involved in its cellular uptake will aid in optimizing its delivery to target cancer cells and improving its efficacy.

Chapter Four

Summary & Conclusion

In this study, we successfully synthesized a DOX conjugate with a bile acid targeting agent using an acid-labile linkage. This conjugate aimed to provide targeted delivery of DOX to liver cancer cells. UDCA-DOX demonstrated high stability in physiological media and exhibited efficient release of DOX in acidic media, mimicking the tumor microenvironment. This release was facilitated by the hydrolysis of the hydrazone linkage present in the conjugate.

Importantly, UDCA-DOX demonstrated reduced toxicity towards noncancerous liver LX2 cells and fibroblast-like 3T3 cells, which can be attributed to possible lower expression levels of bile acid receptors in these cell lines.

In conclusion, utilizing a drug delivery system that utilizes bile acids as targeting agents shows promise for the future management of hepatocellular carcinoma. Further research is necessary to confirm the anticancer activity of the UDCA-DOX prodrug and explore its potential for clinical application in hepatocellular carcinoma treatment.

List of abbreviations

Abbreviation	Meaning
Hep3b cells	Human Liver Carcinoma with hepatitis B gene
HepG2	Human hematoma cancerous cells
LX2	Human normal hepatic cells
3T3	Rodents Fibroblast cell line
$CHCl_3$	Chloroform
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
CA	Cholic Acid
CDCA	ChenoDeoxyCholic Acid
GCA	GlycoCholic Acid
GCDCA	GlycoChenoDeoxyCholic Acid
TCDCA	TauroChenoDeoxyCholic Acid
TCA	TauroCholic Acid
UDCA	UrsoDeoxycholic acid
DOX	Doxorubicin
UDCA-DOX	UrsoDeoxycholic acid-Doxorubicin conjugate
BA	Bile Acid
HCC	HepatoCellular Carcinoma
WHO	Word Health Organization
Et_3N	Trimethylamine
FBS	Fetal bovine serum
Hr/s	Hour or Hours
Ar	Argon gas
H_2O	Water
HCl	Hydrochloride
MeOH	Methanol
Min	Minutes
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

MW	Molecular weight
^{o}C	Degree Celsius
PBS	Phosphate buffer saline
pH	Potential of hydrogen
RPMS	Roswell Park Memorial Institute
DMEM	Dulbecco's Modified Eagle Medium
TEG	Tetraethylene glycol
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
HPLC	High-Performance Liquid Chromatography
λ_{max}	Lambda max
FXR	Farnesoid X receptor
STAT3	Signal transducer and activator of transcription 3
TGR5	G-protein-coupled bile acid receptor

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جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

تصنيع اقترانات من دوكسوروبيسن- حامض يورسوديوكسيكوليك لعلاج سرطان الكبد المستهدف

إعداد

أنسام صوص

إشراف

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قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

2023

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الملخص

سرطان الكبد هو مرض شديد العدوانية مع ارتفاع معدلات الوفيات. تم استخدام الدوكسوروبيسين لعلاج سرطان الخلايا الكبدية بسبب تأثيره المضاد للسرطان واسع النطاق. ومع ذلك، نظرًا لاحتمالية تسمم القلب وتطور المقاومة للأدوية المتعددة، عادةً ما تكون الجرعة محدودة في الاستخدام العلاجي، مما يحد من فائدته على المدى الطويل. حمض أورسوديوكسيكوليك هو حمض الصفراء المحب للماء الذي يرتبط بمستقبلات الحمض الصفراوي بألفة أعلى من أي نوع آخر من الأحماض الصفراوية.

الهدف الأساسي من هذا المشروع هو تطوير اتحاد UDCA-DOX وتقييم قدرات الاستهداف الخاصة به في المختبر لتوصيل DOX إلى خلايا سرطان الكبد على وجه التحديد. مع هذه الأهداف، نحن نهدف إلى استغلال تقارب UDCA لخطوط الخلايا HCC لتقليل سمية DOX على الخلايا الطبيعية، مع الحفاظ على ما يكفي من السمية الخلوية ضد خلايا سرطان الكبد.

تم تصنيع اتحاد UDCA-DOX باستخدام رابط حمضي-متغير، ثم تمت دراسة خصائصه وملف تعريف الإطلاق بواسطة HPLC. بعد ذلك، تم فحص السمية الخلوية لهذا الاتحاد بواسطة اختبار MTS في المختبر في HepG2، وخطوط الخلايا السرطانية الكبدية Hep3B، وخلايا LX-2 والخلايا الكبدية

الطبيعية، والخلايا الكبدية غير الكبدية T33. تمت دراسة الامتصاص الخلوي المستهدف لـ UDCA على وجه التحديد بواسطة خلايا سرطان الكبد بواسطة المجهر الفلوري.

يمكن لـ UDCA–DOX أن يتحلل تلقائيًا في الوسائط الحمضية. أظهر هذا الاتحاد سمية خلوية كبيرة في خطوط خلايا سرطان الكبد (HepG2 وHep3B). تم تأكيد التوصيل الناجح لـ DOX إلى الخلايا السرطانية بواسطة الفحص المجهري الفلوري. ومع ذلك، كانت السمية الخلوية لـ UDCA–DOX محدودة في خطوط الخلايا غير السرطانية (LX-2 وT33)، مما يشير إلى أن توصيل UDCA–DOX إلى الخلايا يعتمد على ميزات محددة في خلايا سرطان الكبد. علاوة على ذلك، كانت السمية الخلوية المرصودة لـ UDCA–DOX أقل من تلك الخاصة بـ DOX الحر، مما يشير إلى أن الدواء وصل إلى الخلية عبر عملية محدودة المعدل، مما يؤيد الفرضية القائلة بأن استيعاب UDCA–DOX يمكن أن يكون من خلال مستقبلات الحمض الصفراوي. الالتقام.

أظهرت هذه الدراسة رؤى قيمة حول إمكانات UDCA–DOX كاستراتيجية علاجية مستهدفة لسرطان الكبد لتقليل الآثار الضارة المرتبطة بعلاج DOX. هناك حاجة إلى مزيد من التحقيقات لاختبار فعالية وسلامة هذا الاتحاد في الجسم الحي.

الكلمات المفتاحية: سرطان الخلايا الكبدية، حمض أورسوديوكسيكوليك، دوكسوروبيسين، استهداف السرطان، الارتباط الحمضي المتغير، الإشعاع.