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Evaluation of cytotoxic potential of loratadine and the combination of loratadine and cisplatin on hepatocellular carcinoma cell lines

Nouran Adly

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Evaluation of Cytotoxic Potential of Loratadine and the Combination of Loratadine and Cisplatin on Hepatocellular Carcinoma Cell Lines

A Thesis Submitted to the Biotechnology Master’s Program
In partial fulfilment of the requirements for the
Degree of Master of Science

By: Nouran Adly, B.Sc (2010)
(Under the Supervision of Professor Suher Zada, Ph.D., Professor of Immunology, Biology Department, SSE, AUC)

September, 2017
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Has been approved by

Thesis Committee Supervisor/Chair

Affiliation ________________________________

Thesis Committee Reader/Examiner

Affiliation ________________________________

Thesis Committee Reader/Examiner

Affiliation ________________________________

Thesis Committee Reader/External Examiner

Affiliation ________________________________

Dept. Chair/Director Date Dean Date
Dedication

This work is dedicated to all cancer patients who was and is still suffering from the side effects of chemotherapy and radiotherapy. To my sister, Nadine Adly, who fought cancer bravely and courageously. Also, to my great family, husband, and beautiful daughter, whom I hope I will inspire someday.
I would like to thank my mother, Gihan Sayed, and my father, Alaa Adly, who helped me tremendously during this journey, for their emotional support, for taking care of my daughter and for helping me through my pregnancies. I would also like to thank my husband, Fadi Sharaf, for his emotional and financial support and for being so patient with me those 2 years. I would like to thank my sister, Alia Adly, for helping me edit this thesis, using LaTeX (www.latex-project.org). Also, I would like to thank my daughter, Joanna, for her patience and would like to apologize for all the days and nights I was too busy to be beside her.

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Finally I would like to thank the AUC for providing research grant which funded this project and for Laboratory fellowship for partially funding my studies.
Abstract

Hepatocellular carcinoma (HCC) has been a major health problem in Egypt with extensive efforts and studies done in enhancing chemotherapeutic drugs for more optimal results. Existing chemotherapeutic drugs are costly with problematic side effects. Recent studies have demonstrated the anticancer potential of antihistamines on different types of cancer, including HCC. Antihistamines have been proven to induce cell cycle arrest and/or apoptosis through different mechanisms depending on their role on different cancer types. The second generation antihistamine, loratadine (LOR), was found to have tumor inhibiting effects on human colon carcinoma cell line. However, no studies were done neither on the influence of loratadine on HCC cells, nor the effect of the combination of loratadine with existing chemotherapeutic drugs to test its potential to improve chemotherapy. Here, the cytotoxic potential of loratadine and the combination of loratadine and cisplatin on HepG2 and SNU449 were investigated. Cell viability assay was performed to show that there is a dose-dependent cytotoxic effect of LOR on both HCC cell lines and that there is a synergistic to additive effects when LOR was introduced to the cells in combination with cisplatin when the IC$_{50}$ of both drugs were used. Loratadine did not show a cytotoxic effect on normal cells when used in low concentrations (<55.6 μM). However, when used in higher concentration (<73.2 μM), LOR showed a high cytotoxic effect. Apoptotic and cell cycle analysis showed that loratadine induced apoptosis and cell cycle arrest in the G2/M phase in SNU449 cells, while combination of loratadine and cisplatin may induce necrosis and cell cycle arrest in G2/M phase. Taken together, loratadine offers a strong basis to be further developed either alone or in combination with cisplatin as a treatment option for advanced hepatocellular carcinoma. Further studies are required to test the effect of loratadine treatment in vivo and perhaps to test the effect of loratadine in combination with sorafenib in vitro and in vivo for the hope to improve HCC therapy.
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List of Abbreviations

Bak  BCL-2 Homologous Antagonist Killer Protein
Bax  BCL-2 Associated X Protein
Casp-3  Caspase 3
Casp-9  Caspase 9
Cdc-2  Cell Division Cycle Protein 2
CDK  Cyclin Dependent Kinase
CI  Combination Index
Chk  Checkpoint
CHOP  Cyclophosphamide, Hydroxydaunomycin, Oncovin and Prednisone
CT  Computed Tomography
CisPt  Cisplatin
CNS  Central Nervous System
CYP 3A4  Cytochrome p450 3A4
Cyt-c  Cytochrome C
DISC  Death-Inducing Signaling Complex
DMSO  Dimethyl Sulfoxide
EGFR  Epidermal Growth Factor Receptor
ESLD  End Stage Liver Disease
ESLC  Egyptian Society of Liver Cancer
FDA  Food and Drug Administration
FITC  Fluorescein Isothiocyanate
<table>
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<th>Abbreviation</th>
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<tr>
<td><strong>FGFR</strong></td>
<td>Fibroblast Growth Factor Receptor</td>
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<tr>
<td><strong>GFR</strong></td>
<td>Growth Factor Receptor</td>
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<tr>
<td><strong>HepG2</strong></td>
<td>Human Hepatoma G2, Human liver cancer cell line</td>
</tr>
<tr>
<td><strong>HGFR</strong></td>
<td>Hepatocyte Growth Factor Receptor</td>
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<tr>
<td><strong>HCC</strong></td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td><strong>HCV</strong></td>
<td>Hepatitis C Virus</td>
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<tr>
<td><strong>1 Br hTERT</strong></td>
<td>Normal human telomerase reverse transcriptase cell line</td>
</tr>
<tr>
<td><strong>IGFR</strong></td>
<td>Insulin Growth Factor Receptor</td>
</tr>
<tr>
<td><strong>LOR</strong></td>
<td>Loratadine</td>
</tr>
<tr>
<td><strong>MAPK</strong></td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td><strong>Mcl-1</strong></td>
<td>Myeloid Leukemia Cell Differentiation</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td><strong>MTT</strong></td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
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<tr>
<td><strong>NSCLC</strong></td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td><strong>PI3K-Akt</strong></td>
<td>Phosphatidylinositol 3 Kinase-Protein Kinase B</td>
</tr>
<tr>
<td><strong>PS</strong></td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td><strong>SNU449</strong></td>
<td>Human Liver cancer cell line</td>
</tr>
<tr>
<td><strong>TACE</strong></td>
<td>Trans-arterial Chemoembolization</td>
</tr>
<tr>
<td><strong>TGFβ</strong></td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td><strong>TRITC</strong></td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td><strong>VEGFR</strong></td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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Introduction

Cancer became an immense burden worldwide as it’s a multifactorial disease. Cancer represents the second main cause of deaths in the world, around 13% of all global deaths [1]. Ways to enhance the diagnosis and/or treatment of cancer has become the highlight of research, as current tools, methods and therapies are still not sufficient to decrease incidence rates. Generally, the available ways for cancer treatment include surgery, chemotherapy and radiotherapy, which showed an increase in the patient’s survival rates of some cancer types. However, such treatments are known to cause life threatening side effects, leaving cancer patients agitated and unsatisfied. Therefore, targeted therapies in which drugs are aimed to target certain proteins and genes that are responsible of cancer growth, can offer a way to improve cancer treatment [1]. Examples of the success of targeted therapies in chemotherapy includes sorafenib for advanced hepatocellular carcinoma (HCC), trastuzumab for breast cancer, bevacizumab for colon cancer, and erlotinib for lung cancer [2].

In Egypt, liver (33.6%) and bladder (10.7%) cancer ranks highest among males while breast (32%) and liver (13.5%) cancer ranks highest among females in Egypt [3]. HCC has become a huge burden in the Egyptian population due to the fact that patients get diagnosed in the advanced stages, where the liver is already cirrhotic. Numerous research studies have been conducted to decrease incidence rates by developing more sensitive, non-invasive methods and techniques to diagnose HCC such as molecular biomarkers [4], and to increase survival rates by developing more targeted therapies to improve chemotherapy, which is the only treatment option for advanced HCC (see Figure 1.2).

Antihistamines have been showing anticancer potential for the past 20 years, where terfenadine, a second generation antihistamine, was first to record sensitizing human breast cancer cells and murine leukemia cells to a chemotherapeutic drug called doxorubicin and therefore restoring sensitivity to resistant cells in
1993 [5]. This allowed looking into the possibility of using antihistamines in chemotherapy as anticancer drugs or to improve the effectiveness of chemotherapy by sensitizing resistant cells to chemotherapeutic drugs or simply causing cell death with minimal side effects, compared to chemotherapeutic drugs used alone. [5, 6, 7]. An advantage of using antihistamines in chemotherapy may lie in the fact that second generation antihistamines had already been through clinical trial phases and therefore is known to be safe with minimal side effects [8] and this could be very beneficial in chemotherapy, as chemotherapy is known to cause serious side effects. Therefore, using already clinically investigated drugs with an anticancer potential offers an advantage in chemotherapy to decrease the severe side effects of chemotherapy.

The aim of this study is to first evaluate the potential of the second generation antihistamine loratadine (LOR) to be used as an anticancer drug by testing its effect individually on HCC cell lines and second, to test the effect of LOR in combination with the known and thoroughly studied chemotherapeutic agent, cisplatin (CisPt) to test the hypothesis of improving chemotherapy. The final aim is to investigate whether LOR and its combination with CisPt will induce apoptosis or necrosis, and whether it will induce cell cycle arrest.
Chapter 1

Literature Review

1.1 Hepatocellular Carcinoma

1.1.1 Epidemiology and Etiology

Hepatocellular carcinoma is the sixth most common cancer [9, 10] and is known to be the third main cause of cancer deaths worldwide [11, 10]. It is the first and second most common cancer in males and females in Egypt, respectively [3, 10]. The incident rate of HCC in Egypt doubled since 2005 and became a burden in the Egyptian population because of its asymptomatic nature in its early stages [11, 7]. As shown in Figure 1.1, hepatitis patients are known to have higher risks of developing HCC, owing to the high and increasing prevalence of HCV in Egypt. Smoking, diabetes mellitus, obesity, alcohol and food contaminated with aflatoxin are also known risk factors of HCC, as demonstrated in Figure 1.5 [11, 12].

1.1.2 Diagnosis and Treatment

The asymptomatic nature of HCC makes it very difficult to be diagnosed. According to Feng et al (2015), 85% of HCC patients are diagnosed in the intermediate and advanced stages [7]. HCV patients or patients with cirrhotic liver, called high risk patients, are usually screened and assessed every 4 months for early detection to undergo liver transplantation once diagnosed with HCC. According to ESLC [14], a patient is diagnosed with HCC if: the tumor size is bigger than
1 cm and the serum alpha fetoprotein, a known HCC marker, is higher than 200 ng/ml, in addition to a triple phase computed tomography (CT) scan to evaluate the liver lesion. MRI (Magnetic resonance imaging) and liver biopsy may also be required for HCC diagnosis [4].

Treatment is given based on the tumor stage, patient’s performance grade and the status of the liver, whether it’s functional or not [4, 14]. Figure 1.2 shows the staging of HCC based on the Barcelona Clinic of Liver Cancer (BCLC) staging system and the treatment options for every stage. Child-Pugh is a system used to assess the status of the liver in cirrhotic patients. For early staging, in which a single tumor of a size less than 2 cm or at most 3 nodules with sizes less than 3 cm is present, treatment options include resection if the patient has a normal portal pressure and bilirubin, liver transplantation if the patient has elevated portal pressure and bilirubin, and if the patient has elevated portal pressure and bilirubin and developed a disease due to the elevation, either percutaneous ethanol injection: use of a needle directed by CT to inject ethanol in the tumor, causing dehydration and protein dehydration or radiofrequency ablation: use of electromagnetic energy to injure the tumor thermally [15] is used. For intermediate stages, where multinodular with sizes larger than 3 cm are present, chemoembolization or TACE (trans-arterial chemoembolization) is used for treatment, in which the chemotherapeutic drug is combined with beads that blocks the blood flow to the tumor, leading to the inhibition of tumor growth. This mixture is then directly injected to the tumor site in the liver, directed by CT, which allows the

Figure 1.1: Stages of HCC development. Adapted from Thornton, 2015. Figure was reused with permission from Reference [13], see appendix 1
treatment to be targeted only to the tumor site [16]. For advanced stages where metastasis begins to take place and the tumor invades the portal vein, chemotherapy using sorafenib, a tyrosine kinase inhibitor which inhibits tumor proliferation and angiogenesis [17] and other multikinase inhibitors are used. As for end stage or terminal HCC, the liver loses its ability to function and the patient’s survival rate decreases tremendously. At this point, only medical treatments of the symptoms can be administered [4, 14, 2].
Figure 1.2: BCLC staging system for HCC and available treatment options. PS: Performance Status. N1: one cancerous lymph node. M1: Metastasis. PEI/RF: Percutaneous ethanol injection/ Radiofrequency. Adapted from Llovet et al, 2008. Figure was reused with permission from Reference [2], see appendix 2.
Given the fact that most HCC cases are diagnosed in the intermediate or advanced stages, immediate treatment is needed. The most effective treatment in these stages is chemotherapy. Even though sorafenib is widely used in chemotherapy for advanced stages, cisplatin is considered one of oldest and most thoroughly studied chemotherapeutic agent. Cisplatin is a platinum based drug that is absorbed highest in the liver, prostate and kidney, and exists in tissues for about 180 days and is eliminated in urine. Cisplatin, (Figure 1.3) is one of the known chemotherapeutic agents used to treat multiple malignant tumors including liver cancer [18]. As presented in Figure 1.4, cisplatin is known to induce apoptosis by forming bifunctional adducts leading to DNA damage leading to apoptosis through several different pathways [19]. Cisplatin activates the cell cycle checkpoints (Chk) and cause a temporary arrest in the S-phase leading to the inactivation of Cdc-2 cyclin A or B kinase in order to cause cell cycle arrest in the G2/M phase. In later events, increased amount of cells gets accumulated in the G1 phase and gets trapped in the G2/M phase as a result of the inactivation of G1 phase CDKs (Cyclin dependent kinases) caused by cisplatin. Moreover, cisplatin affects the p53 (ATR and CHK1) and MAPK pathways, as well as intrinsic (Bax, casp-9, cyt c) and extrinsic apoptotic pathways (casp-8) [20].
Chemotherapy using a combination of anticancer drugs for the purpose of targeting multiple pathways at once is common, especially for cancers like hepatocellular carcinoma which is one of the most chemo resistant tumors [4] as HCC cells carry high load of genetic mutations [22]. In fact, a combination of cisplatin, doxorubicin, 5-flourouracil, and interferon alpha, abbreviated PIAF passed phase II study for advanced hepatocellular carcinoma with a median overall survival of 8.9 months. The one disadvantage of such treatments is the severe side effects it introduces to the patients, such as hematological toxicity and significant myelosuppression [23]. Therefore, ways to improve chemotherapy are highly required, as it is the only treatment option available for patients suffering from advanced hepatocellular carcinoma.
1.1.3 Molecular Pathogenesis of HCC

As illustrated in Figure 1.5, HCC has an extensive display of genetic changes, such as chromosomal deletions or amplifications and somatic mutations, and epigenetic changes, such as histone modifications, upregulation or downregulation of microRNAs and long noncoding RNAs, and chromatin remodeling, making it a very complex and heterogeneous type of cancer.

There are multiple signaling pathways that are known to be dysregulated in HCC. One of these pathways is the WNT/β-catenin or canonical Wnt signaling pathway. The activation of this pathway results from the binding of WNT ligands to frizzled receptors, which causes β-catenin to accumulate in the cytoplasm and gets transferred in the nucleus where it controls certain oncogenes, such as c-myc, cyclin D and survivin [12, 2].

The activation of another important pathway that plays a role in HCC (50% of the cases) is the receptor tyrosine kinase pathway. The activation of this pathway via the binding of ligands to different growth factor receptors (GFR), such as EGFR (epidermal), FGFR (fibroblast), HGFR (hepatocyte), and VEGFR (vascular endothelial), IGFR (insulin), leading to its phosphorylation and therefore the activation of downstream targets: MAPK and PI3K-Akt pathways. The activation of the MAPK pathway or mitogen activated protein kinase (Ras/Raf/MEK/ERK) activates proto-oncogenes and transcriptional factors that cause the transcription of genes responsible of cell differentiation. On the other hand, the PI3K-Akt pathway or phosphatidylinositol 3 kinase-protein kinase B, activation either through (a) IGFR which stimulates carcinogenesis by damaging the mammalian target of rapamycin or mTOR protein, which acts as a controller of cell proliferation, or (b) mutations or epigenetic changes that causes loss of function of PTEN, a tumor suppressor gene [12, 2].

Apoptotic pathways are also known to be escaped in cancer. Extrinsic and intrinsic pathways are the two main pathways of apoptosis. The extrinsic signaling pathway is initiated by the binding of homologous trimeric ligands to death receptors, which recruits a cascade of proteins forming a death-inducing signaling complex or DISC leading to the activation of pro-caspase 8 and caspase 8, triggering apoptosis. The intrinsic signaling pathway involves intracellular or mitochondrial signaling, in which an opening in the inner mitochondrial membrane results in cytochrome c release from the intermembrane space of the mitochondria.
Figure 1.5: Pathogenesis of HCC. Adapted from Dhanasekaran et al, 2016. Figure was reused with permission from Reference [12], see appendix 4.
to the cytosol. Cytochrome C then binds to Apaf-1 and pro-caspase 9, activating both, forming an apoptosome. This cause caspase 9 activation, which triggers apoptosis [24, 2].

Other pathways that have been reported to be activated in HCC include transforming growth factor-beta (TGFβ) pathway, JAK/STAT pathway, and ubiquitin proteasome pathway [12].

1.2 Antihistamines

1.2.1 Background

Antihistaminic drugs are well known for treating different types of allergies. Generally, such drugs target histamines by binding to and blocking histamine receptors: H1 receptor, found in stomach, intestine, bladder, H2 receptors, found in gastric cells, H3 receptors, found in the central nervous system and H4 receptors, found in immune cells, altering the histamine’s action. Antihistamines are categorized into first generation and second generation drugs. First generation antihistamines, such as cyproheptadine, carboxamine and clemastine, are sedating, cause cognitive impairment, cause CNS (central nervous system) effects, and are not fully and optimally clinically investigated, as such drugs were produced before clinical trials were required. On the other hand, second generation antihistamines, such as astemizole, loratadine and terfenadine, are nonsedating, cause no impairment, have minimal CNS effects, and are meticulously studied and examined in clinical trials [8].

As histamine is documented to favor cell proliferation and differentiation and tumor progression via H1 receptor, antihistamines are hypothesized to induce cell death [8, 25, 26]. Even though histamines usually act by binding to H1 receptors, H2, H3, and H4 receptors
1.2.2 Antihistamine’s Role in Cancer

1.2.2.1 Astemizole

Astemizole, an old second generation antihistaminic drug, was discovered in 2011 to decrease tumor proliferation *in vitro* and *in vivo* by targeting and inhibiting Eag1: an essential protein required in cancer progression [27]. In 2015, Chávez-López et al [28] continued to study the effect of astemizole in HCC cells and found that Eag1 expression is elevated in human HCC and astemizole inhibited proliferation of HCC cells and induced apoptosis in 2 HCC cell lines: HepG2 and HuH-7, by blocking and inactivating the Eag1 channels, as shown in Figure 1.6. Moreover, astemizole caused the protein Eag1 perinuclear localization from the cytoplasm, which was observed in other cancer types, such as breast cancer [28].

![Figure 1.6: Proposed effect of Astemizole on HCC. Adapted from Chávez-López et al, 2016. Figure was reused with permission from Reference [4], see appendix 5](image-url)
1.2.2.2 Cyproheptadine Hydrochloride

Cyproheptadine, a first generation antihistamine, was discovered accidently in 2012, by Feng and his group, to achieve total tumor remission when taken with thalidomide in HCC patients with lung metastasis [29], who took cyproheptadine for skin itching. The same group investigated the effect of cyproheptadine on the HCC cell lines, HepG2 and HuH-7, and in 2015 concluded that cyproheptadine suppressed proliferation of HCC cells by inducing cell cycle arrest. The proposed drug’s mechanism of action, based on their study, was through the activation of p38 MAPK facilitating the stimulation of cell regulatory proteins, resulting in cell cycle arrest in both cell lines: G1 phase in HepG2 and G1/S transition in Huh-7, along with apoptosis induction in HuH-7 cell lines, as shown in Figure 1.7 [7].

![Figure 1.7: Proposed mechanism of cyproheptadine (CPH) action on HCC cell lines. Adapted from Feng et al, 2015. Figure was reused with permission from Reference [7], see appendix 6](image)

1.2.2.3 Terfenadine

Terfenadine, a second generation antihistamine, is known for its lack of CNS side effects and therefore is widely studied over the years for its anticancer effect on a number of cancer cell lines, including Hepatocellular carcinoma HepG2, colon cancer cells COLO 205, colorectal adenocarcinoma HT29 and fibroblasts CCD 922SK [30] via G0/G1 cell cycle arrest and apoptosis through cytochrome p450 3A4 (CYP 3A4) inhibition. In addition, melanoma A375 cells was studied
by Jangi et al in 2008 then in 2011 [31], which concluded that terfenadine induced apoptosis and autophagy through different signaling pathways. Moreover, terfenadine was discovered by Wang and his group in 2014 to induce apoptosis in 2 prostate cancer (HRPC) cell lines: PC-3 and DU-145, via the breakage of the induced myeloid leukemia cell differentiation (Mcl-1) protein which further activated the overexpression of Bak (Bcl-2 homologous antagonist killer protein) leading to the cytochrome C release from the mitochondria and therefore inducing DNA damage and apoptosis. Finally, terfenadine was found to have a synergistic effect when combined with epirubicin to prevent the proliferation of non-small cell lung cancer (NSCLC) and stop resistant cells from metastasis both in vitro and in vivo [32].

1.2.2.4 Loratadine

Loratadine (Figure 1.8) is a second generation antihistamine and according to the FDA (Food and Drug Administration), it is absorbed quickly following oral administration of 10mg and and the pharmacokinetics of LOR is not dose-dependent over the dose range from 10mg to 40mg. Moreover, 80% of LOR administered is equally dispersed in urine and feces. LOR is metabolized by cytochrome P450 3A4 (CYP3A4) to descarboethoxyloratadine. LOR was first found to inhibit cell proliferation of human colon cancer (COLO 205) cells in 2006 by Chen and his group, who performed an intensive research that included in vitro and in vivo experiments on LOR effect on COLO 205 cells. They are the first to report that LOR induced cell cycle arrest in G2/M phase by 3 different mechanisms: (1) the upregulation of checkpoint kinase 1 (Chk1), (2) the downregulation of cell division cycle 25C (CDC25C), and (3) inactivation of BAD protein, which is a pro-apoptotic protein, resulting in caspase 9 mediated apoptosis [6]. LOR was further studied from another angle in cancer treatment in 2010 by Soule et al based on the findings of Chen et al in 2006. They hypothesized that since G2/M phase is a radiation sensitive phase, maybe LOR has an effect on the radio sensitivity of human colon carcinoma (HT29) cells. After studying the effect of radiation on LOR-treated cells, cell cycle phase by flow cytometry and studying the expression of cell regulatory proteins by western blot, they established the results that cells pre-treated with LOR did significantly increase radiation induction of cytotoxicity and that it directly induces DNA damage. They finally concluded that LOR is not only a successful chemotherapeutic agent but also a “modifier of radiation responsiveness in the treatment of cancer” [33].
1.2.2.5 Others

An intensive recent study by Ellegaard et al (2016) was conducted to screen the effect of multiple antihistamines, including astemizole, terfenadine, loratadine, desloratadine, ebastine and clemastine, on NSCLC. After cytotoxicity experiments on all the mentioned antihistamines, loratadine and astemizole was found to decrease cancer mortality. They then implemented a study testing the effect of those antihistamines on NSCLC, breast, and prostate cancer patients and found that low concentrations of loratadine, astemizole and ebastine cause NSCLC sensitized to chemotherapy and regressed multidrug resistance in all cancer types [35].
1.3 Hypothesis and Objectives

Given all the mentioned data from research within the past 15 years, one can propose that antihistamines does have an anticancer effect on several different cancer cell lines, including HCC cell lines and could strongly improve chemotherapy.

Even though LOR was thoroughly studied on human colon cancer cells, no studies have been attempted to test the effect of loratadine on HCC cell lines. Moreover, no research was conducted to investigate the combination effect of LOR and CisPt on HCC cell lines.

Therefore, the research hypothesis is that LOR will inhibit the proliferation of HCC cells and there will be a synergetic or an additive effect when LOR is combined with cisplatin. In order to test this hypothesis, the objectives of this study is to

1. Determine the cytotoxic effect of LOR against 2 HCC cell lines: HepG2, originated from a 15 year old Caucasian male with pure liver carcinoma cell line (absence of viral infection) [36, 37] and SNU449, originated from a 52 year old Asian male with grade II-III/IV hepatocellular carcinoma cell line containing hepatitis B virus [38, 39], by cell viability assay.

2. Identify the combination effect of LOR and cisplatin on HepG2 and SNU449 by cell viability assay followed by combination analysis using median effect analysis proposed by Chou and Talalay (2006).

3. Detect if LOR and the combination of LOR and cisplatin will induce early apoptosis or necrosis by annexin V and propidium iodide staining.

4. Examine the effect of LOR and the combination of LOR and cisplatin on the cell cycle in SNU449 cells by flow cytometry.
Chapter 2

Materials and Methods

2.1 Cell Culture

Human HCC cell lines HepG2 and SNU449 (provided by Dr. Mehmet Ozturk from the Department of Molecular Biology and Genetics, Bilkent University, Turkey) were cultured in RPMI 1640 (Lonza, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, USA) and 5% penicillin-streptomycin antibiotic (Invitrogen, USA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

2.2 Preparation of Drugs

According to Feng et al (2015) [7], Loratadine (LOR), purchased from Hangzhou Dayangchem Co., Limited, China, was dissolved in dimethyl sulfoxide or DMSO at a concentration of 100mM, which was then diluted with RPMI media to prepare concentrations of: 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM, 100 µM, and 300 µM.

Cisplatin (CisPt), MYLAN 10mg, was diluted with RPMI media to prepare concentrations of 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM, 100 µM, 300 µM and 1000 µM.
2.3 Cell Viability Assay

MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide has been widely used to determine cell viability and identify cytotoxicity of drugs. It is a positively charged tetrazolium salt which is able to penetrate the eukaryotic cell easily. When MTT is added to cells and incubated for approximately 4 hours, viable cells with active mitochondrial enzymes are able to convert MTT to formazan, a purple colored product, which is accumulated inside the cell as a precipitate, after which DMSO is added to solubilize the formazan product, allowing the measurement of the absorbance of the color by a plate reader at a wavelength of 570nm. Dead cells however, lose their ability to convert MTT to formazan, as they lack the mitochondrial enzymes responsible of the conversion [40].

HepG2 and SNU449 cells were seeded in 96 well plates (Greiner Bio-One, Germany) at 10,000 cells per well and cultured for 24 hrs. The cells were treated with the different concentrations of LOR and CisPt, mentioned in the previous section, for 24 hrs, using untreated cells as a control. MTT (SERVA Electrophoresis, Germany) was added to the cells and incubated for 3 hrs in the dark. DMSO was then added to the cells, plates were shaken for 5 min then the SPECTROstar Nano Microplate Reader, BMG LABTECH, was used to measure the optical density at 570nm. Untreated cells that were used as controls, signified 100% cell viability. A dose-dependent graph was conducted by GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) plotting cell viability vs. drug concentration, after which the IC$_{50}$ or the drug concentration that caused 50% cell death was then calculated for each cell line using the equation:

$$Y = Bottom + (Top - Bottom)/(1+10^{(X-LogIC_{50})})$$

where X is the log of the dose or concentration; Y is the response, decreases as X increases; Top and Bottom is the plateaus in same units as Y, and logIC$_{50}$ is the same log units as X.

2.4 Drug Combination Index

Combination of drugs is a common concept in chemotherapy, where the synergy of different strong anticancer drugs are used to attack the cancer cells vigorously and also as a solution to drug resistance. Chou and Talalay (2006) proposed
Materials and Methods

Table 2.1: Drug Combinational Experimental Design.

<table>
<thead>
<tr>
<th>Combination</th>
<th>0.25 X IC50</th>
<th>0.5 X IC50</th>
<th>IC50</th>
<th>2 X IC50</th>
<th>4 X IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Loratadine (LOR)**

Table 2.1: Drug Combinational Experimental Design.

an effective method for the evaluation of drug combinations quantitatively using a median effect analysis [41]. In order to determine whether the combination effect of the two drugs is synergistic, additive, or antagonistic, the experiment was prepared as follows: for each cell line, the IC₅₀ of each drug individually was calculated, after which a serial dilution was prepared for each drug and combined together with a constant ratio, as illustrated in Table 2.1.

HepG2 and SNU449 cells were seeded in 96 well plates at 10,000 cells per well and cultured for 24 hrs. The cells were treated with LOR, CisPt, and the 5 different combinations, as shown in Table 2.1, for 24 hrs, using untreated cells as a control. MTT was added to the cells and incubated for 3 hrs after which DMSO was added to the wells, plates were shaken for 5 min then the optical density was measured at 570nm using a SPECTROstar Nano Microplate Reader, BMG LABTECH.

CompuSyn software (Paramus, NJ) was used to calculate the combination index or CI, according to the median effect principle of Chou and Talalay, to determine whether the effect of the combination is synergistic, additive or antagonistic.

### 2.5 Cytotoxicity of LOR, CisPt, and Combination IC₅₀ on Normal Cells

To evaluate the cytotoxicity of the IC₅₀ of LOR and CisPt, and Combination IC₅₀ on normal cells, 1 Br hTERT- human immortalized skin fibroblasts (gifted from Dr. Andreas Kakarougkas, University of Sussex, UK) was used as a control.
Materials and Methods

2.6 Annexin V Apoptotic Assay

Phosphatidylserine (PS) is a phospholipid that is located in the inner membrane of the cell. During early stages of apoptosis, PS gets translocated to the outer membrane of the cell and is considered to be a hallmark of apoptosis. Annexin V is a known PS-binding protein that is used to track apoptosis [24].

Rotitest® Annexin V kit (Carl Roth, Germany) uses the binding efficiency of annexin V to PS to label damaged cells and also uses propidium iodide to counterstain the nuclei of cells with opened membranes, which is an indication of necrosis. This gives an advantage of not only tracking apoptosis, but also for distinguishing apoptosis from necrosis.

SNU449 cells were cultured in 6 well plates at a density of 500,000 cells per well for 24 hrs. Cells were treated with: IC$_{50}$ of LOR, IC$_{50}$ of CisPt, and Combination IC$_{50}$ in FBS-free media, for 24 hrs with untreated cells as controls. According to the manufacturer’s instructions, a single cell suspensions were prepared by trypsinization and washed with PBS twice. Annexin V binding buffer was diluted (10-folds) and added to the cells, after which Annexin V-FITC conjugate (5 μM) was added and slowly mixed. Propidium iodide (5 μM) was then slowly added to the mixture then was incubated for 15 min in the dark at room temperature. Diluted Annexin V binding buffer was added again then placed on a glass slide and covered with a glass coverslip after which the cells were observed under a fluorescence microscope (OLYMPUS IX70) using FITC and TRITC channels.

2.7 Cell Cycle Analysis by Flow Cytometry

A flow cytometer is a laser-based instrument that quantifies the fluorescence physical characteristics of single cells, which are first dyed with propidium iodide,
a DNA binding fluorescent dye. As illustrated in Figure 2.1, the single cell suspension passes through a sheath that causes a laminar flow allowing the cells to individually pass till those cells intersect with an argon-ion laser beam. The radiated light is collected by collection optics to guide the light to dichroic mirrors (mirrors with different properties at 2 different wavelengths) and 3 filters for the purpose of separating different wavelength bands. The resulting light signal is then detected and digitized for computer analysis, transforming the signal into a histogram [45].

Figure 2.1: Flow Cytometer Schematic. Adapted from Brown & Wittwer, 2000. Figure was reused with permission from Reference [45], see appendix 7

This technology is used for numerous applications, including cell cycle analysis. The cell cycle (2.2) consists of multiple phases for the division and duplication of the cell into two daughter cells: G phase (cell growth), S phase (DNA synthesis) and M phase (mitosis) [46]. Flow cytometry quantifies cells in each phase, this can serve the purpose of identifying in which phase did a particular drug cause cell cycle arrest in order to further study the genes/proteins or mechanism in which the drug acts upon.
According to Feng et al (2015), SNU449 cells were seeded in 6 well plates (Greiner Bio-One, Germany) at 500,000 cells per well and cultured for 24hrs. Cells were treated with: IC$_{50}$ of LOR, IC$_{50}$ of CisPt, and Combination IC$_{50}$ in FBS-free media, for 24 hrs with untreated cells as controls. Single cell suspensions were prepared by trypsinization and re-suspending in PBS or phosphate buffered saline, then were fixed with cold 70% ethanol at 4°C overnight. Fixed cells were washed twice with PBS, centrifuged at 500 g for 5 minutes and the supernatant was discarded. The cells were first treated with 1mg/ml RNase A (Thermo Scientific, USA) to get rid of any RNA, then stained with 5 µg/ml propidium iodide (Carl Roth, Germany) for 30 min in the dark at room temperature. [7, 47]. Cells were analyzed on a COULTER EPICS XL Flow Cytometer (Beckman COULTER, CA) by the software: Elite XL.

### 2.8 Statistical Analysis

Experiments were done in triplicates. Data were expressed as mean ± standard deviation. Differences between treated and untreated control groups were analyzed by one-way ANOVA followed by Bonferonni’s post test using GraphPad Prism 5.0 software. Statistical significance was considered at a P-value <0.05(*), <0.1 (**), and <0.001(***).
Chapter 3

Results

3.1 Cytotoxic Evaluation of LOR and CisPt individually on HepG2 and SNU449

In order to evaluate the cytotoxic potential of LOR, cell viability assay was performed by using a range of concentrations of LOR from 0.1 μM to 300 μM for 24 hours. As shown in Figure 3.1, LOR has shown a dose dependent effect on both HepG2 and SNU449. A semi-log plot was graphed by GraphPad Prism 5.0 software for the identification of IC$_{50}$ of LOR on both cell lines. The IC$_{50}$ of HepG2 and SNU449 were calculated to be 55.6 μM and 73.2 μM, respectively.

A similar plot was graphed to confirm the known effect of CisPt on both HCC cell lines and to identify the CisPt IC$_{50}$ of HepG2 and SNU449, using a range of concentrations of CisPt from 0.1 μM to 1000 μM for 24 hours. Figure 3.2 shows and confirms the cytotoxic effect of CisPt on HepG2 and SNU449, with IC$_{50}$ calculated to be 53.9 μM and 116 μM for HepG2 and SNU449, respectively.
Results

Figure 3.1: Cytotoxic effect of LOR on HepG2 and SNU449. Concentration-viability plots attained after exposure of HepG2 and SNU449 cells to LOR (0.1 - 300 µM) for 24 hrs and the corresponding IC₅₀ for both cell lines. IC₅₀ values were expressed as mean ± standard deviation (SD) of three independent experiments, each carried out in 4 replicates (n=4).

Figure 3.2: Cytotoxic effect of CisPt on HepG2 and SNU449. Concentration-viability plots attained after exposure of HepG2 and SNU449 cells to CisPt (0.1 - 1000 µM) for 24 hrs and the corresponding IC₅₀ for both cell lines. IC₅₀ values were expressed as mean ± standard deviation (SD) of three independent experiments, each carried out in 4 replicates (n=4).
Results

Table 3.1: The Combination Effect of LOR and CisPt on HepG2. Data represents the mean ± standard deviation (SD) of three independent experiments, each carried out in 4 replicates (n=4).

<table>
<thead>
<tr>
<th>Loratadine (uM)</th>
<th>Cisplatin (uM)</th>
<th>Inhibition rate (%)</th>
<th>CI value</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.9</td>
<td>0</td>
<td>7.2 ± 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.8</td>
<td>0</td>
<td>18.2 ± 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55.6</td>
<td>0</td>
<td>44.7 ± 10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>111.2</td>
<td>0</td>
<td>96.6 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>222.4</td>
<td>0</td>
<td>99.1 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.5</td>
<td>15.0 ± 10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>27</td>
<td>41.4 ± 24.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>53.9</td>
<td>52.3 ± 5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>107.9</td>
<td>58.2 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>215.6</td>
<td>66.9 ± 8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.9</td>
<td>13.5</td>
<td>20.0 ± 9.5</td>
<td>1.65</td>
<td>Antagonism</td>
</tr>
<tr>
<td>27.8</td>
<td>27</td>
<td>48.4 ± 16.0</td>
<td>1.09</td>
<td>Additive</td>
</tr>
<tr>
<td>55.6</td>
<td>53.9</td>
<td>83.1 ± 3.6</td>
<td>0.85</td>
<td>Slight Synergism</td>
</tr>
<tr>
<td>111.2</td>
<td>107.9</td>
<td>99.3 ± 1.9</td>
<td>0.45</td>
<td>Synergism</td>
</tr>
<tr>
<td>222.4</td>
<td>215.6</td>
<td>99.3 ± 2.6</td>
<td>0.90</td>
<td>Slight Synergism</td>
</tr>
</tbody>
</table>

3.2 Combination Effect of LOR and CisPt on HepG2 and SNU449

To test the potential of LOR to improve chemotherapy, LOR was combined with CisPt in constant ratios and the effect was tested by cell viability assay on HCC cell lines. Combination Index (CI) values were calculated using CompuSyn software (Paramus, NJ), and according to Endo et al (2011), CI >1.3 = antagonism; CI 1.1-1.3 = moderate antagonism; CI 0.9-1.1 = additive effect; CI 0.8-0.9 = slight synergism; CI 0.6-0.8 = moderate synergism; CI 0.4-0.6 = synergism; CI 0.2-0.4 = strong synergism.

Tables 3.1 and 3.2 summarizes the drug concentrations used (0.25 X IC₅₀, 0.5 X IC₅₀, IC₅₀, 2 X IC₅₀, 4 X IC₅₀) for each drug, the corresponding cell death percentage, and the CI value and the corresponding effect of the combination of each ratio on HepG2 and SNU448, respectively.

For HepG2, the CI values (0.45-0.9) suggested synergistic effects when high concentrations of both drugs are used (IC₅₀, double and quadruple the IC₅₀) and
Result...
was cytotoxic in high concentrations (73.2 µM) resulting in 93% cell death, while upon using lower concentrations (55.6 µM), cytotoxicity was lower resulting in only 25% cell death (Figure 3.3B). CisPt was not cytotoxic neither in low (53.9 µM) nor in high (116 µM) concentrations, resulting in 20-30% cell death. As for the combination of the IC$_{50}$ of both drugs, severe cytotoxicity (94% cell death) was observed when high concentration ratios of both drugs were used (Figure 3.3A). Lower concentration ratios of both drugs resulted in lower cytotoxicity (60% cell death) to immortalized skin fibroblasts (Figure 3.3B).

![Figure 3.3: Comparison of cytotoxicity of LOR, CisPt, and Combination on HCC cell lines and hTERT.](image)

**3.4 Apoptosis Analysis on SNU449**

Apoptotic assay was done in order to examine if LOR, CisPt and the combination of the IC$_{50}$ of both drugs, cause cell death via apoptosis or necrosis. As chemotherapy is the only standard treatment option for advanced HCC patients, SNU449 cell lines was used as a representative of advanced HCC stages. Cells that are positive for annexin V (green fluorescence) indicate early apoptosis, while cells that are positive for PI (red fluorescence) indicates necrosis. Cells that are positive for both stains giving yellow fluorescence indicates late apoptosis.

Untreated SNU449 cells (Figure 3.4A) was negative for annexin V (1/58) and PI staining (1/58) (Figure 3.4B and Table 3.3), indicating that the cells were
viable. LOR (73.2 μM) -treated cells (Figure 3.4C) was positive for annexin V staining (3/6) but not PI staining (Figure 3.4D and Table 3.3), indicating that the cells were undergoing early apoptosis. CisPt (116 μM) - treated cells (Figure 3.4E) showed increased red fluorescence (20/85) than green (2/85) and yellow (1/85) fluorescence (Figure 3.4F and Table 3.3). In addition, CisPt- treated cells showed change in morphology, where the cells displayed blebs and has a bubbly appearance (Figure 3.4E), indicating that the cells were undergoing necrosis. As for cells treated with a combination of 73.2 μM LOR and 116 μM CisPt (Figure 3.4G), cells showed red (6/18), green (1/18), and yellow fluorescence (2/18), indicating cells were undergoing necrosis (Figure 3.4H and Table 3.3).
Results
Results
Results

CisPt

E

F
Figure 3.4: Annexin V/ PI Staining for Untreated and Treated SNU449 Cells. Green Fluorescence visualized by the FITC (Fluorescein isothiocyanate) Filter indicates early apoptosis, red fluorescence visualized by the TRITC (Tetramethylrhodamine isothiocyanate) filter indicates necrosis, and yellow fluorescence indicates late apoptosis. (A,C,E,G) represents untreated SNU449 cells, SNU449 cells treated with LOR, SNU449 cells treated with CisPt, and SNU449 cells treated with a combination of the IC$_{50}$ of LOR and CisPt, respectively. (B,D,F,H) represents merged FITC and TRITC filters for untreated cells, LOR-treated cells, CisPt-treated cells, and Combination-treated cells.
Table 3.3: Number of viable cells, cells undergoing early apoptosis, late apoptosis, and necrosis for each condition, according to annexin V and PI staining.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Viable Cells</th>
<th>Early Apoptosis</th>
<th>Late Apoptosis</th>
<th>Necrosis</th>
<th>Total Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>LOR</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>CisPt</td>
<td>62</td>
<td>2</td>
<td>1</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>Combination</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

3.5 Cell Cycle Analysis on SNU449

To explore the effect of LOR, CisPt and the combination of the IC$_{50}$ of LOR and CisPt on the cell cycle of SNU449 cells, cells were stained with annexin V and propidium iodide (PI), after which flow cytometry was done. Histograms showed a statistically significant decrease in the number of cells in the Go/G1 phase in CisPt and Combination treated groups compared to control cells (Figure 3.5), followed by a statistically significant increase in the G2/M phase in all treated groups, indicating cell cycle arrest in the G2/M phase.
Figure 3.5: Effects of LOR, CisPt, and Combination on the cell cycle in SNU449.

(A) Histograms shows the percentage of cells in the phases: sub G1 ("F"), Go/G1 ("G"), and G2/M ("Q"/"P"). (B) Comparison of cells percentage in the different phases upon treatment. (C) Table summarizing Flow cytometry analysis. Data represents the mean (%) ± standard deviation (SD) of two independent experiments. Statistical comparison were performed by GraphPad Prism 5.0 software using One-way ANOVA with Bonferonni’s post-test. P-value <0.05(*).
Chapter 4

Discussion and Conclusion

HCC mortality rate is high in the world, especially in Egypt due to the high incident rates of HBV and HCV [11]. The pathophysiology of HCC is complex as it is a multifactorial type of cancer. However, common molecular pathways that are altered in hepatocellular carcinoma includes the WNT/β-catenin, receptor tyrosine kinase, MAPK, and PI3K-Akt pathways [2], [12]. This makes HCC a complex and heterogenous event. As most patients are diagnosed in the advanced stage because of the asymptomatic nature of HCC in its early stages, chemotherapy is the only standard treatment option [11, 7, 14]. Therefore, ways to improve chemotherapy is highly required to decrease HCC mortality rate and increase survival rates.

Antihistamines have been studied for over 15 years in vitro and in vivo and have shown anticancer potential in some cancer cell lines [27], [7], [6], [28], [5], [48], [31]. Loratadine, a second generation antihistamine, was thoroughly examined for its cytotoxic potential against human colon cancer cells (COLO 25) in 2006. LOR was found to inhibit the proliferation of COLO 25 by inducing cell cycle arrest in the G2/M phase and by inducing caspase 9 mediated apoptosis [6]. In 2010, LOR was further studied from another angle by Soule et al. Their hypothesis was based on the findings of Chen et al. in 2006: LOR may have an effect on the radio sensitivity of human colon carcinoma cells due to the finding that LOR did induce cell cycle arrest in the G2/M phase, which is a radiation sensitive phase. They concluded that cells pre-treated with LOR did significantly increase radiation induction of cytotoxicity and that it directly induces DNA damage in human colon carcinoma cells [33]. Moreover, Ellegard et al intensively studied the effect of multiple antihistamines on non-small cell lung cancer (NSCLC) in 2016 and found that LOR not only decreased cancer mortality, but also caused NSCLC
sensitization to chemotherapy and reverted resistance [35]. Therefore, LOR offers multiple advantages and showed a potential of being a successful chemotherapeutic agent, a modifier of radiation responsiveness in the treatment of cancer, and caused sensitization to chemotherapy and reverted resistance. This led to the interest in studying the effect of LOR on certain hepatocellular carcinoma cell lines to test the potential of LOR to act as a chemotherapeutic agent and to also test the potential of LOR to improve chemotherapy by studying the effect of the combination of LOR with CisPt.

Here, the effect of LOR was evaluated, along with its combination with cisplatin, in HCC cell lines: HepG2 and SNU449, a representative of early stage and advanced stage of HCC, respectively. Consistent with the results of Chen et al (2006) on colon cancer cells, COLO 205 cells, LOR showed a dose-dependent cytotoxicity in both HCC cell lines, with a higher IC_{50} in SNU449 (73.2 \mu M) than HepG2 (55.6 \mu M), which may be due to the fact that SNU449 cell line is a more advanced stage of HCC than HepG2. Therefore, SNU449 was chosen for further analysis.

Combination therapy is commonly used in chemotherapy for the purpose of decreasing the development of resistant cells and to be able to use lower doses to produce less side effects, which is nephrotoxicity in the case of CisPt [49]. Examples of using combination therapy includes rituximab and CHOP (cyclophosphamide, hydroxydaunomycin, Oncovin, and prednisone) chemotherapy for non-Hodgkin’s lymphoma and taxol and Herceptin for breast cancer [50]. Synergism is defined as a greater net therapeutic effect of the combination of both drugs than the total sum of effects produced by each drug individually, while additive effect produces combination therapeutic effect that is equal to the total sum of effects caused by each drug individually [51]. Antagonism, however, is when the net therapeutic effect of the combination is less than the sum of effects of each drug alone. Thus, the optimum drug combination is that which provides additive to synergistic effects.

In HepG2, the combination of LOR with CisPt produced an antagonistic effect when used in low concentration ratios (13.9 \mu M LOR:13.5 \mu M CisPt). However, as higher concentration ratios were used, the combination effects became more synergistic. In SNU449, no antagonism was produced upon using all combination ratios, producing additive to synergistic effects. These results indicates that a greater effect is generated when high concentration ratios of LOR and CisPt
are used (double and quadruple the IC$_{50}$ of each drug) than the total effect of LOR and CisPt alone. This could serve as a great advantage in chemotherapy as dose-dense chemotherapy is known to improve survival and is used in multiple cancer types [52]. Therefore, the combination of 55.6 $\mu$M LOR with 53.9 $\mu$M CisPt, resulting in 83% cell death in HepG2, and the combination of 73.2 $\mu$M LOR with 116 $\mu$M CisPt, resulting in 78% cell death in SNU449 were used for further analysis as both combinations gave synergistic to additive effects with higher percentage of cell death. Further studies could be needed to inspect the effect of inconstant ratios, for example, to test the effect of the combination of low concentrations of CisPt and high concentrations of LOR to further explore the possibility that LOR can improve chemotherapy effectiveness by decreasing the nephrotoxicity caused by cisplatin as well as causing optimal cell death.

The IC$_{50}$ values of LOR, CisPt, and Combination of IC$_{50}$s of both drugs were tested against 1 Br hTERT cells. 1 Br hTERT cells are skin fibroblasts that are immortalized, which gives them the ability to proliferate in culture. hTERT was used here as a control/normal cells to give an indication of the cytotoxicity of the drugs on normal cells. The hypothesis was that LOR will not show any cytotoxicity as it was clinically tested and was proven to be safe by going through clinical trials. Unexpectedly, high concentrations of LOR (73.2 $\mu$M) and high ratios of both drugs (73.2 $\mu$M LOR and 116 $\mu$M CisPt) showed severe cytotoxicity to hTERT (94% cell death), while lower concentrations of LOR (55.6 $\mu$M) and both CisPt concentrations (53.9 $\mu$M and 116 $\mu$M) didn’t exhibit any cytotoxicity.

The cytotoxicity of LOR and its combination with CisPt on hTERT may be explained by the fact that hTERT shares some cancer-like properties when it was immortalized, as immortalized cells have increased telomere expression and could induce tumor growth [53]. Moreover, the study by Chen et al. in 2006 compared concentrations of LOR between 10 - 50 $\mu$M in humun colon cancer cells and primary normal cells, but did not evaluate higher concentrations of LOR in normal cells, even though 75 $\mu$M of LOR was studied in cancer cells [6]. Consequently, searching for drugs that exhibits higher toxicity on cancer cells with minimal toxicity on normal cells are attempted. Therefore, further studies are required to test the effect of the IC$_{50}$ of LOR and different ratios of both drugs on primary normal hepatocytes.

To elucidate the mechanism by which CisPt and Combination IC$_{50}$ induced cell death and inhibited tumor growth, apoptotic assay and cell cycle analysis
were performed. Apoptotic analysis showed that LOR treated cells were positive for annexin V staining, which may indicate that LOR induces early apoptosis. CisPt- treated cells and cells treated with a combination of the IC\textsubscript{50} of both drugs were positive for PT staining, indicating necrosis. However, expression analysis of markers/genes of apoptosis, such as caspase 8, caspase 9, caspase 3, PARB and necrotic markets such as HMGB1 or High mobility group protein B1 by real time PCR and western blotting, as well as TUNEL assay are necessary to confirm the above result.

Finally, cell cycle analysis was performed to indicate whether treatment with LOR, CisPt, or combination of the IC\textsubscript{50} of both drugs will cause cell cycle arrest. The cell cycle starts with the G1 phase, where cells grow (2N) then cells begin to replicate in the S phase which is DNA synthesis phase after which cells continue to grow in the G2 phase and finally divide into 2 daughter cells in the M phase (4N). A histogram is used to represent the percentage of cells in each of these stages. The histograms generated in the NSA Diagnostic Labs(Cairo, Egypt) showed that treatment with LOR, CisPt and Combination IC\textsubscript{50} lead to an increase in the percentage of cells in the G2/M phase: 22.9\% ± 0.2, 24.5\% ± 2.9, and 24.6\% ± 1.6, respectively, compared to the untreated SNU449 cells, 14.4\% ± 0.00, which is an indication that cells are accumulated in the G2/M phase and could not complete the cell cycle. Moreover, there was a decrease in the percentage of cells in the Go/G1 phase: 68.8\% ± 0.1, 65.3\% ± 3.6, and 65.8\% ± 0.57, respectively, compared to the untreated SNU449 cells, 74.2\% ± 0.14, which confirms the hypothesis of the cell cycle arrest in the G2/M phase, as cells were not able to complete the cycle and therefore the percentage of cells decreased in the following phase, which is Go/G1. Consistent with other studies [6], [33], LOR-treated colon cancer cells also resulted in cell cycle arrest in the G2/M phase.

In conclusion, the findings displayed in this thesis signifies the anticancer potential of loratadine in 2 HCC cell lines: HepG2 and SNU449. As LOR is already reported to be a released antihistamine that is prescribed to children and adults, this had driven further interest to explore its capacity to improve chemotherapy. Cisplatin in combination with LOR resulted in synergistic to additive effects when the IC\textsubscript{50} of both drugs were used and resulted in higher percentage of cell death. This can open doors to investigate the possibility of evaluating the use of antihistamines in cancer \textit{in vivo} and even clinically.
Future Directions

1. Investigate the anticancer potential of LOR on Huh7 cell line (HCC cell line derived from HCV) could shed light on its effect on HCC derived from HCV, which represents most of the cases in Egypt.

2. Investigate the effect of inconstant concentration ratios of LOR and CisPt, i.e. low concentration of CisPt in combination with high concentration of LOR, on HCC cell lines.

3. Investigate the effect of LOR in combination with sorafenib could give a clearer indication of the potential of LOR to improve chemotherapy, as sorafenib is used in chemotherapy for advanced HCC.

4. In order to elucidate the mechanism in which LOR and the combination of loratadine and chemotherapeutic drugs, RNA sequencing would be very beneficial to examine the effect of the therapy on the transcriptome level in order to identify all the genes and pathways that got deregulated by such therapy.

5. More studies are required to test the effect of higher concentrations of LOR (>50 μM) on primary normal cells.

6. More In vitro studies, such as expression analysis of apoptotic markers and cell regulatory genes are necessary to confirm the effect of LOR and combination therapy on HCC cell lines and to examine the method of action of LOR and its combination with cisplatin.

7. In vivo studies are necessary to confirm the effect of LOR and combination therapy on animal models.
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References


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Yu-Min Fang,1 Chin-Han Fang,2 Syue-Yi Chen,2 Hsiao-Yen Hsia,1 Yu-Hsin Chen,2 and Chang-Da Hsu1,2

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