# EFFICACY AND MECHANISM OF ACTION OF A NOVEL CLASS OF ANTI-CANCER DRUGS

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**Abstract**

The incidence of cancer worldwide has increased over the years, and gastrointestinal cancers (G.I.) are amongst the most common forms of cancer. Nevertheless, there is still no curative treatments for this group of tumors. Nucleoside analogues are widely used in cancer treatment. The prevailing compounds are Gemcitabine (used for pancreatic cancer and other carcinomas), 5-Fluorouracil (used in breast, colon, and other cancers), Cytarabine and Clofarabine (used in leukemias). Gemcitabine, the current standard of care for various forms of solid tumors, has a limited efficacy against pancreatic cancer. The objective of this project was the development of effective drugs against pancreatic cancer. We focused on a novel class of nucleoside analogues designed to bypass the most common cellular road blocks and resistance mechanisms. After an extensive screen for cell killing activity, two lead molecules were exclusively studied: LCB2151 and LCB2132. These two molecules showed high efficacy in killing human cancer cells from three different human G.I. cell lines: BxPC3 and Capan-2, two pancreatic cell lines representative of K-Ras positive and negative tumors, as well as the liver cell line HepG2. LCB2151 showed high efficacy in killing Gemcitabine-resistant cancer cells, and a low toxicity in normal cells. Interestingly, results show that these prodrugs can efficiently bypass key resistance mechanisms developed by cancer cells. The results obtained in this project are promising and could pave the way for a more effective treatment of pancreatic cancer.

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| | |
|---|---|
| 5-FU | 5-Fluorouracil |
| ATP | Adenosine Tri-phosphate |
| BCLC | Barcelona-Clinique Liver Cancer |
| CDA | Cytidine Deaminase |
| CDK | Cycline-dependent Kinase |
| cDNA | complementary DNA |
| dATP | deoxy-Adenosine tri-phosphate |
| dCK | deoxy-Cytidine Kinase |
| ddH20 | double distilled water |
| DNA | deoxy-nucleic acid |
| dNTP | deoxy-nucleotide tri-phosphate |
| EGFR | Epidermal Growth Factor Receptor |
| HCC | Hepatocellular Caricnoma |
| hENT1 | human Equilibrative Nucleoside Transporter |
| IGFR | Insulin-like Growth Factor Receptor |
| mAB | monoclonal Antibody |
| MAPK | Mitogen-activated protein Kinase |
| MEK | Mitogen-activated protein Kinase kinase |
| mRNA | messenger RNA |
| MRP1/2 | Multidrug resistance-associated protein 1/2 |
| NA | Nucleoside Analogue |
| NaOV | Sodium Orthovanadate |
| PDGFR | Platelet-derived Growth Factor Receptor |
| PEG | Polyethinol Glycol |
| PMSF | phenylmethylsulfonyl fluoride |
| qPCR | quatitative Polymerase Chain Reaction |
| RIPA | Radioimmunoprecipitation assay |
| RIPK1 | Receptor-interacting serine-threonine protein kinase |
| RNA | ribonucleic acid |
| RNR | Ribonucleotide Reductase |
| ROS | Reactive Oxygen Species |
| RT-PCR | Reverse Transcription-Polymerase Chain Reaction |
| SDS | Sodium Dodecyl Sulfate |
| SOD | Superoxide Dismutase |
| VEGFR | Vascular Endothelial Growth Factor Receptor |

# 1. Introduction

## 1.1.Gastrointestinal Cancers

In 2013, cancer caused over 8 million deaths worldwide, and has become the second leading cause of morbidity behind cardiovascular diseases. Gastrointestinal cancers affect the digestive system and include cancer of the esophagus, gallbladder, liver, pancreas, stomach, small intestine, bowel (large intestine or colon and rectum), and anus (Global Burden of Disease Cancer Collaboration *et al*., 2015).

### 1.1.1. Liver Cancer

#### 1.1.1.1.Epidemiology

Hepatocellular carcinoma (HCC) is the most common hepatic malignancy in the world. The most vulnerable subsets of the population are patients with cirrhosis, and/or with chronic hepatitis B (Clark, Maximin, Meier, Pokharel, & Bhargava, 2015). Although patients with hepatitis C-related cirrhosis are the most susceptible to acquire HCC in North America, patients from Africa and Asia were shown to have a higher incidence of HCC related to hepatitis B (Bruix, Sherman, & American Association for the Study of Liver Diseases, 2011).

There are also cases of HCC in noncirrhotic liver. One subtype of this kind is fibrolamellar HCC, which is predominant in young adults in Europe and North America and accounts for up to one third of the cases of noncirrhotic HCC. Nevertheless, the incidence of fibrolamellar HCC in the United States is approximately 0.02 cases per 100,000 individuals, almost 100 times lower than cirrhosis-associated HCC (Gaddikeri *et al*., 2014). However, there are several conditions that can influence the development of

HCC without underlying cirrhosis. They include Hepatitis B and C, alcohol consumption, exposure to *Aspergillus flavus* aflatoxin B1, chemical and industrial carcinogens, tissue iron overload, inherited diseases, metabolic syndromes, hepatic adenoma to carcinoma, and hepatic vascular abnormalities. These conditions generally cause alterations in cell cycle regulation, oxidative stress, and increased levels of tumorigenic growth factors (Gaddikeri *et al*., 2014).

Obesity has also been shown to cause a higher risk of HCC. The obesity related inflammatory mechanisms are thought to be linked to the development of HCC, specifically through the elevated production of IL-6 and tumor necrosis factor. It has even been suggested that IL-6, C-Reactive Protein (CRP), C peptide, non-high molecular weight adiponectin, and Glutamate Dehydrogenase (GLDH), which are also elevated in obese patients, could be used as potential biomarkers to determine the probability of cancer (Aleksandrova *et al*., 2014; Park *et al.*, 2010).

#### 1.1.1.2. Characteristics

Liver cancer has a wide morphological diversity. In order to provide patients with a more personalized therapy, the histology-based definition of the morphological heterogeneity of liver cancer has been modified several times. The World Health Organization classified liver cancers as HCC, intrahepatic cholangiocarcinoma (ICC), and combined hepatocellular and cholangiocarcinoma (cHCC-CC) (Flejou, 2011). The histological types of HCC are thin trabecular type, thick trabecular type, pseudo-glandular type, and compact type. The cellular phenotypes of HCC are: clear cell type, fat-rich type, spindle cell type, and undifferentiated type ( Li & Wang, 2015). Liver cancer heterogeneity has also been classified by tumor differentiation status, tumor growth patterns and

pathological features of paratumor tissues (Flejou, 2011). The differentiation status of HCC is divided into well differentiated, moderately differentiated, poorly differentiated, and undifferentiated. On the other hand, tumor growth patterns are classified as invasion of ambient normal liver tissue, invasion of capsule, generation of satellite nodules, intrahepatic metastasis, and formation of tumor thrombi. Moreover, the inflammation grade (G) and fibrosis stage (S) in the surrounding tissues of the tumor are classified from G1 to G4 and from S0 to S4 (Li & Wang, 2015).

#### 1.1.1.3. Diagnosis and Treatment

Classic HCC is normally detected during a routine checkup of patients with cirrhosis, normally through ultrasonographic surveillance. This facilitates an early diagnosis, before clinical symptoms become evident (Trevisani *et al*., 2010). Noncirrhotic HCC is however more difficult to detect since its patients do not have a known underlying liver disease. Therefore the tumors are detected at a more advanced stage and can cause symptoms like abdominal pain, distention, weight loss, anorexia, and chest pain (Gaddikeri *et al.*, 2014).

Once a patient has been diagnosed with HCC, it is important to stage it in order to start the appropriate treatment. The Barcelona-Clinic Liver Cancer (BCLC) staging system (Forner *et al*. , 2010) has become widely accepted in clinical practice and is also used in many clinical trials of new drugs (Bruix *et al.,* 2011). According to this system, resection is still the first option for patients who are in stage 0 of HCC, in which there is only one nodule smaller than 2 cm. Although patients with higher stages could also benefit from resection, the rate of mortality is higher. For this reason, liver transplantation or ablation is recommended. In HCC stage B and C, BCLC system recommends chemoembolization and

Sorafenib respectively (Bruix *et al*., 2011). Sorafenib (Nexavar, Bayer HealthCare Pharmaceuticals–Onyx Pharmaceuticals) stops tumor-cell proliferation and tumor angiogenesis, and increases the rate of apoptosis in various tumor models. It has been shown to inhibit Raf-1 and B-Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 and platelet-derived growth factor receptor β (PDGFR- β). However, Sorafenib increases the overall survival of patients with advanced HCC by only three months (Llovet *et al*., 2008; Wilhelm *et al.*, 2004). Patients in stages B and C have the highest mortality rates. The 5-year survival rates for these patients is 7% for stage B and 2% for stage C patients (American Cancer Society, 2016). There is still no curative treatment that increases the probabilities of a complete recovery (Table 1).

### 1.1.2. Pancreatic Cancer

#### 1.1.2.1. Epidemiology

Pancreatic cancer is one of the most lethal forms of cancer, and a highly unmet medical need. In 2014, it was predicted that in 2015 approximately 40,560 people will be diagnosed with pancreatic cancer in the United States, and around 85% of them will not survive (Table 1). Its incidence has increased over the years and it has become the 4$^{th}$ leading cause of cancer-related deaths (Kaltsas *et al.* , 2014). In Canada, 4590 people died of pancreatic cancer in 2015, being the 4$^{th}$ leading cause of cancer related deaths both for male and female (Government of Canada: Canadian Cancer Statistics, 2015). The high mortality rate is due to two factors: the pancreas is located in an inaccessible location within the abdomen, which makes the diagnosis more difficult than other gastrointestinal cancers (Lowenfels & Maisonneuve, 2004). Also, at early stages pancreatic cancer exhibits few or

no symptoms at all, which is the reason why this cancer is often detected at a late stage or when the tumor has metastasized (Chang *et al.* , 2014). Due to its delayed detection, only 10 to 20% of the patients with pancreatic cancer have resectable tumors, and less than 20% of these patients survive longer than 5 years (Jordheim *et al.*, 2013a).

Pancreatic cancer is mostly diagnosed in people over 60 years of age, and almost half of the patients are 75 years or older at the time of diagnosis. Although surgery is the best option for increased survival, many of the patients have increased risk of complications due to their older age (Lowenfels & Maisonneuve, 2004).

The incidence of pancreatic cancer is expected to increase, based upon the rise of the elderly population older than 65 years in many countries. Another major risk factor for pancreatic cancer is smoking. In Japan the frequency of pancreatic cancer has increased at the same level as the smoking population. Conversely, in the USA the smoking population has decreased and the incidence of pancreatic cancer is declining (Lowenfels & Maisonneuve, 2004). Smoking is the strongest environmental risk factor that causes pancreatic cancer. The carcinogens derived from tobacco are thought to be absorbed from the lungs and passed to the bloodstream en route to the pancreas. There is also a possibility that the products of the tobacco that are ingested reach the pancreas after reflux into the pancreatic ductal system from the duodenum. This would explain the high amount of pancreatic cancers that arise in the head of the pancreas (Doll *et al.*, 1994; Lowenfels & Maisonneuve, 2004; Mack *et al.*, 1986).

At the genetic level, many studies have determined an increased predisposition for the relatives of pancreatic adenocarcinoma patients of up to three fold. Nevertheless, pancreatic cancer that is linked to a familial setting has a penetrance of less than 10%

compared to a higher penetrance in other types of cancer (Lynch *et al.*, 1996). Furthermore, genetic defects seem to have a great influence in families that inherit pancreatic cancer as an autosomal-dominant trait with high penetrance. Such syndrome has been identified in one family. It has a penetrance of almost 100%, and seems to be associated with diabetes, pancreatic exocrine insufficiency and pancreatic adenocarcinoma (Bardeesy & DePinho, 2002; Eberle *et al*., 2002).

**Table 1. Liver and Pancreatic Cancers Worldwide Statistics.**

| Cancers | Treatments | Cases/year | Deaths/year | 5 year survival rate |
|---|---|---|---|---|
| **Liver *** | Tumor resection/Sorafenib | 792000 | 818000 | 28% |
| **Pancreas**$^{+}$ | Tumor resection/ Gemcitabine/FOLFIRINOX | 338000 | 257000 | 5% |

*****(Global Burden of Disease Cancer Collaboration et al., 2015; Harlan, Parsons, Wiggins, Stevens, & Patt, 2015). $^{+}$ (Kaltsas *et al*., 2014).

#### 1.1.2.2. Characteristics and Diagnosis

The symptoms start when the tumor is large enough to compress other structures or organs, or when the tumor metastasizes, thus depending on the tissue into which the cancer cells have infiltrated into (Chang *et al*., 2014).

When a patient has been diagnosed with pancreatic cancer, doctors usually look at different prognostic factors that could help determine the best treatment to use and how the patient is going to react to the treatment. Some of these factors include "overall tumor stage, lymph node metastasis, status of resection margins, tumor histology, tumor size, lymphovascular invasion, perineural invasion, and possibly even liver function test" (Kim *et al*., 2011). Nevertheless, when the cancer is at an advanced stage and a resection cannot be performed, other predictive markers would be helpful to treat this aggressive malignancy.

The high expression of human Equilibrative Nucleoside Transporter 1 (hENT1) has been shown to be associated with a longer overall survival. In a study from the University of Barcelona (Perez-Torras *et al*., 2008), adenoviral-mediated overexpression of hENT-1 in a pancreatic cell line, proved to enhance the response to the prevailing drug used for pancreatic cancer in mice. This report and many others have suggested that the levels of expression of this gene could be a reliable prediction marker to response to treatment for pancreatic cancer.

### 1.2.Current standard of care

Gemcitabine, a nucleoside analogue, is the chemotherapeutic treatment most widely used, and the current standard of care. Its cytotoxic activity is due to its incorporation into DNA and RNA, and its inhibition of various nucleic acid synthesis enzymes (Fig. 1). Unfortunately, this molecule is not efficiently curative, and in many cases the tumors develop resistance (Galmarini *et al.*, 2002).

A study in the University of Texas showed that operative resection is underutilized in patients with localized pancreatic cancer, regardless of the benefit that the surgery offers. It was found that 70% of patients with resectable cancer in the study population did not receive this curative treatment (He *et al.*, 2015).

Given that cancer has become such a common disease, there have been many attempts to develop treatments with higher efficacy than the current standard of care. Most of them have focused on adjuvant molecules for Gemcitabine or combination treatments with this compound. These adjuvant molecules would be able to target a certain pathway in order to increase the effect of Gemcitabine, or they could have a synergistic effect with

this drug. However, the majority of these attempts have failed to yield better results than the ones from the standard method of care.

Tyrosine kinases are a group of enzymes essential in key pathways involved in cell proliferation, differentiation, migration, metabolism, and programmed cell death. These enzymes are often implicated in neoplastic development, enhancing aberrant signaling that benefits cancer cells (Paul & Mukhopadhyay, 2004). For this reason, many studies have focused on blocking members of this group of enzymes through Tyrosine kinase inhibitors. Erlotinib, an Epidermal Growth Factor Receptor (EGFR) inhibitor that binds to its ATP-binding site, showed positive results in a Phase III clinical trial involving 569 patients with pancreatic cancer. Co-treatment with Gemcitabine and Erlotinib improved the overall survival of patients by 1 year, a significant result for pancreatic cancer patients. Erlotinib is thus the only targeted therapy approved for metastatic pancreatic cancer (Moore *et al*., 2007). Cetuximab, a monoclonal antibody (mAb) that binds to the ligand-binding domain of the same receptor, did not show high efficiency as an anticancer drug. A Phase III clinical study involving 745 patients with locally advanced metastatic pancreatic cancer tested the results of Gemcitabine treatment alone versus co-treatment with Cetuximab. However the combination treatment did not improve the overall survival of patients compared to Gemcitabine by itself (Philip *et al.*, 2010). Sorafenib is a Raf-1 and B-Raf inhibitor that blocks the VEGFRs and the PDGFR-β. As mentioned earlier, it is used to treat hepatocellular carcinoma patients, although Llovet *et al*. (2008) reported that it increases the overall survival of patients with advanced hepatocellular carcinoma by only 3 months.

Pancreatic cancer cells overexpress insulin-like growth factor receptor (IGFR) and its ligands IGF-1 and 2 (Bergmann *et al.*, 1995). IGF1R inhibitors have been synthesized to stop the growth of cancer cells. Ganitumab, a mAb that inhibits IGF-1 and 2 from binding to their receptor, has been tested in human pancreatic cancer xenograft models by itself and in combination with Gemcitabine. Kindler *et al.* (2012) reported a 12 month survival rate of 39% with co-treatment of Ganitumab and Gemcitabine, compared to 23% by Gemcitabine alone in patients with metastatic pancreatic cancer. However, these results do not significantly exceed the ones from Gemcitabine by itself, making them not viable for an alternate treatment.

Antibiotics synthesized by *Streptomyces spp.*, Anthracyclines, have been shown to have a strong anti-cancer effects. Doxorubicin, one of the main antibiotics used for cancer treatment, was isolated in the 1960s and has been widely used for the treatment of many types of tumors. Doxorubicin intercalates in the DNA, causing its deformation and the stabilization of the topoisomerase II-DNA complex producing double strand DNA breaks. Furthermore, Anthracyclines are known for the formation of free radicals through the reduction of the Anthracycline ring that produces a semiquinone free radical, and thus the release of a hydroxyl free radical. Unfortunately, free radical damage is also related to the high cardiotoxicity produced by this anticancer treatment (Hortobagyi, 1997).

The discovery of doxorubicin and its effective mechanism of action lead to the development of another topoisomerase II inhibitor, Etoposide. This drug has been used for almost three decades to treat a variety of tumors (Liu, 1989). However, its mechanism of action is not restricted to cancer cells, and can cause several malignancies. Additionally, it has not showed a strong effect on pancreatic cancer and other types of tumors (Iwasa *et al*.,

2010). Furthermore, several drug resistant alleles have been identified that decrease its efficacy (Jiang, 2005).

Another anticancer drug that has been used for over four decades to treat many types of solid tumors is Cisplatin. This drug is a platinum derived compound that was discovered by accident in the 1960's by Dr. Rosenberg at Michigan State University (Rosenberg *et al.*, 1965). Cisplatin gets activated inside the cells through replacement of one of the two chlorine groups by water. Then it binds to the purine bases of guanine and adenine to form monofunctional or bifunctional adducts. Normally these adducts are located adjacent to one another, and are known as intrastrand adducts, or crosslinks. These unwind or bend the DNA, and result in an apoptotic cell death due to inhibition of replication and transcription of the DNA (Siddik, 2003). Although this drug was proven effective to help cancer patients live longer, many of them develop resistance to this drug. Additionally, this drug is highly toxic to the kidneys and gastrointestinal tract. Cisplatin analogues have been developed to decrease its toxicity; one of them is Carboplatin, which has a more stable chloride group. Even though Carboplatin is less toxic for the gastrointestinal tract and the kidneys, it causes thrombocytopenia, due to the larger concentrations that has to be used in anticancer treatments (Harrap, 1985; Kelland, 2007). Oxaliplatin, another analogue, was found to have higher anticancer efficacy than Cisplatin. Today, it is used in combinatory treatments with Leucovorin and 5-Fluorouracil, known as FOLFOX, and it is the standard first line of care for colorectal cancer. However, this treatment only increases the overall survival of patients by a few months compared to untreated patients, and its neurotoxicity is dose-limiting (Alian *et al.*, 2012).

Finally, antimitotic treatments have been developed in order to stop the proliferation of cancer cells, without affecting non-dividing or quiescent cells. Many compounds have been synthesized targeting distinct proteins involved in mitosis (Chan *et al.*, 2012). Anti-microtubular drugs inhibit normal spindle formation, and chromosome alignment. Paclitaxel is an antitumor drug that has shown interesting results in several types of cancer including breast, lung, and ovarian cancer. In pancreatic cancer patients, paclitaxel increased the median progression-free survival from 3.7 months with Gemcitabine to 5.5 months with paclitaxel (Von Hoff *et al*., 2013). Its mechanism of action involves the polymerization of tubulin, and the reorganization of the cytoskeleton of the cells, blocking the cells in the G2/M phase. (Horwitz, 1994). Although these types of drugs are meant to target actively dividing cells, interphase cells can also be damaged, given the role of microtubules throughout the cell-cycle. For this reason, these drugs cause high myeloid toxicity and neurotoxicity (Jordan & Wilson, 2004).

The conclusions from the 2014 ASCO Gastrointestinal Cancers Symposium are that there is still an urgent need for new chemotherapy combinations. There are many more types of treatments that are currently being tested. Many of them have Gemcitabine as a backbone because of its efficacy and its wide range of usage. There are also many types of therapies in development, including immunotherapies, gene therapies, and inhibitors of different pathways. But until then patients will be waiting for a drug that gives them a higher survival rate and lower toxicity to cure this deadly disease.

### 1.2.1. Nucleoside Analogues: Characteristics and Mechanism of Action

Nucleosides are the essential building blocks in pathways vital for cellular metabolism, such as DNA and RNA synthesis, cell signaling, and enzyme regulation.

Nucleoside analogues (NAs) are synthetic compounds that mimic nucleosides and interfere with the cellular metabolism of their target. For this reason, these molecules are key components of therapeutic regimens in various indications (Jordheim *et al.*, 2013a). Nucleoside analogues consist of a nucleobase (purine or pyrimidine) linked to a sugar moiety. Nucleotide analogues have a phosphate group that is bound to the sugar ring at the C-5' position hydroxyl group (Jordheim *et al.*, 2013b).

Cytotoxic nucleoside analogues work as antimetabolites that inhibit and stop the synthesis of nucleic acids. Their mechanism of action is based on their incorporation into the nascent DNA or RNA strand, and/or the alteration of the pool of endogenous nucleosides (Galmarini *et al*., 2002).


**Figure 1. Mechanism of Action of Nucleoside Analogues.** Nucleoside analogues enter cells through nucleoside transporters. Once inside the cell, they get phosphorylated by dCK, and other kinases until they reach the triphosphorylated stage, which is their active form. Then they can inhibit RNR, or get incorporated into the DNA and stop DNA

synthesis. NA-C: Nucleoside Analogue-Cytidine; NA-U: Nucloside Analogue-Uridine; hENT: human Equilibrative Nucleoside Transporter; MRP: Multidrug Resistance Protein; CDA: Cytidine Deaminase; dCK: deoxycytidine kinase; NA-CMP: Nucleoside Analogue-Cytidine Monophosphate; NA-CDP: Nucleoside Analogue-Cytidine Diphosphate; NA-CTP: Nucleoside Analogue-Cytidine Triphosphate; NTP: Nucleoside Triphosphate; RNR: Ribonucleotide Reductase; dNTP: deoxynucleotide Triphosphate.

Nucleoside analogues are hydrophilic molecules that require an active transport using specific membrane transporters to enter the cells as seen in Figure 2 *(Galmarini et al*., 2002). Once inside the cells, the drugs are subsequently phosphorylated by a specific nucleoside kinase, deoxy cytidine kinase (dCK) (Huang *et al.*, 2003; Johansson & Eriksson, 1996; Sabini *et al.,* 2007; Saran & Chatterjee, 1980). Then, a nucleoside monophosphate kinase, and finally a nucleoside diphosphate kinase (Huang *et al*., 2003; Jordheim *et al*., 2013a) complete the enzymatic cascade leading to the formation of the active metabolite of the drug, the nucleoside triphosphate. As such, it can inhibit important intracellular enzymes like polymerases or ribonucleotide reductase, and be incorporated into newly synthesized DNA and RNA (Jordheim *et al.,* 2013a).

Gemcitabine is the current standard of care for non-resectable pancreatic cancer. This deoxycytidine analogue, requires as well a process of intracellular phosphorylation to become an active drug. In this case, the diphosphorylated and the triphosphorylated forms are both active metabolites (Heinemann *et al.*, 1988). Gemcitabine is commonly used in the treatment of various types of cancers (non-small-cell lung cancer, adenocarcinoma of the pancreas, breast cancer, ovarian cancer, etc.) (Cerqueira *et al.*, 2007). Gemcitabine became the standard of care for pancreatic cancer after it was shown to improve the median overall survival from 4.4 months with 5-FU to 5.6 months. Then, a phase 3 clinical trial showed that Gemcitabine median overall survival ranged from 5 to 7.2 months (Conroy *et*

*al.*, 2011b). However, the vast majority of pancreatic adenocarcinomas progress due to an acquired chemoresistance to this treatment (Nakano *et al*., 2007).

5-Fluorouracil (5-FU), a uracil analogue with a fluorine atom at the C-5 position of the nucleobase (Wohlhueter *et al.*, 1980), is commonly used to treat colorectal, pancreatic, breast and head and neck cancers (Galmarini *et al*., 2002). It is part of a new therapeutic regimen called FOLFIRINOX. This is a combinatory treatment that includes Oxaliplatin (platinum-based antineoplastic agent), Irinotecan (topoisomerase I inhibitor), Fluorouracil and Leucovorin (folic acid derivative) (Conroy *et al*., 2011a). Pre-clinical studies have shown that Irinotecan has synergistic activity in pancreatic cancer cells, when it is administered 24 hours before Fluorouracil and Leucovorin. It was shown that after 24 hours of treatment with Irinotecan, 57% of cancer cells had accumulated on the S phase, sensitizing them to Fluorouracil treatment (Azrak *et al.*, 2004; Ueno *et al*., 2007). Furthermore, Oxaliplatin and Fluorouracil have good synergistic effects (Mans *et al,*, 1999), as well as Oxaliplatin and Irinotecan (Ducreux *et al*., 2004). A French study between 2005 and 2009 that treated metastatic pancreatic cancer patients with FOLFIRINOX or Gemcitabine, found that FOLFIRINOX increased the overall survival of patients from 6.8 to 11.1 months. However, FOLFIRINOX was more toxic than Gemcitabine, causing grade 3 or 4 neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, sensory neuropathy, and grade 2 alopecia (Conroy *et al*., 2011a).

The mechanism of action of 5-Fluorouracil (5-FU) involves three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). When 5-FU enters the cell, it is directly converted to fluoridine monophosphate (FUMP) by orotate

phosphoribosyltransferase (OPRT), or indirectly by uridine phosphorylase (UP) and uridine kinase (UK). Then, FUMP is phosphorylated to FUDP, which can be further phosphorylated to become FUTP, or converted to FdUDP by ribonucleotide reductase. FdUDP can then be further phosphorylated or dephosphorylated to become FdUTP or FdUMP. Dihydropyrimidine dehydrogenase (DPD) is the rate limiting enzyme for deactivation of the drug, it deactivates 5-FU by converting up to 80% of it to dihydrofluorouracil (DHFU) (Longley *et al.*, 2003). As mentioned earlier, 5-FU is used in the treatment of various forms of cancer, but unfortunately it is not a curative treatment.

### 1.2.2. Common side effects and toxicity of anti-neoplastic treatments

Although novel antineoplastic agents developed in the last decade show improved potency and selectivity, they still exhibit significant side effects caused by off-target activity. To make matters worse, Gemcitabine, the first line of treatment for pancreatic cancer, is administered at very high doses (0.8-1.25g/$m^2$). It is given intravenously weekly as a 30-minute infusion for 3 weeks, followed by one week of rest. It causes numerous side effects, the main ones being: mild myelosuppresion, nausea and vomiting, influenza-like syndrome, and rash (Patel *et al.*, 2001). These side effects, while uncomfortable, are mostly reversible after termination of the treatment. However, a more concerning and irreversible side effect is cardiovascular toxicity. The latter constitutes a third of drug failure in late clinical trials, causing the withdrawal of drugs from development and/or from the market (Laverty *et al.*, 2011).

Our laboratory has developed extensive expertise in chemotherapy-induced cardiotoxicity over the years. We showed that at least two classes of chemotherapy, anthracyclines and tyrosine kinase inhibitors cause inhibition of a master regulator of

cardiomyocyte survival, GATA-4 (Aries *et al.,* 2004; Maharsy *et al.,* 2014). These studies and others that followed were important milestones and a reminder of the importance of early stage evaluation of cardiotoxicity in our quest for the development of a novel anticancer agent.

### 1.2.3. Recurrent Resistance Determinants to Nucleoside Analogues

The clinical use of nucleosides analogues is hampered by the development of resistance. Over time, tumors develop various mechanisms of resistance against nucleoside analogues. Understanding the molecular mechanisms underlying these resistances is crucial to overcome them and for the successful development of a new generation of nucleoside analogues.

#### 1.2.3.1. Deamination

Deaminases, and specifically cytidine deaminase (CDA) are the main hurdle nucleoside analogues have to face *in vivo*. CDA is mainly responsible for the poor bioavailability of nucleoside analogues such as Gemcitabine. CDA metabolizes deoxynucleoside analogues by converting them to the corresponding inactive uridine derivatives (Gilbert *et al.*, 2006). The negative effect of CDA in cancer treatment with NA was confirmed when cancer cells transfected with CDA became more resistant to Cytarabine than their parental cells (Boivin *et al.,* 2004). To date, two different polymorphisms have been described in the CDA gene that provide different sensitivities to Gemcitabine. The recombinant protein carrying the missense variant L27Q (79ANC) has a better affinity for Cytarabine and Gemcitabine (Gilbert *et al*., 2006). On the other hand, the threonine variant of A70T (208GNA) has a lower affinity for these nucleosides, which causes higher toxicities in patients treated with chemotherapy (Yue *et al*., 2003). As with

the nucleoside transporter gene, it was found that the mRNA expression and activity of CDA is negatively correlated, to the overall survival of patients with pancreatic adenocarcinoma (Bengala *et al*., 2005). Finally, Bardenheuer *et al.* took advantage of this knowledge and proved that CDA could be used for the benefit of cancer patients. They transferred a retroviral gene in hnuman hematopoietic cells to overexpress CDA, protecting them from the cytotoxicity of NA (Bardenheuer *et al*., 2005).

#### 1.2.3.2. Nucleoside transporter

Nucleoside transporters are the first proteins to interact with nucleosides at the cellular level. Therefore, they play an important role in the biological efficacy of nucleoside analogues. Various studies (Farrell *et al*., 2009; Hodge *et al.*, 2011; Kim *et al*., 2011; Mori *et al*., 2007; Perez-Torras *et al*., 2008) have found that the expression of human Equilibrative Nucleoside Transporter 1 (hENT-1) in the tumors significantly increases the life span of pancreatic cancer patients treated with Gemcitabine. Conversely, one of the mechanisms of resistance found in cells treated with Gemcitabine, is the depletion of hENT-1 expression. Quantitative RT-PCR results, where hENT-1 mRNA expression was measured, showed that its levels were conclusively linked with the clinical outcome (Giovannetti *et al.*, 2006). These results were further confirmed by a study in California, which evaluated the expression of hENT-1 using immunohistochemistry of 229 patient tumor samples. The results showed that Gemcitabine-treated patients with combined low and high hENT-1 levels had a longer overall survival than patients with no h-ENT-1 (Farrell *et al.*, 2009).

Besides changes in gene expression, other studies have unveiled site-specific mutagenesis and random mutagenesis of the hENT-1 gene in transformed yeasts. For

instance, a study by Visser *et al*. (2002) showed that the amino-acid at position 33 in hENT-1 and hENT-2 altered the sensitivity of both proteins to Dilazep and Dipyridamole (vasodilator drugs, and inhibitors of ENTs). Later on, it was discovered that the same amino-acid is involved in the transport of nucleosides (Visser *et al*., 2005). Thus, mutations of this gene could also be associated with the down-regulation of the protein's function (Jordheim & Dumontet, 2007).

#### 1.2.3.3. Ribonucleotide Reductase

The Ribonucleotide Reductase (RNR) enzyme is key to the regulation of the pool of deoxynucleotide diphosphate and triphosphates through a *de novo* pathway (Nordlund & Reichard, 2006). dNTPs are essential for DNA replication and DNA repair. RNR has been described as an alpha(6)beta(2) complex formed by 6 M1 subunits and 2 M2 subunits (α6β2), and can be activated or inhibited by the binding of ATP or dATP respectively. Rofougaran *et al.* found that at physiological levels, this is the major form of RNR, the other form been α2β2 (Rofougaran *et al.*, 2006). The M1 subunit has the allosteric regulation sites, while the M2 subunit has a binuclear iron cofactor and is responsible for the reductive activity (Duxbury *et al.*, 2004). Normal cells that have a low proliferation rate express low levels of RNR. However, neoplastic cells have a high proliferation rate, thus overexpress RNR in order to provide enough dNTPs for DNA synthesis. Nevertheless, M1 and M2 subunits are not overexpressed equally, with each acting differently in cancer (Zhou *et al*., 2013). For example, the overexpression of M2 subunit is involved in the transformation and tumorigenic potential by cooperating with activated oncogenes (Fan, Villegas, Huang, & Wright, 1998). Meanwhile, the overexpression of M1 subunit has the opposite effect: tumor-suppressing (Fan *et al.*, 1997).

The correlation between RNR and the effect of deoxynucleotide analogues has called the attention of many scientists. Various studies have been performed to investigate this relationship, most of them involving Gemcitabine (Jordheim & Dumontet, 2007). Using knockdown approaches with a lung adenocarcinoma cell line, it was demonstrated that the expression of the M1 subunit correlates with the sensitivity to Gemcitabine (Bepler *et al.*, 2006).

### 1.3. Development of new anticancer drugs: Gemcitabine derivatives

Nucleoside analogues have been used to treat viral infections and various types of cancers for a long time. Nevertheless, nucleoside analogues have not been entirely curative for cancer patients. Consequently there is a medical need to improve the potency of the currently used anti-neoplastic drugs that belong to this family of molecules (Jordheim *et al.*, 2013a).

As mentioned earlier, Gemcitabine is clinically used for a wide range of solid tumors. However, the biggest issue with this drug is the development of resistance over time. This problem was found to be attenuated using chemical modifications that usually give the molecule a more sustained effect (Moysan *et al.*, 2013).

Polyethylene glycol (PEG) is a polyether compound that is used in many areas from industrial manufacturing to medicine. PEG conjugation to Gemcitabine and other nucleoside analogues is one of the approaches used to overcome some of the resistance determinants (Pasut & Veronese, 2012). PEGylation is considered one the most successful techniques to prolong the plasma half-life of the drug, prevent degradation by cytidine deaminase, increase the water solubility, increase accumulation in tumor zones thanks to

the “enhanced permeability and retention” effect (EPR) (Caliceti & Veronese, 2003) and sometimes even increase the cytotoxicity in cancer cells (Harris & Chess, 2003). The addition of PEG on Gemcitabine has been tested by various research teams. In one study, PEG-Gemcitabine was synthesized by conjugating the amino groups at the 4-(N)-position of Gemcitabine to N-hydroxysuccinimide derivative of PEG. As studied in an animal model, PEG-Gemcitabine showed a prolonged circulation time in plasma (Vandana & Sahoo, 2010). Confocal analysis of cells treated with PEG-Gemcitabine have shown their co-localization in the lysosome and endosome after 24 hours incubation, and their retention in cancer cells after 3 days of incubation (Chuang *et al.*, 2010). PEG-drugs are absorbed by the cells through endocytosis and they are retained in transport vesicles in the endolysosomal scaffold. Then, the acidic nature of the endolysosomal transport vesicles allows the cleavage of the amide bonds between PEG and Gemcitabine, prolonging its release (Chuang *et al*., 2010). However, a drug encapsulated or solubilized with pegylated agents can activate the complement system, which is a major contributor to infusion reactions, or complement-activation related pseudoallergy (CARPA) (Szebeni, 2005). For this reason, although this technique provides the drug with some advantages, the risk of getting an immune reaction makes this a risky approach.

The addition of Valproic Acid to Gemcitabine via an amide bond at 4(N)-position, led to the molecule LY2334737. This prodrug was developed to help Gemcitabine bypass the hydrolysis stage in enterocytes, and showed an increased half-life compared to Gemcitabine. LY2334737 is an oral drug that resists the passage through the gastrointestinal tract, with a 21% degradation at pH=1 (Bender *et al.*, 2009).

The addition of a lipophilic group is another approach that has been tested in order to improve the stability and cytotoxic activity of Gemcitabine (Moysan *et al.*, 2013). Co-encapsulation of Gemcitabine with other anticancer drugs has also been promoted to obtain a synergistic effect. Besides improving a drug's cytotoxic activity, encapsulation also extends their time of circulation in the blood through the addition of PEG on the surface (Cattel *et al.*, 2003). However, despite all these benefits, this new version of Gemcitabine with a lipophilic moiety has not shown better antitumor activities than Gemcitabine (Sloat *et al*., 2011).

### 1.4. Nucleoside Analogue Chemistry:

#### 1.4.1. Synthesis and Conformational Aspects

Anticancer nucleoside analogues' structural variety depend on the modifications made to either the nucleobase, the sugar moiety, or the use of a prodrug. These modifications include halogenation, saturation, methylation, and others (Jordheim *et al.*, 2013b).

According to Vorbruggen and Ruh-Pohlenz in their Handbook of Nucleoside Synthesis, there are three methods that can be used to synthesize nucleosides: the fusion reaction, the metal salt procedure, and the Hilbert-Johnson reaction. In all three methods, the cyclic substrate (sugar) reacts in different ways with the nucleobase to form nucleosides (p.4-9). A novel methodology was developed in Dr. Guindon's Lab at IRCM in Montreal, which uses an acyclic precursor that already contains the nucleobase. This new approach introduces silylated nucleobases diastereoselectively onto acyclic substrates that are subsequently cyclized. This strategy gives access to either D or L nucleosides with *trans*

or *cis* stereochemistry. One of the advantages of this strategy comes from the use of a wider pool of starting material, hence a wider scope of molecular diversity. This method represents an important advance in the synthesis of novel nucleoside drugs (Chapdelaine *et al.,* 2009; Tambutet *et al*., 2014). This group has also provided an efficient and selective way to introduce all-carbon stereogenic quaternary centers into nucleoside analogues (Duplessis *et al.*, 2009).

Guindon's lab is in the process of developing a new generation of nucleoside analogues that could overcome the recurrent resistance determinants of cancer cells. The hypothesis of this project is that these new proprietary prodrugs having a lipid moiety attached to the nucleobase, could enter the cancer cells through passive diffusion, without the need of nucleoside transporters. This molecule should thus bypass a first mechanism of resistance while protecting the molecule against CDA induced degradation. These nucleoside analogues would then have a higher efficiency at killing cancer cells. Hopefully these new compounds will be able to help cure the millions of people that are victims of cancer every year.

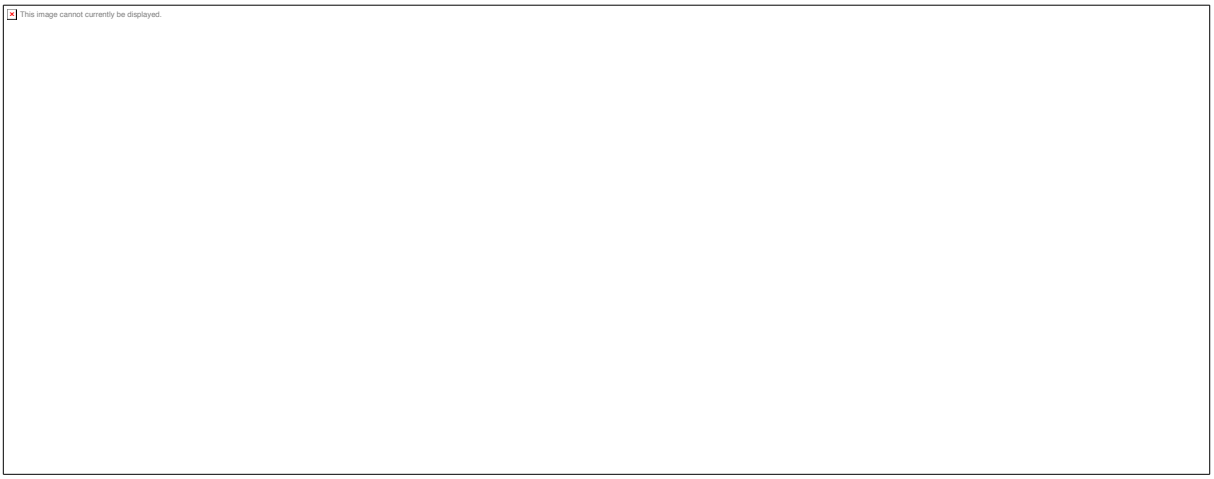



**Figure 2. Hypothetical mechanism of action of the novel nucleoside analogues synthesized in the Guindon Lab.** The nucleoside analogue would enter the cell without

the need of a nucleoside transporter, thanks to the lipophilic group. Then, the compound could have a direct effect on the cells, or get phosphorylated and stop DNA replication, or get cleaved, phosphorylated and stop DNA replication, all of them leading to cell death.

## 2. Materials and Methods

### 2.1. Cell culture

BxPC3, Capan-2 and HepG2 cell lines were obtained from ATCC. BxPC3 cell line comes from a 61 years old woman with pancreatic adenocarcinoma. These are epithelial adherent cells, that are suitable for transfection. Furthermore, they express pancreas cancer specific antigen, and develop tumors in 21 days in nude mice (Tan *et al*., 1986). Capan-2 is another pancreatic cancer cell line. It comes from a 56 years old Caucasian male with pancreatic adenocarcinoma. These are polygonal adherent cells, with a karyotype of 69 chromosomes. It expresses mucin, and has a K-Ras mutation, present in 90% of pancreatic cancer tumors. Finally, this cell line is tumorigenic in nude mice (Dahiya *et al*., 1993). On the other hand, HepG2 is a widely used human liver cancer cell line. These cancer cells belonged to a 15 years old Caucasian adolescent male. They are epithelial adherent cells, that express insulin-like growth factor II, but they are not tumorigenic. Furthermore, HBV have not been detected in their genome (Morris et al., 1983).

These cell lines were cultured in 150x25 mm culture dishes. HepG2 was cultured in Eagle's Minimum Essential Medium (ATCC); Capan-2 was cultured in McCoy's 5A Medium (ATCC); and BxPC3 was cultured in RPMI 1640 (ATCC). All these media were supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The cells were kept in a humidified chamber at 37˚C with 5% $CO_2$. Once a culture dish reached 90% confluence, the cells were washed with 1X PBS and detached from the bottom of the plate with 0.05% trypsin. A fraction of the total cells would then be passed to a new culture dish with new culture medium. For HepG2 and BxPC3 cells, 25% of the cells were passed to a

new culture dish with new media. For Capan-2 cells, 33% of cells were passed to a new culture dish with new media.

### 2.2. Dilution of the Drugs

The different drugs tested in this project were synthesized in Dr. Yvan Guindon's Laboratory at IRCM and they were sent to Dr. Nemer's Laboratory at Roger Guindon Hall. Once the drugs were received, they were diluted in DMSO (as vehicle) to a concentration of 50 mM and stored at 4˚C.

### 2.3. Growth Inhibition Determination

Cells (10,000 per well) were plated in 96 well plates. After 24 hours of incubation, the cells were treated in triplicates with increasing concentrations of the drugs. As a control, cells were treated with vehicle (DMSO) using their equivalent concentration (4.9mM was the maximum concentration used). The plates were then incubated for 96 hours in a humidified chamber at 37˚C with 5% $CO_2$. After the incubation time, the plates were analyzed using the Cell TiterGlo Assay (Promega, WI), a cell viability assay that detects living cells by the quantitation of ATP present. The 96 well plate is first incubated at room temperature for 30 minutes, and then 200 µL of the Cell TiterGlo reagent is added to each well. This lyses the cells and generates a luminescent signal proportional to the amount of ATP. The plates were put for 10 minutes in the rotator for the reagent to mix well. Finally, plates are read in the luminometer and the GLOMAX software was used to estimate the number of cells present in each well. These numbers were put in an Excel table in which the average of cells present under each concentration was calculated. These averages were then compared to the results from the respective DMSO concentrations and the growth

inhibition was calculated. The graphs were then created using the inhibitor logarithm equation with four parameters on the program GraphPad.

### 2.4. Selectivity Index Determination

Splenocytes were extracted from a wild type mouse spleen using mechanical dissociation followed by Red Blood Cell (RBC) lysis and incubated in DMEM with 10% deactivated serum. RBC lysis consists on incubating the cells with a solution of ammonium chloride, potassium carbonate, and EDTA; after centrifugation, the splenocytes should be separated from the red blood cells. Splenocytes were then plated in a 24 well plate and treated with increasing concentrations of LCB2151, or vehicle for 24 hours in a humidified chamber at 37˚C with 5% $CO_2$. Then the cells were stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit. Samples were then run in the Flow Cytometer and results were analyzed using Kaluza software.

### 2.5. Combination Treatments

Cells were plated in 96 well plates according to the plating methodology described in section 2.3. After 24 hours of incubation, the cells were co-treated in triplicates with increasing concentrations of Gemcitabine and a fixed concentration of LCB2151. As a control, cells were treated with vehicle (DMSO) using the same concentrations as the drugs. The plates were then incubated for 96 hours at a humidified chamber at 37˚C with 5% $CO_2$. After the incubation time, the plates were analyzed using Cell TiterGlo Assay (Promega, WI).

### 2.6. Consecutive Treatments

Cells were plated in 96 well plates according to the plating methodology described in section 2.3. After 24 hours of incubation, the cells were treated in triplicates with

increasing concentrations of Gemcitabine. As a control, cells were treated with vehicle (DMSO) using the same concentrations as for the drugs. The plates were then incubated for 72 or 96 hours at a humidified chamber at 37˚C with 5% $CO_2$. After these incubation times, the media were changed and the remaining gemcitabine resistant cells were treated with 15 μM LCB2151, 200 nM Gemcitabine or the respective vehicles for 48 or 24 hours. The plates were analyzed using Cell TiterGlo Assay (Promega, WI).

### 2.7. Quantitative RT-PCR

Cells were plated in 10 cm plates according to the plating methodology described in section 2.3. In this case, 1 million cells were plated in 10cm plates. Twenty-four hours after plating, the cells were treated with the concentration that corresponds to the $IC_{50}$ of the drugs. Triplicates were done for each concentration of the drug (3x 10 cm plates), and all of the treatments were done for 3 incubation time points: 3, 6 and 12 hours. After each incubation time, the plates were taken out of the incubator and the RNA was extracted with Trizol (Invitrogen). Once the RNA is quantified, cDNA was synthesized using QuantiTect Reverse Transcription kit (QIAGEN). RT-PCR was performed using QIAGEN Multiplex PCR Kit and quantified in Corbett Research PCR Rotorgene 6000. All experiments were performed in triplicate. The mRNA levels pro and anti-apoptotic genes were analyzed in two different experiments with three replicates each. The other qPCR experiments were performed once with three triplicates. RNPS1 gene was used as an endogenous control, and results were expressed as fold change relative to cells treated with vehicle. The conditions of the qPCR are the following: Hold Temperature: 95 °C for 5 min., and 35 cycles of 95 °C for 5 seconds and 60 °C for 10 seconds.

| Genes | | Primers |
|---|---|---|
| ABCC1 | S | ACTTCGTTCTCAGGCACATC |
| | AS | TGATCCGAAATAAGCCCAGG |
| ABCC2 | S | TCATCGTCATTCCTCTTGGC |
| | AS | ACGGATAACTGGCAAACCTG |
| SCL29A1 | S | CCACTCTATCAAAGCCATCCTG |
| | AS | ATGAAGTAACGTTCCCAGGTG |
| P21 | S | GTCCAATCCTGGTGATGTCCGAC |
| | AS | GCGTCTCCGTGACGAAGTCAAAG |
| P53 | S | GCCATCTACAAGCAGTCACAG |
| | AS | TCATCCAAATACTCCACACGC |
| BIK | S | CTGGGTCTGGCTTTCATCTAC |
| | AS | CTGTTCGCAGGACACCC |
| BAX | S | GACATGTTTTCTGACGGCAAC |
| | AS | AAGTCCAATGTCCAGCCC |
| BBC3 | S | CGACCTCAACGCACAGTAC |
| | AS | CCTAATTGGGCTCCATCTCG |
| DIABLO | S | TTGGTCTTTCAGAGATGGCAG |
| | AS | GTGATTCCTGGCGGTTATAGAG |
| ENDOG | S | CCCCACCTCAACCAGAATG |
| | AS | ATTTCCCATCAGCCTCTGTC |
| MCL1 | S | AAGGACAAAACGGGACTGG |
| | AS | ATATGCCAAACCAGCTCCTAC |
| XIAP | S | GCACGGATCTTTACTTTTGGG |
| | AS | GGGTCTTCACTGGGCTTC |
| BCL-XL | S | GTGGAAAGCGTAGACAAGGAG |
| | AS | CTGCATTGTTCCCATAGAGTTC |
| BCL-2 | S | TTGTGGCCTTCTTTGAGTTCGGTG |
| | AS | GGTGCCGGTTCAGGT ACTCAGTCA |
| P67 | S | GCATCAACAGAGACAAGCACTC |
| | AS | CAATACCTCACAGGCAAACAGC |
| SOD1 | S | ACAAAGATGGTGTGGCCGAT |
| | AS | AACGACTTCCAGCGTTTCCT |
| RNPS1 | S | ACCCATGGTAGTTGCTGCTC |
| | AS | AGCTGGCTCTCCACTCACTC |

**Table 2. List of Primers used.**

### 2.8. Statistical Analysis of qPCR results.

qPCR results from cancer cells treated with the different drugs were compared to the results from the cancer cells treated with the vehicle (DMSO) as control. We used Student's *t*-test to determine the statistical significance of the mRNA levels detected from a drug treatment with respect to its control. A result was considered significant when *p* was less than 0.05.

### 2.9. Whole Cell Protein Extraction

Cells were plated in 6 well plates (500,000 cells per well) according to the plating methodology described in section 2.3. After 24 hours of plating, the cells were treated with different concentrations of the drugs tested. Cells were then incubated for different amounts of times: 3, 6, 12, and 24 hours. After each time point, the medium was removed from the plates and the wells were rinsed with 1 mL of PBS at room temperature. From this point, all of the steps were done on ice. 600 µL of 5X RIPA buffer (250 mM Tris pH 7.5; 750 mM NaCl; 5% NP-40; 2.5% desoxycholate; 0.5% SDS; $ddH_2O$) was added to each well and the cells were scraped and passed through a syringe fitted with a 21-gauge needle and transferred to an Eppendorf tube. 0.5 mM PMSF, 1 mM Apr and 1 mM NaOV were added to each sample and they were incubated for 30 minutes on ice. Finally the samples were centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant containing the total proteins was transferred to another microcentrifuge tube and stored at -20°C. The Thermo Scientific Pierce BCA Protein Assay was used to quantify the amount of protein extracted.

### 2.10. Western Blot

Whole cell protein extracts (30 µg) were loaded onto 15% acrylamide gels and resolved by electrophoresis. Proteins were then transferred to nitrocellulose membrane.

The membrane was blocked with 5% milk blocking buffer (5% Bovine Serum Albumin [BSA] blocking buffer was used for p53 detection). Antibody incubation for Bax (Abcam, ab7977; 1:500), Bbc3 (Abcam, ab33906; 1:500), Bcl-2 (Cell signaling, 2876S; 1:500), Bcl-X (Cell signaling, 2764S; 1:500), and GAPDH (Abcam, ab8245; 1:500) was done in 1% milk blocking buffer and 1% BSA blocking buffer for p53 (Cell signaling, 2525S; 1:500) detection.

### 2.11. Cell Death Determination

Cells were plated in two 96 well plates according to the plating methodology described earlier. After 24 hours of incubation, the cells were treated in triplicates with increasing concentrations of LCB2151. Half of the first plate was treated with only LCB2151, the other half was treated with LCB2151 in combination with 25nM of caspase 3 inhibitor DEVD (Calbiochem, 235423), and half of the second plate was treated with LCB2151 in combination with 400nM of RIPK-1 inhibitor Necrostatin-1 (Abcam, ab141053). As a control, cells were treated with vehicle (DMSO) using the same concentrations as the drugs. The cells were then incubated for 24 hours in a humidified chamber at 37˚C with 5% $CO_2$. The cells were analyzed using Cell TiterGlo Assay (Promega, WI).

### 2.12. Immunofluorescence

Cells (50,000 per well) were plated in 8-well slides. After 24 hours, the cells were treated in duplicate with 8 μM LCB2151, 1 μM Gemcitabine, and vehicle. Twelve hours later, cells were washed and fixed with 4% PFA, then permeabilized with 0.2% Triton X-100, and blocked with filtered 5% BSA. Antibody incubation with anti-cyclophilin D (Calbiochem, AP1035) and anti-LC3 (Cell Signaling, 2775S) was done in 1% BSA. Alexa

fluor 488 (Z-25302) and Alexa fluor 546 (Z-25004) were the dyes on the secondary antibodies used in 1% BSA. Finally, 100 µL of ProLong® Gold Antifade Mounting solution with DAPI was added to each slide and covered with a cover-slip. The next day, images of the stained cells were taken using the Zeiss AxioObserver.D1 (Inverted), and Axiovision software.

### 2.13. Cell Entry Determination

Cells were plated in three 96 well plates. After 24 hours of incubation, cells were treated in triplicate with increasing concentrations of LCB2151, LCB2132, or Gemcitabine. Half of the first plate was treated only with LCB2151, the other half was treated with LCB2151 in combination with 10µM of human equilibrative nucleoside transporter inhibitor NBMPR (Sigma-Aldrich, N2255), and the same procedure for the other drugs, on the other two plates. As a control, cells were treated with vehicle (DMSO) using the same concentrations as the drugs. The plates were then incubated for 96 hours in a humidified chamber at 37˚C with 5% $CO_2$. The plates were analyzed using Cell TiterGlo Assay (Promega, WI).

### 2.14. Detection of Metabolites through High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS).

HepG2 cells were plated in 10 cm plates. After 24 hours of incubation, cells were treated in triplicates at the corresponding $EC_{50}$ concentration of LCB2151 (8 µM), lipoate (8 µM and 40 µM), or the parent nucleoside of LCB2151, LCB1180 (8 µM). After four different time-points (3, 6, 12, and 24 hours), cells were washed with 1X PBS and lysates were prepared and ran as previously described by (Stuart *et al.*, 2014). Supernatants and

pellets were then sent to the IRCM in Montreal, to be run by the Proteomics Discovery Platform using HPLC techniques.

The cell pellets were resuspended in 500 µL of cold 60% methanol and incubated overnight at -20 ºC. The samples were centrifuged at 14 000 rpm for 5 min, and the supernatants were collected and dried using a SpeedVac concentrator, then stored at 4ºC for 24 hours before to be analyzed by LC/MS.

### 2.14.1. LC-SRM assay development

The optimization of the assay was performed for each drug by identifying the 3 best (most intense signals) transitions (i.e. a pair of parent and fragment ions specific to a drug), the optimal collision energy (CE) and the retention time for each drug.

### 2.14.2. LC-SRM-MS analysis (SRM: Selected Reaction Monitoring)

Cell extracts were suspended in 50 µL of a buffer containing 95% water, 5% ACN, 0.01% formic acid, 10 mM ammonium acetate and two internal standards (peptides TGGFL and TGGFM). Sixteen µL aliquots were injected into the LC/MS instrument. Drugs were separated on a Luna C18 HPLC column (150 mm x 2 mm, 3 µm particule size) from Phenomenex, (Torrance, CA). This column was installed on the Accela 1250 HPLC system and coupled to the TSQ Vantage (ThermoFisher Scientific, Bremen, Germany). The buffers used for chromatography were 95% water / 5%ACN / 0.01% FA / 10 mM ammonium acetate (buffer A) and 95% ACN / 5% water / 0.01% FA / 10 mM ammonium acetate (buffer B). Extracts were loaded on-column and eluted with a single slope gradient at a flow rate of 500 µL/min. Solvent B increased from 16.6 to 100% in 12 min, and the column was reconditioned in 16.6% B for 7 minutes. The TSQ Vantage was operated in

SRM mode with collision gas pressure set at 1.2 m Torr, Q1 and Q3 Peak width (FWHM) at 0.70 and a 0.800 second cycle time. The mass spectrometer was interfaced with an Ion Max source with a heated electrospray ionization probe (Thermo Fisher Scientific). The capillary temperature was set to 360ºC, vaporizer temperature to 380ºC and spray voltage at -3000 V in negative polarity mode.

### 2.14.3. Statistical analyses

Raw mass spectrometric data was imported into and analyzed with Pinpoint 1.3.0 (Thermo Fisher Scientific). The instrument performance and reproducibility were monitored using the internal standards. Drug abundances in each sample was calculated using the external standard technique.

## 3. Results

### 3.1. Antiproliferative Efficacy of New Proprietary Nucleoside Analogues

Nucleoside analogues (150) synthesized in the Guindon Lab were screened in order to assess their anti-neoplastic effects. These compounds were tested in three different human cancer cell lines: BxPC3, Capan-2, and HepG2.

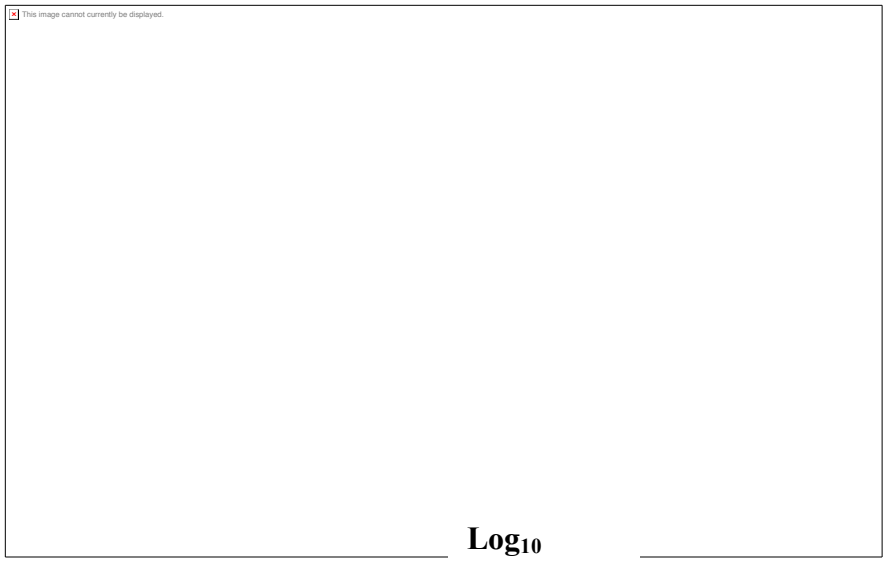
The growth inhibitory effect of the compounds was measured using Cell TiterGlo Assay (Promega, WI) and their dose-response curves were established. LCB2151 and LCB2132 showed the highest anti-neoplastic potency, causing 99% growth inhibition in all three cell lines after 4 days of incubation (Fig. 3A and 3B). Their $EC_{50}$, or the concentration at which they were 50% effective, was 8 μM for LCB2151, and 12.5 μM for LCB2132. These two molecules contain the same lipoeitic moiety, and the same nucleobase, but differ in their carbohydrate part.

To determine their mechanism of action, the most potent molecule was chosen, LCB2151 (Fig. 3C).

In parallel, the effect of Gemcitabine, a nucleoside analogue clinically used against several solid tumors (Galmarini *et al*., 2002), was tested as a positive control. Shown in Fig. 3D, BxPC3 was the most sensitive cell line, while Capan-2 was the most resistant cell line, with only 30% growth inhibition after 96 hours of incubation. This result contrasts those reported by Tang *et al*. (2011), where Capan-2 was found to have an intermediate resistance to Gemcitabine, getting up to 80% growth inhibition at 1 μM.

These results show the potency of LCB2151 and LCB2132 at inhibiting the growth of gastrointestinal cancer cells.

**A.**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**B.**


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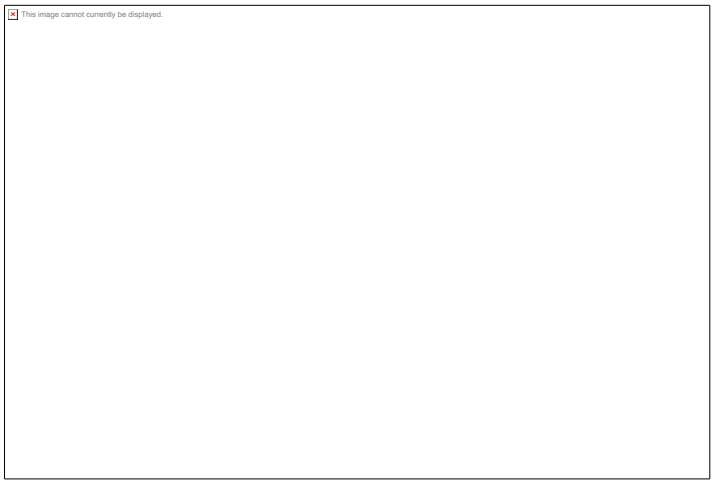
**C.**

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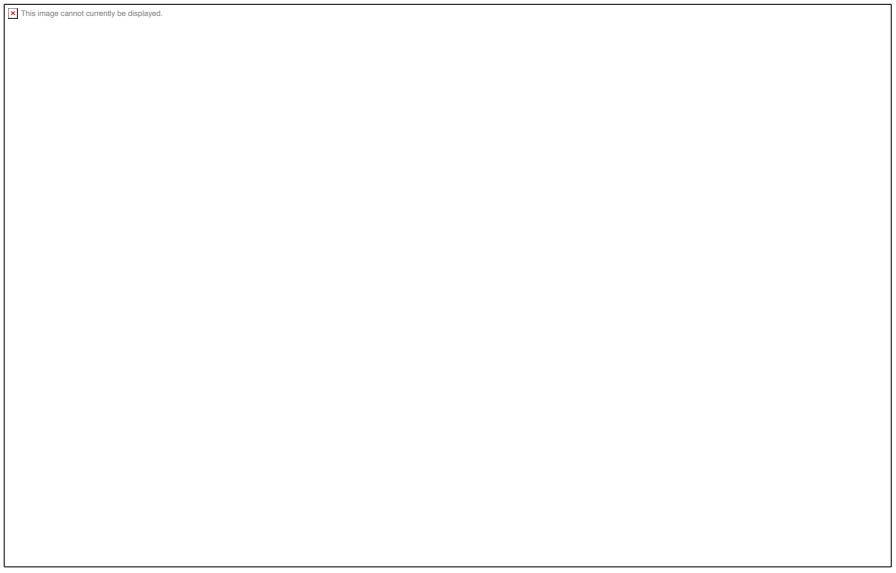



**Figure 3. Screening Study. A.** Percentage of Growth Inhibition of LCB2151 in three human cancer cell lines: BxPC3, Capan-2, and HepG2. **B.** Percentage of Growth Inhibition of LCB2132 in the same cell lines. **C.** $EC_{50}$ and $IC_{50}$ values of LCB2151 and LCB2132 on the three cell lines used. **D.** Percentage of Growth Inhibition of Gemcitabine in the three human cancer cell lines used. This figure shows the potency of LCB2151 and LCB2132, where the two molecules reach 99% growth inhibition in the three mentioned cell lines.

### 3.2. Effect of LCB2151 on non-tumor cells.

Given the high efficacy at inhibiting the growth of hepatocellular carcinoma and pancreatic adenocarcinoma cells, it was important to determine if LCB2151 is toxic to non-cancerous cells. Splenocytes were chosen given the importance of immune cells in cancer treatment. Since the nineteenth century, scientists have studied the role of immune cells to provide some protection from cancer. Immune cells can promote the destruction of cancer cells identifying certain characteristics on the cell membrane of cancer cells. Nevertheless, immune cells can also trigger the growth and development of tumors by producing certain cytokines and promoting inflammation (Alderton & Bordon, 2012).

For this experiment, splenocytes were extracted from a spleen of a wild type mouse and treated with high concentrations of LCB2151 for 24 hours. The results show almost no cytotoxic effect on splenocytes even at high concentrations of the new drug (Fig. 4). When splenocytes were treated with 30 times the concentration required to inhibit 99% of cancer cells, there were only 10% dead cells.

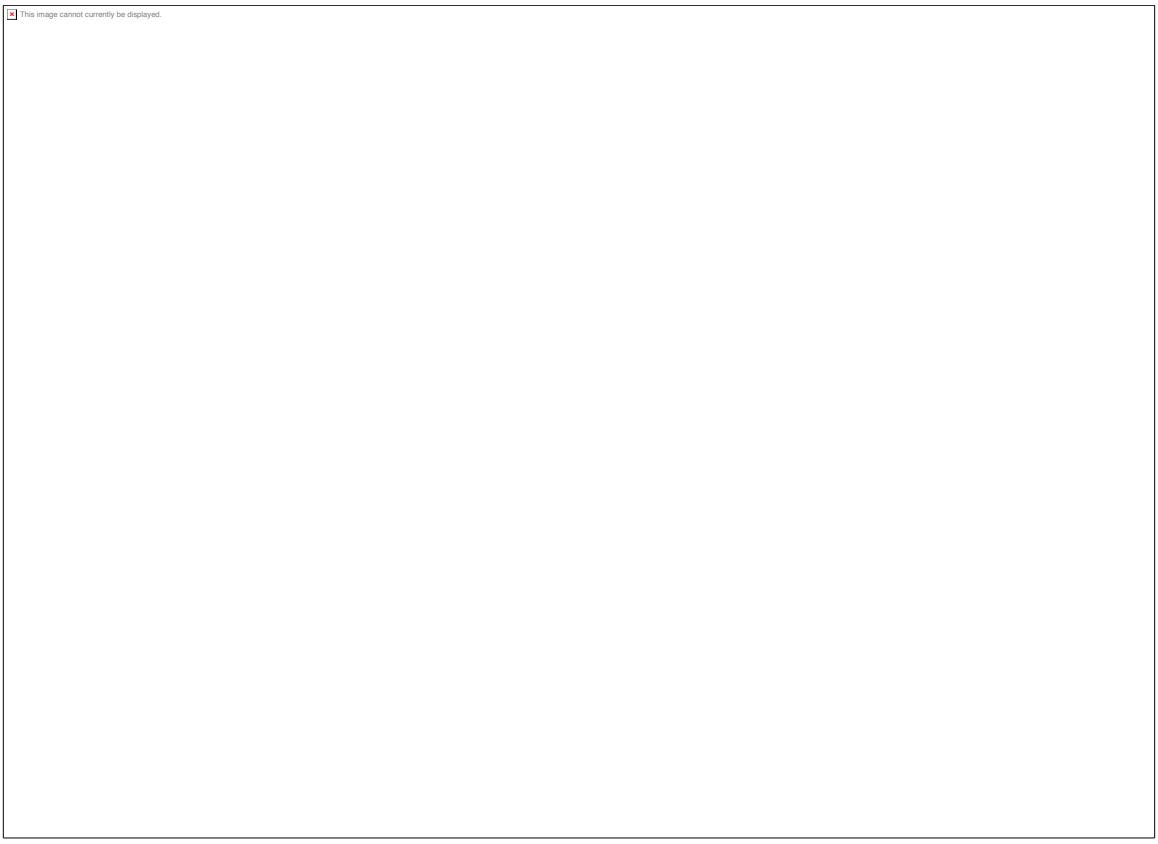



**Figure 4. Selectivity Index Determination.** Percentage of live and dying primary splenocytes treated with increasing concentrations of LCB2151 and the amount of vehicle that corresponds to the amount of drug added at each concentration. This experiment was performed once (n=1) with 4 repetitions. These results show that LCB2151 is not toxic on primary splenocytes.

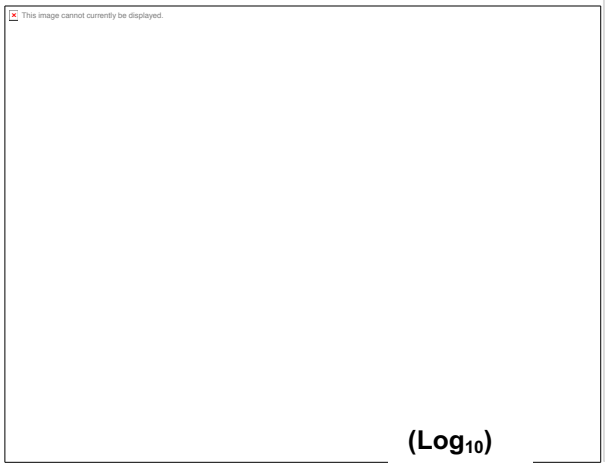
### 3.3. LCB2151 can kill Gemcitabine resistant cells.

In this study, HepG2 and Capan-2 were proven to be resistant to Gemcitabine's treatment (Fig. 5D). Capan-2 showed the highest resistance profile. This cell line was extracted from a pancreatic adenocarcinoma patient of 56 years old. The tumor was located in the head of the pancreas and infiltrated the muscularis of the duodenal wall distal to the ampula and the peripancreatic fibroadipose tissue posteroinferiorly (Kyriazis *et al.*, 1986). This cell line has been shown to have a high doubling time (Kyriazis *et al*., 1986) which interferes with Gemcitabine's effect that depends on the cells being in the S phase of the cell cycle (Nakano *et al.*, 2007). Furthermore, Capan-2 cell line carries a K-RAS mutation, very common in almost all primary tumors of pancreatic cancer (Deer *et al*., 2010). This mutation leads to the activation of signaling pathways that promote "cell proliferation, metabolic reprograming, anti-apoptosis, remodeling of the tumor microenvironment, evasion of the immune response, cell migration and metastasis" (Eser *et al.*, 2014). These characteristics make Capan-2 a good cell line to test the efficacy of new molecules.

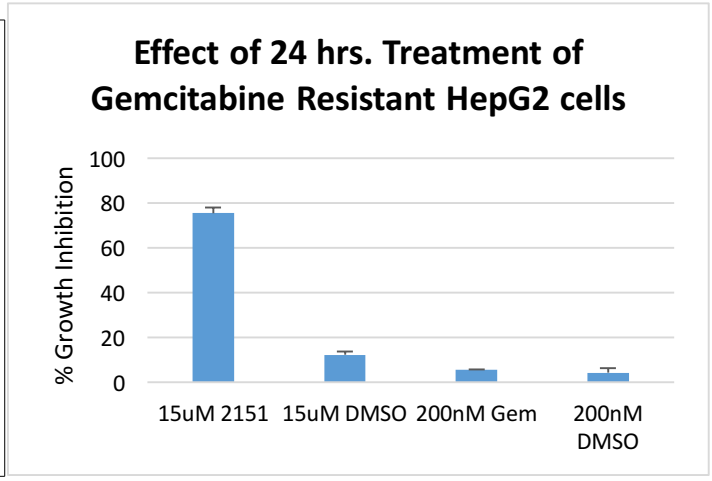
Consecutive treatments were carried out in order to determine if LCB2151 could kill resistant cells that survive Gemcitabine treatment. HepG2 and Capan-2 cells were treated with Gemcitabine for 72 and 96 hours; surviving cells were subsequently treated with 15μM of LCB2151, 200nM Gemcitabine, or their respective vehicles. The results show that LCB2151 is able to induce death in Gemcitabine resistant cells (Figs. 3 and 4). HepG2 cells treated with Gemcitabine for 96 hours show a growth inhibition of up to 60%, the remaining 40% (gemcitabine resistant cells) treated with LCB2151 for 24 hours show a growth inhibition of more than 70%, having an overall of 88% of cancer cells killed; while the re-administration of Gemcitabine had no effect (Fig. 5A and 5B). Furthermore,

when cells were treated with Gemcitabine for 72 hours only 30% of cells were killed; then with LCB2151 treatment for 48 hours, more than 95% of the resistant population was killed (Fig. 5C and 5D). The results of the same experiments performed in Capan-2 confirmed that LCB2151 is able to kill Gemcitabine resistant cells, though to a lower degree. When Capan-2 cells were treated with Gemcitabine for 96 hours, 20% of cells were killed, then treatment with LCB2151 for 24 hours killed 55% of the 80% of cells left, having an overall of 64% of cells killed. On the other hand, 72 hours treatment with Gemcitabine killed 20% of Capan-2 cells, and 48 hours treatment with LCB2151 killed 60% of the 80% of cells left, getting an overall of 68% of cancer cells killed. In both experiments, further treatment with Gemcitabine was not effective at killing the remaining cancer cells (Figs. 6A, 6B, 6C and 6D). In any case, LCB2151 is able to evade the mechanisms of resistance developed by the cancer cells against Gemcitabine. These results show how LCB2151 has a greater efficacy than Gemcitabine at killing highly resistant pancreatic cancer cells.

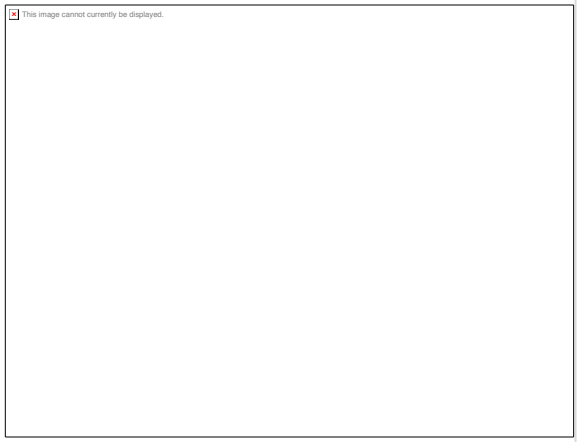
A.




B.




C.



D.

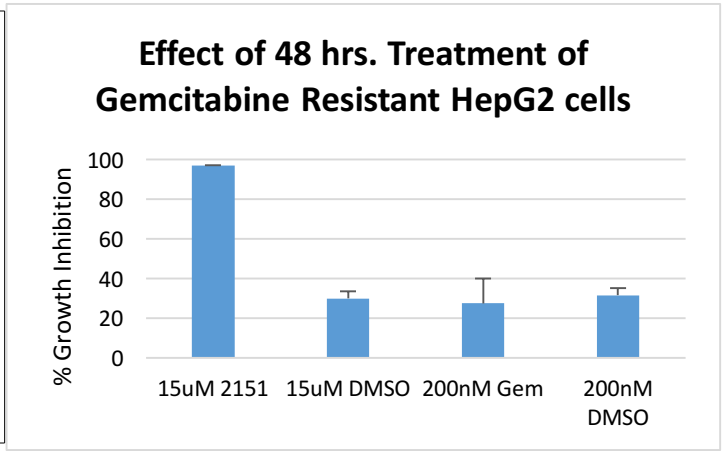



**Figure 5. Consecutive Treatments in HepG2 cells. A.** Percentage of growth inhibition of HepG2 cells treated with increasing concentrations of Gemcitabine for 96 hours. **B.** Percentage of growth inhibition of the remaining HepG2 cells from Fig. 5.A treated with 15 µM 2151, 200 nM Gemcitabine, or vehicle for 24 hours. **C.** Percentage of growth inhibition of HepG2 cells treated with increasing concentrations of Gemcitabine for 72 hours. **D.** Percentage of growth inhibition of the remaining HepG2 cells from Fig. 5.C treated with 15 µM 2151, 200 nM Gemcitabine, or vehicle for 48 hours. LCB2151 is able to kill 95% of gemcitabine resistant cells after 48 hour treatment.

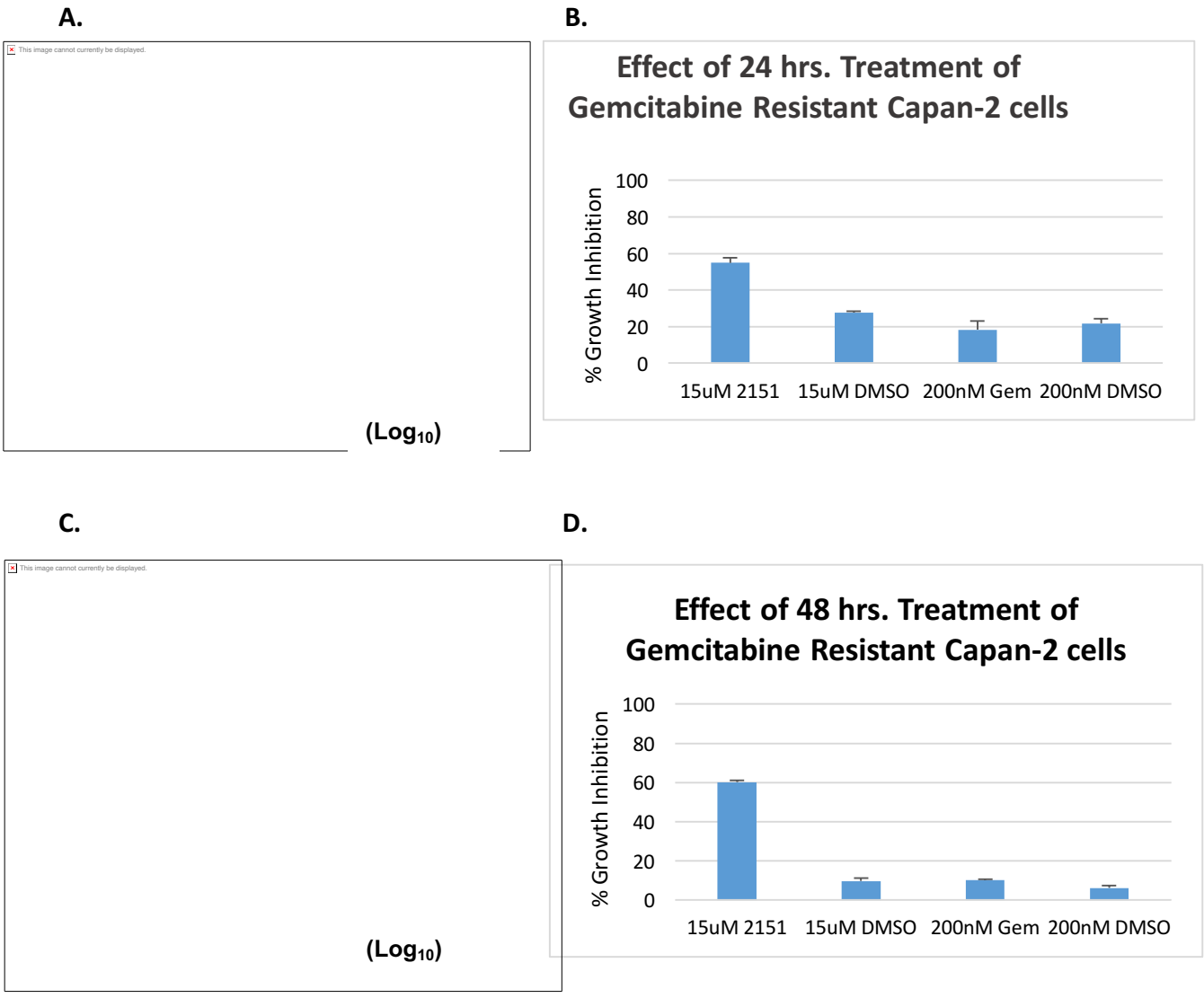


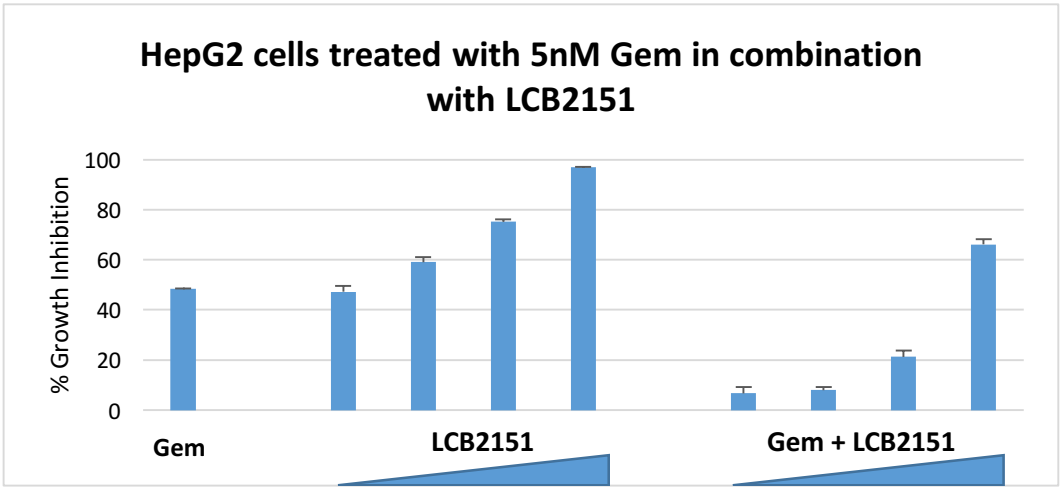

**Figure 6. Consecutive Treatments in Capan-2 cells. A.** Percentage of growth inhibition of Capan-2 cells treated with increasing concentrations of Gemcitabine for 96 hours. **B.** Percentage of growth inhibition of the remaining Capan-2 cells from Fig. 6.A treated with 15 µM 2151, 200 nM Gemcitabine, or vehicle for 24 hours. **C.** Percentage of growth inhibition of Capan-2 cells treated with increasing concentrations of Gemcitabine for 72 hours. **D.** Percentage of growth inhibition of the remaining Capan-2 cells from Fig. 6.C treated with 15 µM 2151, 200 nM Gemcitabine, or vehicle for 48 hours. LCB2151 can kill up to 60% of gemcitabine resistant cells after 48 hour treatment.

### 3.4. Effect of LCB2151 on Gemcitabine's action.

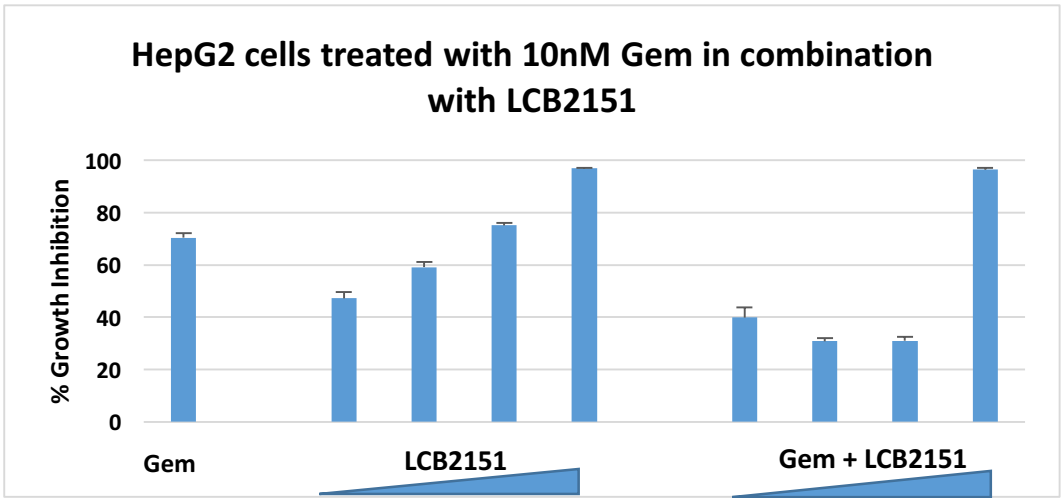
The previous results suggest that LCB2151 and Gemcitabine have distinct mechanisms of action. We tested whether Gemcitabine and LCB2151 could synergize in combinatory treatments. HepG2 and Capan-2 cells were treated with combinations of varying concentrations of Gemcitabine and LCB2151. The results show an inhibitory effect. When HepG2 cells were treated with 5 nM Gemcitabine (Fig. 7A), there was a 50% growth inhibition. On the other hand, when cells were treated with 5 μM of 2151, there was also about 50% growth inhibition. Surprisingly, when HepG2 cells were treated with 5 nM of Gemcitabine in combination with 5 μM of 2151, growth inhibition goes down to less than 10%. The same effect is seen when the same cells are treated with 10 nM or 25 nM of Gemcitabine in combination with different concentrations of LCB2151 (Figs. 7B and 7C). The same trend was observed in Capan-2 cells (Figs. 8A, 8B and 8C).

The results shown in Figs. 5 and 6 suggest that LCB2151 and Gemcitabine do not synergize in combinatory treatments. In fact, LCB2151 has a higher potency by itself. The reason behind this inhibitory effect between Gemcitabine and LCB2151 may have something to do with the different mechanisms of action of both compounds. Treatment with LCB2151 might be increasing the mechanisms of resistance of cancer cells involving key enzymes of Gemcitabine's metabolism. The expression of hENT may be down-regulated or saturated with LCB2151, inhibiting the entrance of Gemcitabine to the cell. Furthermore, the expression of multidrug resistance proteins MDR1 and 2 could be increased with this combinatory treatment, promoting the efflux of both drugs outside the cells.

A.


HepG2 cells treated with 5nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

B.


HepG2 cells treated with 10nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

C.

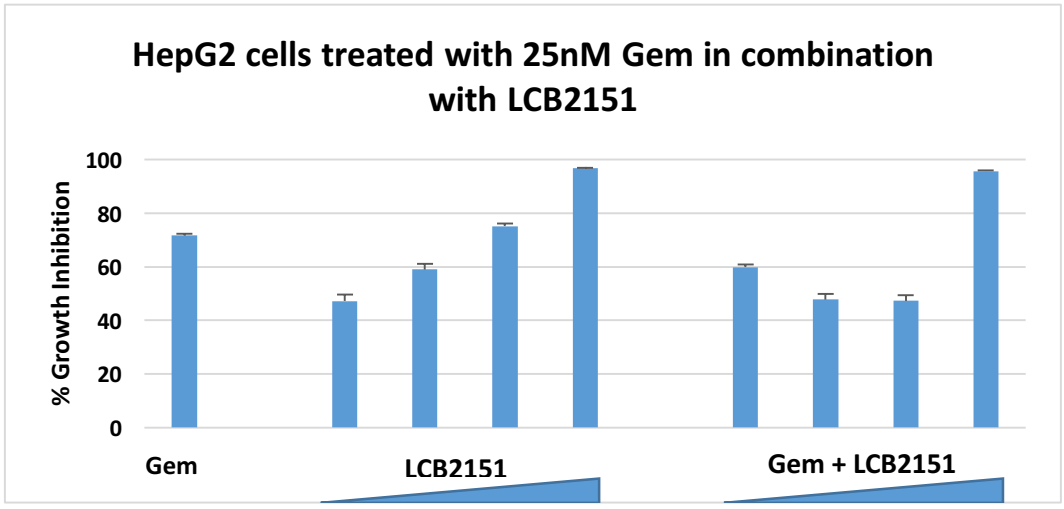
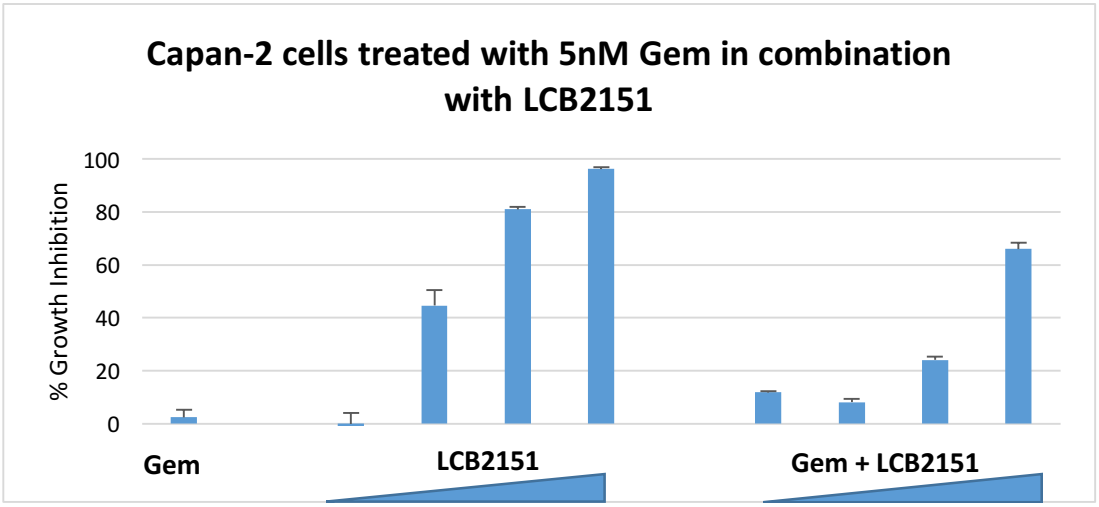
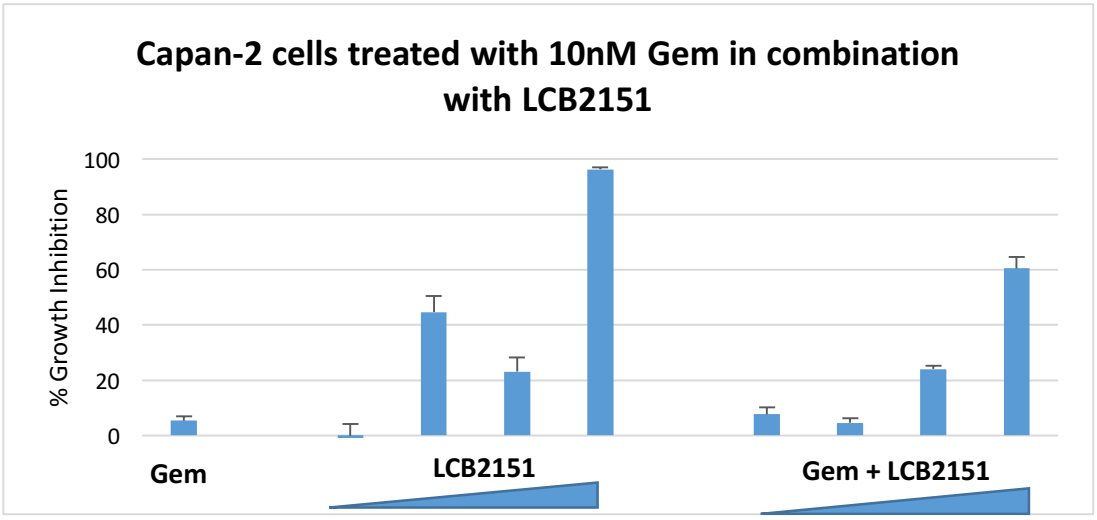

HepG2 cells treated with 25nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

**Figure 7. Combination Treatment in HepG2. A.** Percentage of Growth Inhibition of HepG2 cells treated with 5nM Gemcitabine, **B.** 10nM Gemcitabine and **C.** 25nM Gemcitabine alone and with increasing concentrations (5μM, 7.5μM, 10μM, or 15μM) of LCB2151 for 96 hours.

A.


Capan-2 cells treated with 5nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

B.


Capan-2 cells treated with 10nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

C.

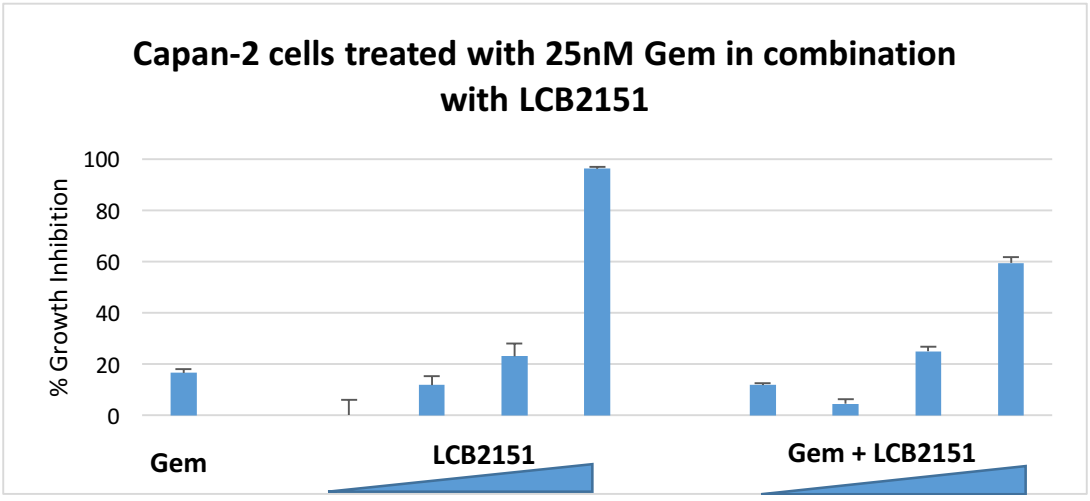

Capan-2 cells treated with 25nM Gem in combination with LCB2151
% Growth Inhibition
100
80
60
40
20
0
Gem
LCB2151
Gem + LCB2151

**Figure 8. Combination Treatment in Capan-2. A.** Percentage of Growth Inhibition of Capan-2 cells treated with 5nM Gemcitabine, **B.** 10nM Gemcitabine and **C.** 25nM Gemcitabine alone or with increasing concentrations (5μM, 7.5μM, 10μM, or 15μM) of LCB2151 for 96 hours. LCB2151 shows more potency and efficacy by itself, than in combination with Gemcitabine.

### 3.4.1. Effect of LCB2151 and LCB2132 on the mechanism of resistance of cancer cells

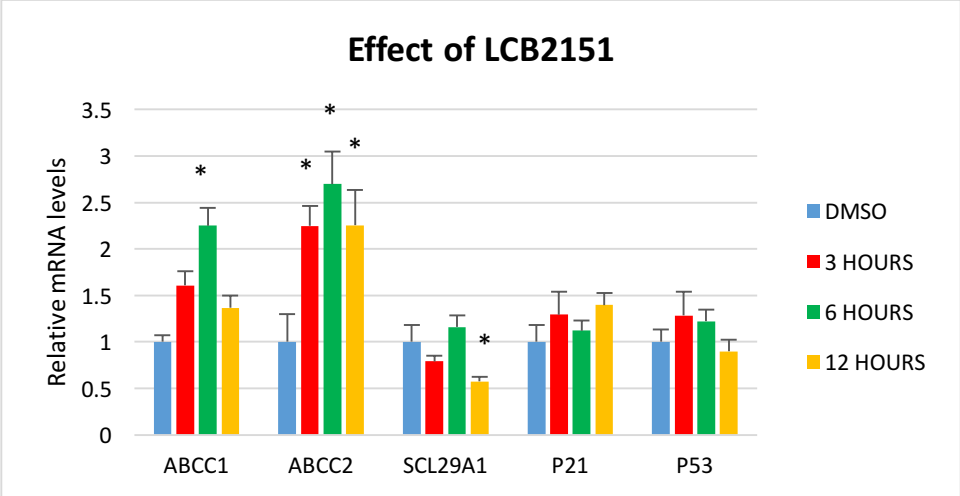
In order to test the possible causes of the inhibitory effect between Gemcitabine and LCB2151, RNA assays were performed on HepG2 cells treated with either LCB2151 or LCB2132 for 3, 6, or 12 hours. qPCR analysis was done to determine the effect of these two molecules on the mRNA levels of some of the genes that could be inhibiting the effect of Gemcitabine during combinatorial treatments: Multidrug resistance protein 1 and 2 (MRP1/2) (ABCC1 and ABCC2 genes) that affect drug depletion from the cell, and human equilibrative nucleoside transporter 1 (SCL29A1 gene) that affects cellular drug uptake. Tumor protein p53 and cyclin-dependent kinase inhibitor 1 protein p21 mRNA levels were also analyzed given that they are activated after Gemcitabine's treatment (Hill *et al*., 2013). The up-regulation of p53 protein levels promote the expression of several genes related to the apoptotic pathway, one of them p21, which then facilitates G1 growth arrest by inhibiting the activity of cyclin-dependent kinases (CDKs) (Abbas & Dutta, 2009).

The results from the qPCR experiment were normalized to the housekeeping gene RNPS1, and compared to results from DMSO treated cells. Figure 9A shows that LCB2151 treatment causes a fast and significant up-regulation of the mRNA levels of both MRP1 and 2. By 12 hours after treatment, hENT levels are significantly down-regulated. This could explain the inhibitory effect between LCB2151 and Gemcitabine that was seen previously in Figs. 7 and 8. LCB2151 appears to elevate the mechanisms of resistance of cancer cells increasing the deamination of the drugs and inhibiting the uptake of Gemcitabine. LB2132 treatment display the same trend as LCB2151 (Fig. 9B). On the other hand, Gemcitabine's treatment does not seem to have such an effect on these genes

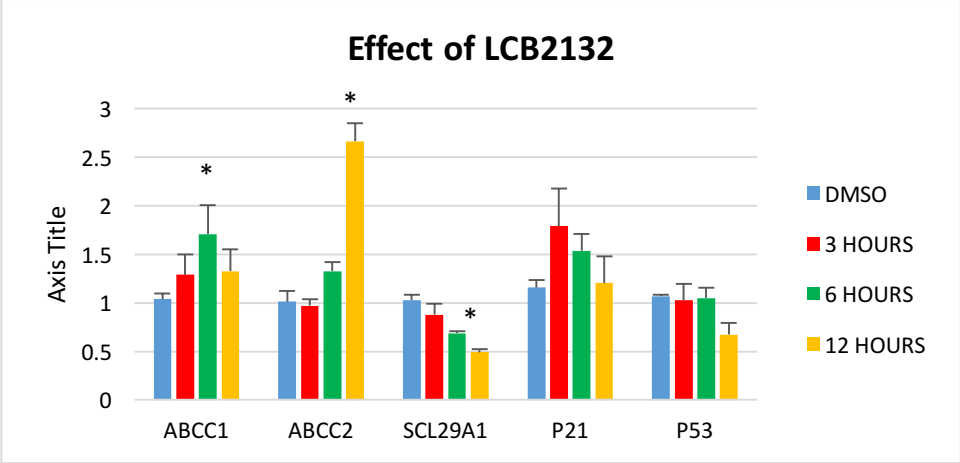
involved in the acquired resistance of cancer cells (Fig. 9C). At 6 hour treatment, we can see a small up-regulation of MRP1 mRNA levels, but they go back to normal at 12 hours treatment. The results suggest that LCB2151 induced changes in transporters may decrease the cellular concentration of Gemcitabine and affect its efficacy.

On the other hand, the mRNA levels of p53 and p21 were not affected by LCB2151 or LCB2132. Meanwhile, Gemcitabine's treatment (Fig. 9C) for 6 hours display an increase of mRNA levels of p53 gene of more than two fold compared to vehicle. Furthermore, p21 transcriptions show that Gemcitabine's treatment promotes a substantial effect on this gene. Its mRNA levels were up-regulated 7 fold after only 3 hours of treatment, and more than 22 fold at 6 and 12 hour treatment. These results suggest that LCB2151 and LCB2132 act through a different pathway than Gemcitabine.

**A.**


Effect of LCB2151
Relative mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
ABCC1
ABCC2
SCL29A1
P21
P53
DMSO
3 HOURS
6 HOURS
12 HOURS

**B.**


Effect of LCB2132
Axis Title
3
2.5
2
1.5
1
0.5
0
ABCC1
ABCC2
SCL29A1
P21
P53
DMSO
3 HOURS
6 HOURS
12 HOURS

**C.**

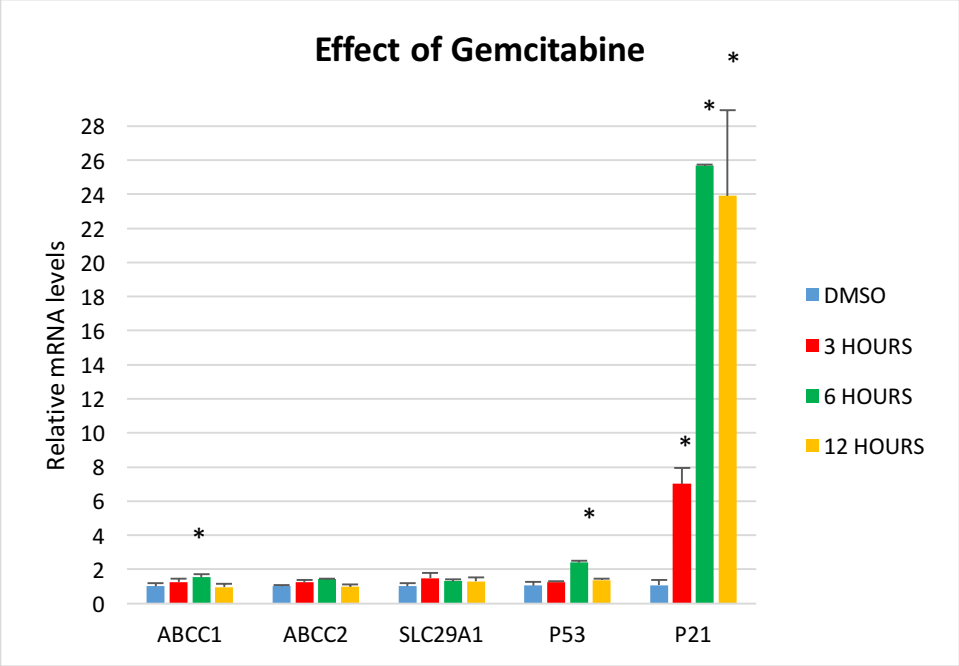



**Figure 9. Genetic changes in cancer marker genes.** mRNA levels of cancer marker genes in HepG2 treated with **A.** LCB2151, **B.** LCB2132 and **C.** Gemcitabine, for 3, 6, and 12 hours. This experiment was performed once (n=1) in triplicates. Results were normalized to the housekeeping gene (RNPS1), and calibrated to the results with DMSO. * represents statistical significance where $p < 0.05$ using t-test, where values are the means of triplicate samples from three independent experiments. LCB2151 and LCB2132 promote an up-regulation of genes related to the mechanism of resistance of cancer cells.

### 3.4.2. Effect of hENT down-regulation on LCB2151, LCB2132 and Gemcitabine.

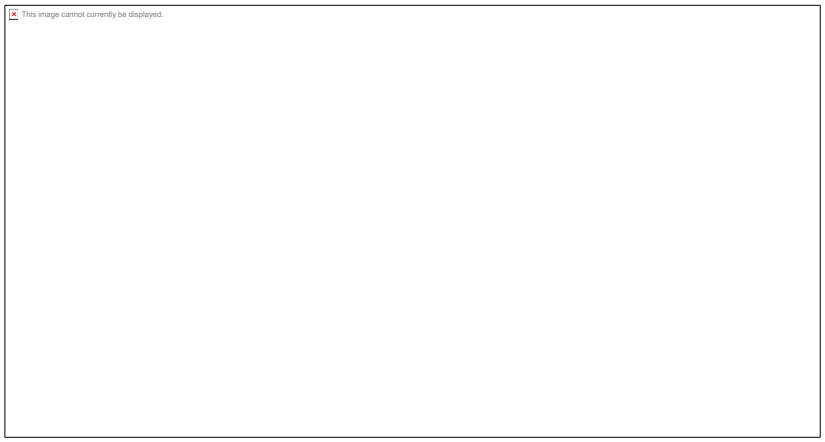
The previous results show that hENT mRNA levels are significantly down-regulated after treatment with LCB2151 and LCB2132. As stated earlier, these molecules were designed to bypass this standard mechanism of resistance of cellular nucleoside uptake. Consequently, the down-regulation of hENT would be expected to affect Gemcitabine's action, but not LCB2151/LCB2132 actions. In order to determine whether the inhibition of nucleoside transporters affects the efficacy of our compounds, they were used in combination with a nucleoside transporter inhibitor.

HepG2 cells were treated with LCB2151, LCB2132, Gemcitabine, or vehicle, for 96 hours with or without the presence of pan-nucleoside transporter family inhibitor NBMPR. All killing activity was determined using ATP level measurement. The dose response curves of the two newly synthesized nucleoside analogues did not vary in the presence of the inhibitor (Figs. 10A and 10B), suggesting that the biological effects of these drugs are independent of these transporters. Conversely and as expected, the potency of Gemcitabine was significantly affected in the presence of NBMPR (Fig. 10C).

The above results were confirmed in Capan-2 cells under the same conditions (Figs. 11A, 11B and 11C). LCB2151 and LCB2132 activity was unchanged in the presence of NBMPR. These exciting results suggest that our prodrugs can evade a key chemotherapy-induced resistance mechanism in cancer cells and could explain their ability at killing Gemcitabine resistant tumor cells.

**A.**


**B.**




**C.**

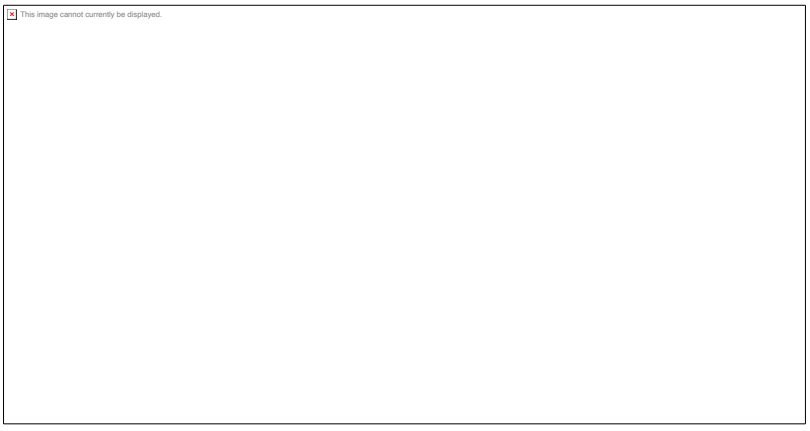
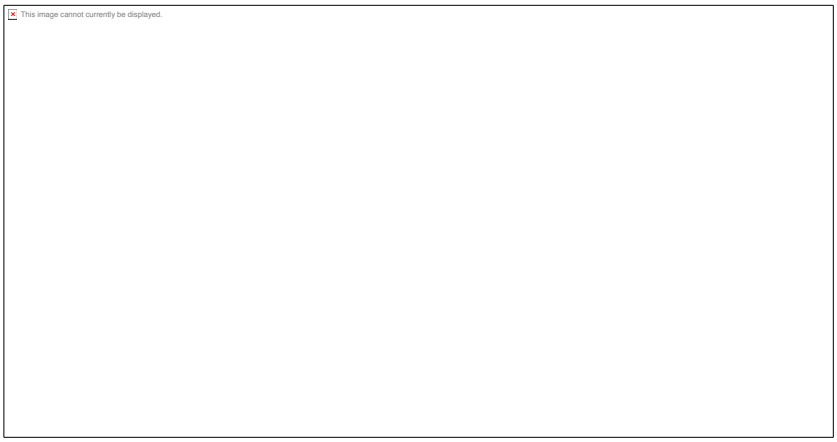
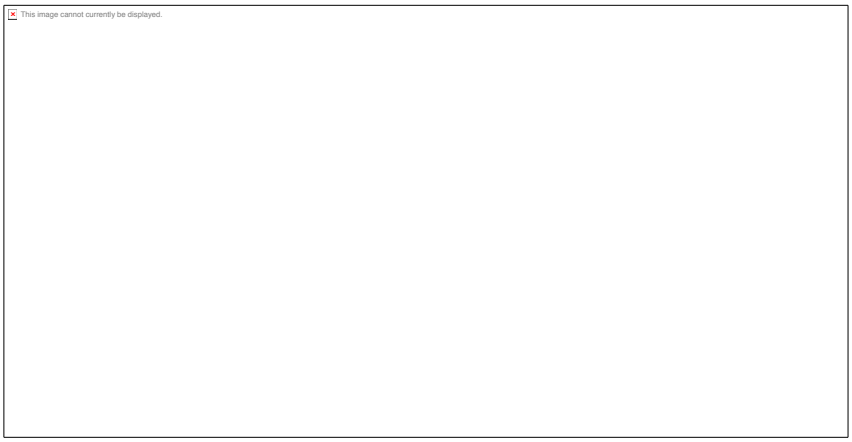



**Figure 10. Inhibition of hENT1 in HepG2 cells.** Percentage of Growth Inhibition of HepG2 cells treated with increasing concentrations of **A.** LCB2151, **B.** LCB2132, or **C.** Gemcitabine by themselves, or in the presence of 10 μM of NBMPR inhibitor, and NBMPR by itself.

A.




B.




C.

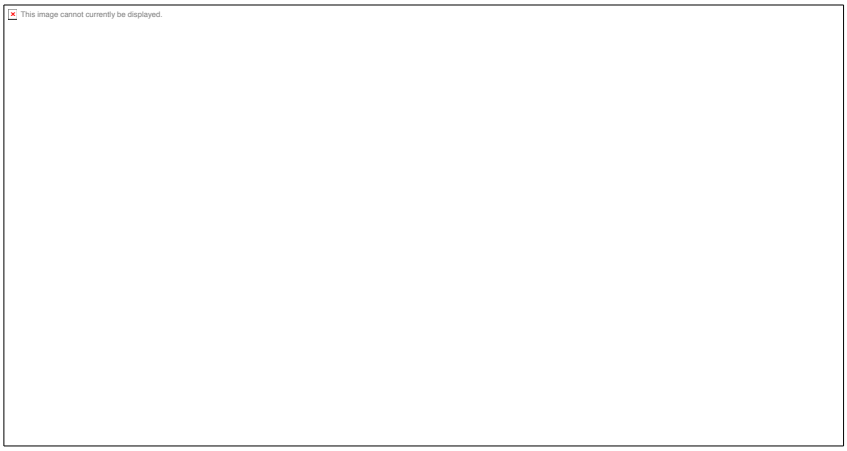



**Figure 11. Inhibition of hENT1 in Capan-2 cells.** Percentage of Growth Inhibition of Capan-2 cells treated with increasing concentrations of **A.** LCB2151, **B.** LCB2132, or **C.** Gemcitabine by themselves, or in the presence of 10 μM of NBMPR inhibitor, and NBMPR by itself. LCB2151 and LCB2132, unlike Gemcitabine, are able to enter cells without the need of nucleoside transporters.

### 3.5. Mechanism of Action

The results shown until now suggest that LCB2151 and LCB2132 have a distinct mechanism of action that differs from that of Gemcitabine. Furthermore, they have shown a high efficacy to kill Gemcitabine-resistant cells and bypass some of the mechanisms of resistance of cancer cells. These characteristics render LCB2151 and LCB2132 interesting compounds for further investigations.

#### 3.5.1. Effect of LCB2151 and LCB2132 on proliferative pathways.

##### 3.5.1.1. Genetic changes in pro-apoptotic genes suggest that cells are undergoing apoptosis.

Several chemotherapeutic agents trigger a mitochondrial pathway of cell death, which is controlled by the BCL-2 (B-cell lymphoma 2) family of proteins. These proteins interact with each other through blocks of sequence homology, known as BH domains. Two sets of proteins can be differentiated in this family: the pro-survival family members include BCL-2, BCL-XL, BCL-W, MCL1, BFL1 AND BCL2L10; and the pro-apoptotic family members include BAX, BAK, BAD, BIM, BIK, BID, BBC3 AND BOK (Czabotar *et al.*, 2014; Elmore, 2007). The apoptotic pathway to cell death involves the interaction of subgroups of this family of proteins: BH3 (BCL-2 homology 3) only proteins, or initiators (pro-apoptotic) that include BIM, BBC3, BIK, BAD and BID, the pro-survival cells (mentioned above), and the pro-apoptotic effector proteins, such as BAX and BAK (Czabotar *et al*., 2014).

The intrinsic pathway of apoptosis starts when a stimulus causes the activation of initiator proteins that inhibit pro-survival BCL-2 like proteins, allowing the activation of

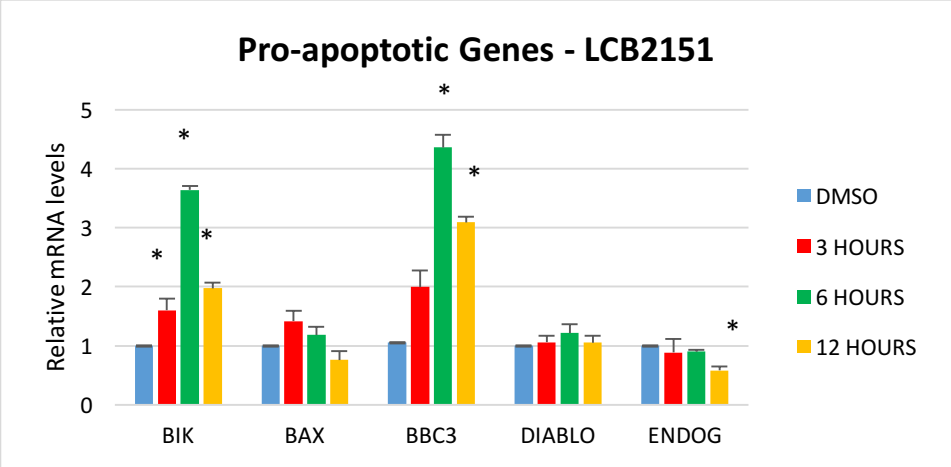
pro-apoptotic effector proteins, which disrupt the mitochondrial outer membrane. Cytochrome c is released from the mitochondria and promotes the activation of caspase 9. At the same time, DIABLO (or SMAC, second mitochondria-derived activator of caspases) blocks caspase inhibitor XIAP (x-linked inhibitor of apoptosis protein). The extrinsic pathway of apoptosis is activated when a death receptor ligand of the tumor necrosis factor (TNF) family engages death receptors on the cell membrane, which leads to caspase 8 activation, and TNFR-associated death domain protein (TRADD). Caspase 8 also generates the truncated form of BID (tBID), which can also promote the apoptotic response. The intrinsic and extrinsic pathways converge at the activation of effector caspases (caspase 3, 7 and 6) (Czabotar *et al.*, 2014).

Having established the importance of all of these proteins in cell death in other systems, it was important and necessary to determine the effect of LCB2151 and LCB2132 on their mRNA levels. mRNA was extracted from HepG2 cells treated with the concentration corresponding to the $IC_{50}$ of LCB2151, LCB2132, or their respective vehicles, for 3, 6, or 12 hours. qPCRs were done on reverse transcribed cDNA using primers specific to pro-apoptotic genes (Figs. 12A and 12B). Results normalized to the housekeeping gene RNPS1, and compared to results from DMSO treated cells, show that the genes BIK and BBC3 are significantly up-regulated in cells treated with either LCB2151 or LCB2132. This up-regulation starts as early as 3 hours post treatment and it increases after 6 hours. This result suggests that initiator proteins are being activated and that apoptotic pathways might be activated by our molecules (Westphal *et al.*, 2011). BAX and DIABLO did not show any regulation after either treatment, while ENDOG was slightly down-regulated after 12 hour treatment with LCB2151.

Levels of mRNA of anti-apoptotic (pro-survival) genes were also analyzed in cells treated with LCB2151, LCB2132, or their respective vehicles, at the same time points (Figs. 13A and 13B). The results show a significant up-regulation of MCL-1 in prodrug treated cells in a time dependent manner. Moreover, cells treated with LCB2151 showed a significant up-regulation of XIAP mRNA levels 3 hours post treatment, but the levels decreased at later time points; no changes in either BCL-XL or BCL-2 were detected (Fig. 13A). Neither XIAP nor BCL-XL transcript levels were changed in the presence of LCB2132, but BCL-2 levels were transiently up-regulated. This pattern of transient up-regulations of anti-apoptotic genes is often seen in cells undergoing apoptosis and may reflect cellular compensatory mechanisms.

Treatment with LCB2151 and LCB2132 clearly affects the levels of mRNA of pro and anti-apoptotic genes from the BCL-2 family of proteins. The loss of balance of these two kinds of proteins could mean an activation of apoptosis (Westphal *et al.*, 2011).

**A.**




**B.**

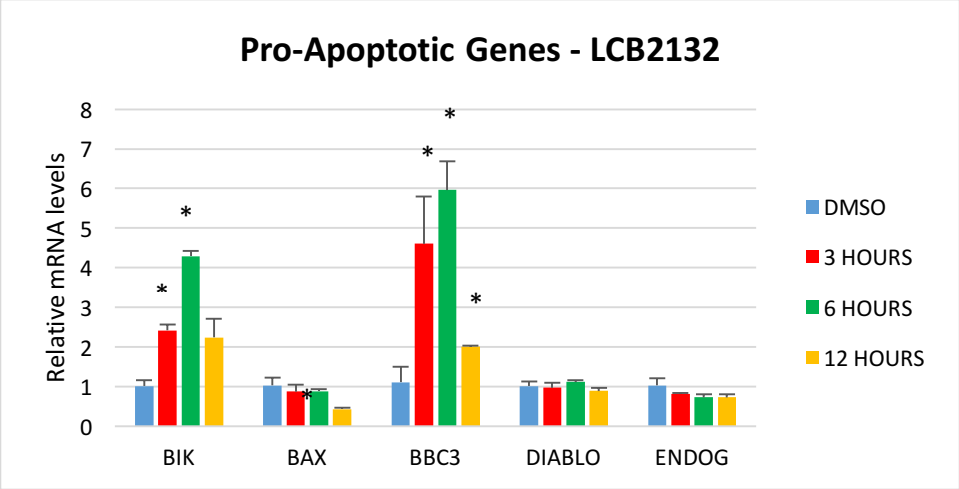
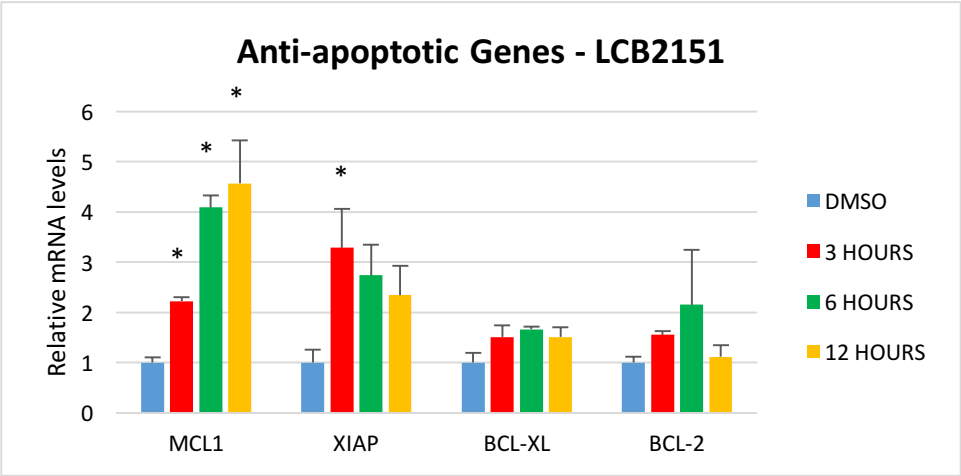



**Figure 12. Genetic Changes in pro-apoptotic genes.** mRNA levels of pro-apoptotic genes in HepG2 cell line treated with **A.** LCB2151 and **B.** LCB2132 for 3, 6, and 12 hours. Results were normalized to the housekeeping gene (RNPS1), and calibrated to the results with DMSO. * represents statistical significance compared to treatment with vehicle, where $p < 0.05$ using t-test, where values are the means of triplicate samples from six independent experiments. LCB2151 and LCB2132 promote the up-regulation pro-apoptotic genes.

**A.**




**B.**

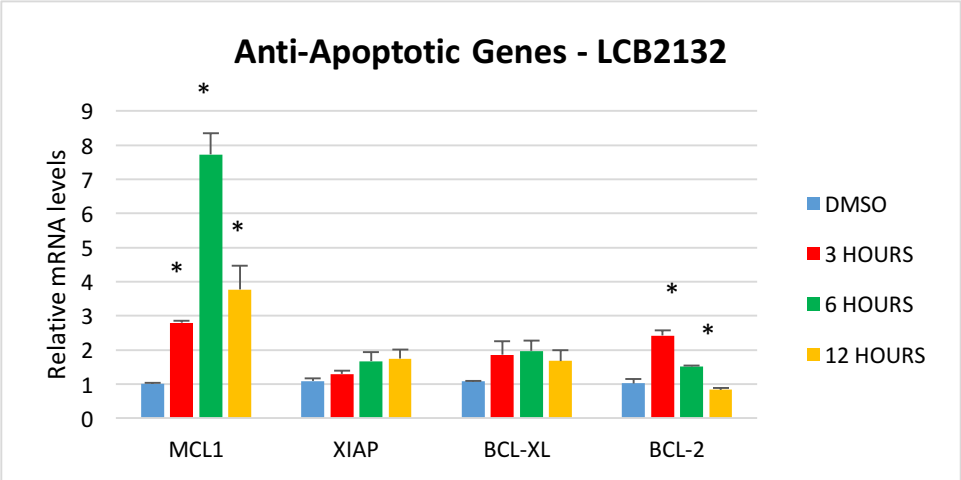



**Figure 13. Genetic Changes in anti-apoptotic genes.** mRNA levels of anti-apoptotic genes in HepG2 cell line treated with **A.** LCB2151 and **B.** LCB2132 for 3, 6, and 12 hours. Results were normalized to the housekeeping gene (RNPS1), and calibrated to the results with DMSO. * represents statistical significance compared to cells treated with vehicle, where $p < 0.05$ using t-test, where values are the means of triplicate samples from six independent experiments. LCB2151 and LCB2132 promote the up-regulation of anti-apoptotic genes that may reflect a compensatory mechanism of cancer cells.

#### 3.5.1.2. LCB2151 induces changes in protein levels of key apoptotic factors.

qPCR results displayed an increase in expression of pro-apoptotic genes which suggests that LCB2151 treatment triggers an apoptotic response in cancer cells. It was then important to evaluate the effects of this treatment on gene expression at the protein level. A series of Western Blots were done using whole cell extracts obtained from HepG2 cells treated with LCB2151, Gemcitabine, or their vehicles for 12 hours. The results in Fig. 14 show an increased regulation of BBC3 levels in cells treated with LCB2151, confirming the qPCR results. On the other hand, LCB2151 caused a significant down-regulation of BCL-2 levels. These results support the hypothesis that LCB2151 promotes the mitochondrial apoptotic pathway.

On the other hand, LCB2151 treatment had no effect on p53, also confirming the mRNA results (Fig. 9). Conversely, Gemcitabine showed a clear activation of the p53 gene. These results again suggest that LCB2151 and Gemcitabine activate different cell death mechanisms in cancer cells.

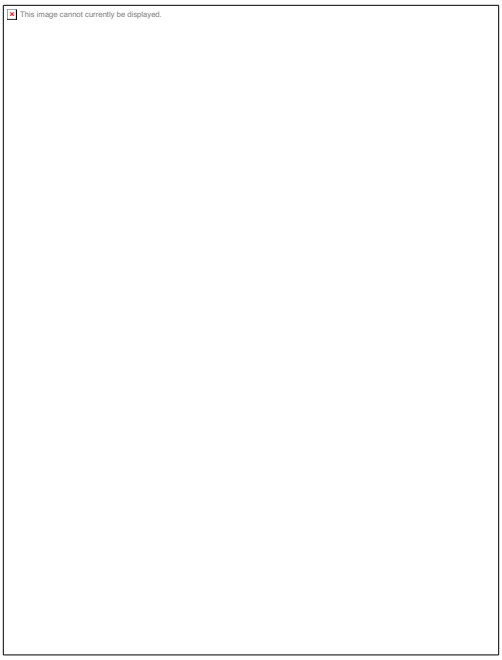


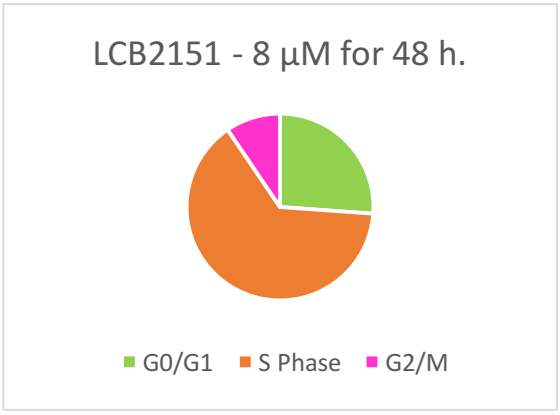

**Figure 14. Western Blot Analysis.** Whole cell extracts of HepG2 cells treated with either LCB2151 10μM, Gemcitabine 1μM, DMSO, or untreated, separated on a 15% acrylamide gels and blotted using the following anti-bodies: BAX, BBC3, P53, BCL-XL, BCL-2 and GAPDH. These are interesting preliminary results that show a regulation of BBC3 and BCL-2 proteins under LCB2151 treatment, that confirms the qPCR results. On the other hand, there was an absence of p53 activation in LCB2151 treated cells, which shows that LCB2151 and Gemcitabine act through different pathways.

### 3.5.1.3. Effect of LCB2151 on cell cycle progression.

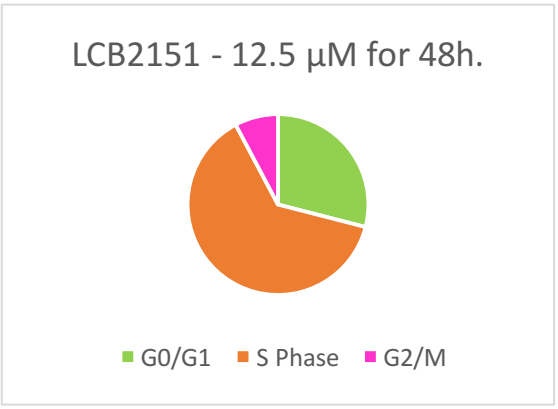
Studies on Gemcitabine's mechanism of action have determined that its main activity is to induce cell cycle arrest and cell death by getting incorporated into DNA during the S phase stage of the cell cycle (Hui & Reitz, 1997).

It was interesting to see whether LCB2151 also caused an effect on cell cycle progression. HepG2 cells were treated with LCB2151, Gemcitabine, or vehicles for 48 hours. Two different concentrations were used with LCB2151, 8 uM which corresponds to the EC50, and 12.5 μM, which is the concentration at which the drug has reached its maximum effect on the cells, almost 100% growth inhibition (Fig. 3A). For Gemcitabine, cells were treated with 1 μM, the EC50 for this cell line (Fig. 3D). After 48 hours of treatment, cells were fixed and stained with Vybrant DyeCycle Ruby stain kit. Stained cells were analyzed in the Flow Cytometer, and the number of cells in each cell cycle was measured. The distribution of cells in the different stages of the cell cycle was similar between the cells treated with LCB2151 and the cells treated with vehicle (Figs. 15A, 15B, and 15C). On the other hand, consistent with published results, cells treated with Gemcitabine had a lower number of cells in the S phase, and a higher number of cells in the G0/G1 phase (Fig. 15D). Similar results were reported by (Cappella *et al*., 2001). Again these results show that LCB2151 and Gemcitabine activate different pathways in cancer cells.

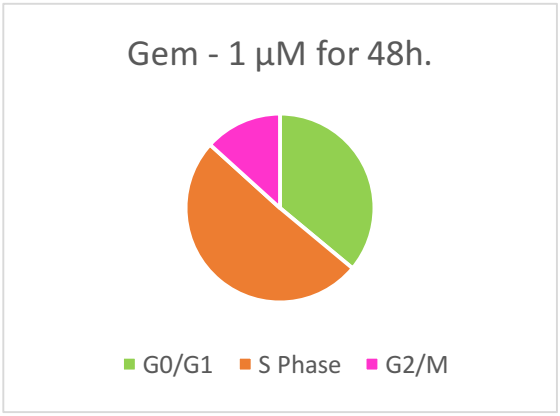
**A.**


LCB2151 - 8 µM for 48 h.
G0/G1
S Phase
G2/M

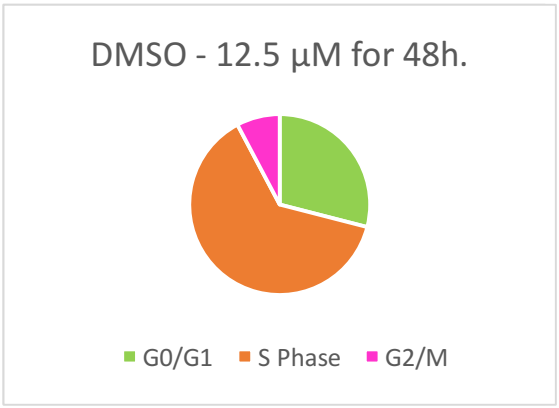
**B.**


LCB2151 - 12.5 µM for 48h.
G0/G1
S Phase
G2/M

**C.**


Gem - 1 µM for 48h.
G0/G1
S Phase
G2/M

**D.**


DMSO - 12.5 µM for 48h.
G0/G1
S Phase
G2/M

**E.**

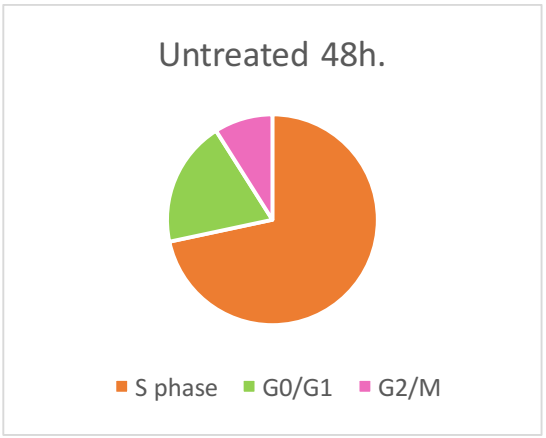

Untreated 48h.
S phase
G0/G1
G2/M

**Figure 15. Effect of LCB2151 and Gemcitabine on Cell cycle progression.** Effect of different concentrations of LCB2151 on cell cycle progression. **A.** 8μM LCB2151, **B.** 12.5μM LCB2151, **C.** 1μM Gemitabine, and **D.** 12.5μM DMSO, **E.** Untreated, on cell cycle progression after 48 hour treatment. Cells were fixed and stained with Vybrant DyeCycle Ruby stain. This experiment was performed once (n=1) in duplicates. The results shown suggest that LCB2151 does not have an effect on cell cycle progression, its action is only cytotoxic.

### 3.5.2. Effect of LCB2151 and LCB2132 on cell killing.

#### 3.5.2.1.Cancer cells treated with LCB2151 undergo apoptosis.

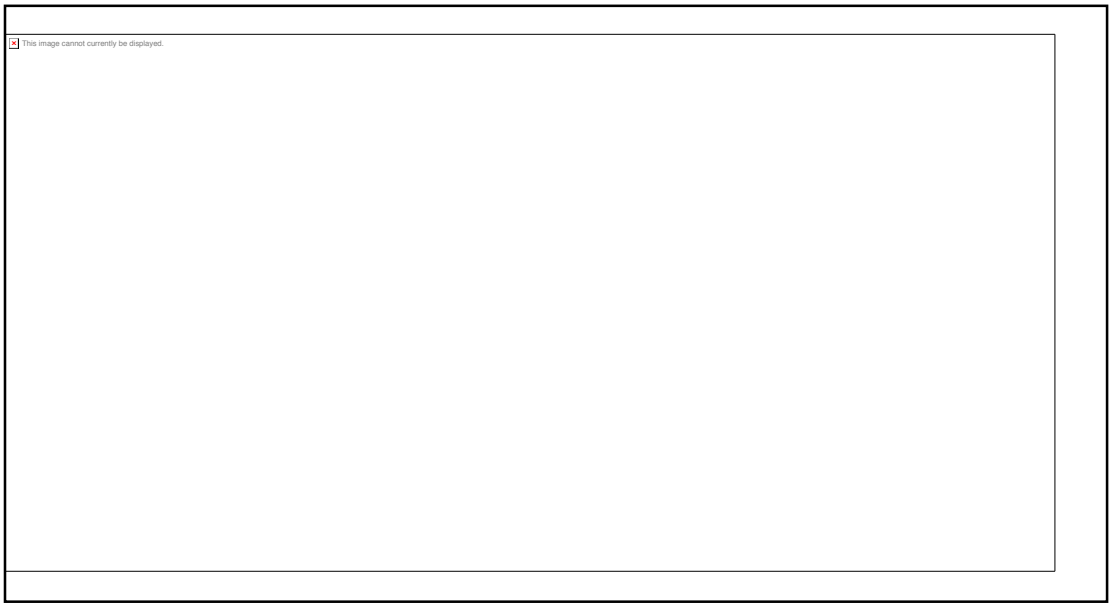
The results obtained so far suggest that LCB2151 treatment may cause apoptosis in cancer cells. mRNA levels of pro-apoptotic genes were up-regulated following LCB2151 treatment, and the results were confirmed at the protein level with whole cell extracts. However, p53 was not activated following this treatment. In order to confirm the mechanism of cell death of cancer cells treated with LCB2151, it is important to discard other possibilities, such as necrosis and autophagy. Necrosis is a complex cell death mechanism that has been elucidated in the last few years, and has many possible pathways that can even involve some of the proteins that are typical in apoptotic cell death, thus termed necroptosis. This mechanism often involves the inhibition of Caspase 8 activation by Receptor-interacting serine/threonine-protein kinase 1 (RIPK-1), which inhibits the activation of apoptosis, and leads the cell towards a necroptotic pathway (Vanden Berghe *et al.*, 2014). In contrast, autophagy is a process by which autophagosomes engulf cytoplasmic components and digest them, and it is dependent on Microtubule-associated protein 1A/1B-light chain 3 (LC3) (Tanida *et al.*, 2008). Autophagy has also been linked to cancer by providing cancer cells a mechanism of recycling to maintain mitochondrial function and energy homeostasis (White *et al*., 2015).

Apoptosis and necroptosis were tested in HepG2 cells that were treated with LCB2151 alone or in combination with either the caspase 3 inhibitor DEVD, or RIPK-1 inhibitor Necrostatin-1 for 24 hours. ATP levels showed that caspase 3 inhibition significantly shifts the response curve of LCB2151 (Fig. 14A).

Furthermore, immunofluorescence on fixed HepG2 cells treated with 8 μM LCB2151 or vehicle for 12 hours, showed absence of LC3 (normally present in autophagic cells), and an up-regulation of Cyclophilin D, which is normally present in apoptotic cells, as can be also seen in the cells treated with Gemcitabine (Figs. 14B and 14C).

These results, combined with the qPCRs and the Western Blot data, confirm that LCB2151 treatment induces the mitochondrial apoptotic pathway in cancer cells.

**A.**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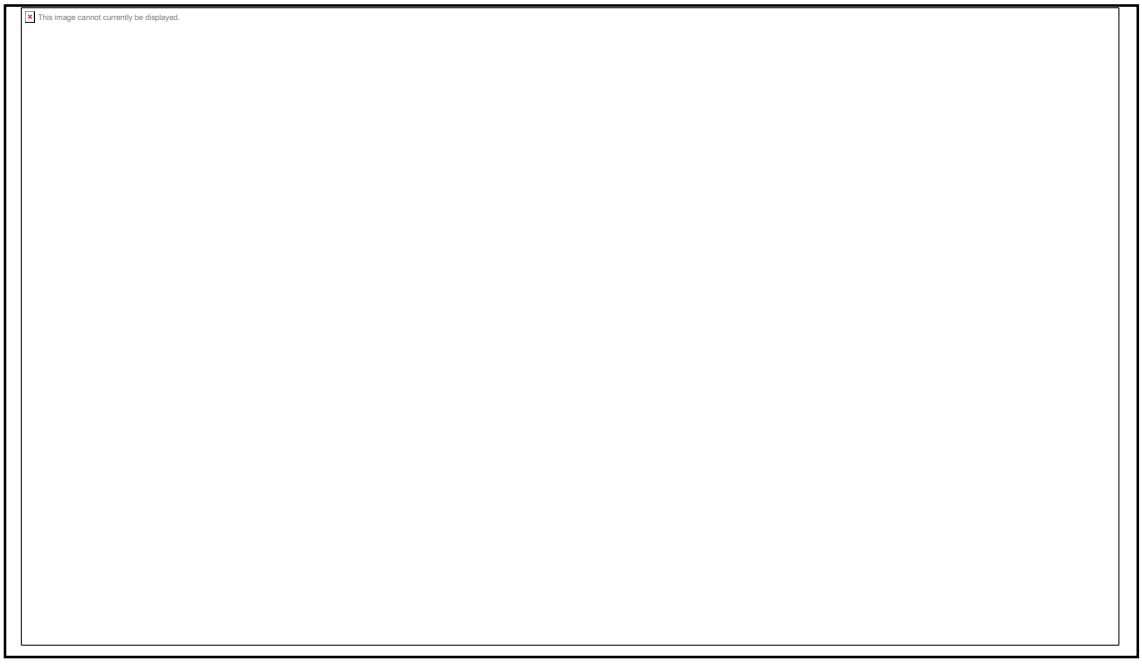
**B.**


This image cannot currently be displayed.

**C.**


**Figure 14. Determination of mechanism of cell death. A.** Percentage of Growth Inhibition of HepG2 cells treated with LCB2151 +/-400 nM RIPK-1 inhibitor Necrostatin-1, or +/- 25 nM of caspase-3 inhibitor DEVD for 24 hours. **B.** Immunofluorescence of HepG2 cells treated with LCB2151 or vehicle for 24 hours, and stained for DAPI, LC3 and Cyclophilin D. **C.** Immunofluorescence of HepG2 cells treated with Gemcitabine or vehcle for 24 hours, and stained for DAPI, LC3 and Cyclophilin D.

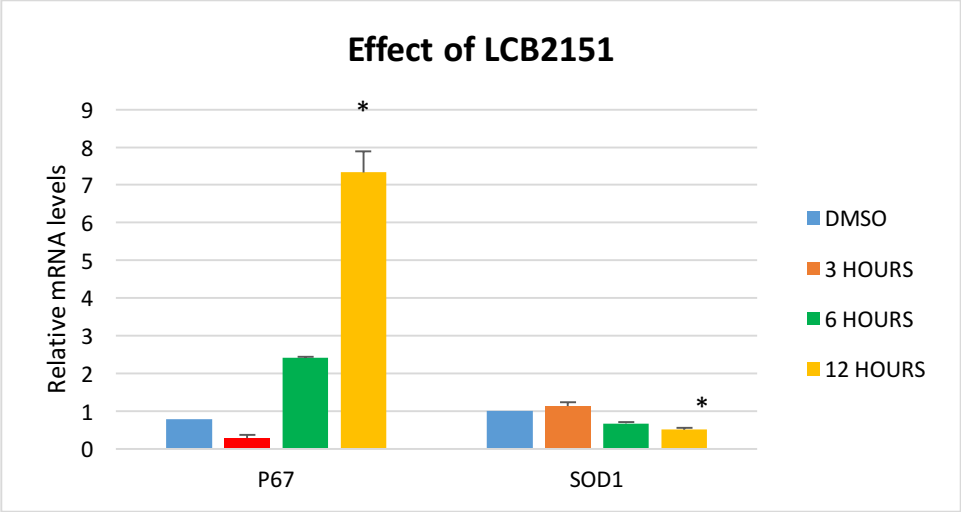
### 3.5.2.2. Effect of LCB2151 and LCB2132 on stress markers.

Cancer cells have an increased energy production, macromolecular biosynthesis and need to maintain a certain redox balance. Mutations in oncogenes and tumor suppressor genes cause alterations in the metabolism of cancer cells (Cairns *et al.*, 2011). Reactive oxygen species (ROS), known as harmful for cells at certain concentrations, are useful in cell signaling and essential for many biological processes. Cancer cells usually have an increased ROS production due to mutations and a need for blood supply. Interestingly, many anti-cancer therapies use the increase of ROS production as a mechanism to kill cancer cells increasing the expression of NADPH oxidase, the major source of ROS; while other therapies function as antioxidants, increasing the expression of Superoxide dismutase (SOD1) (Wang & Yi, 2008).

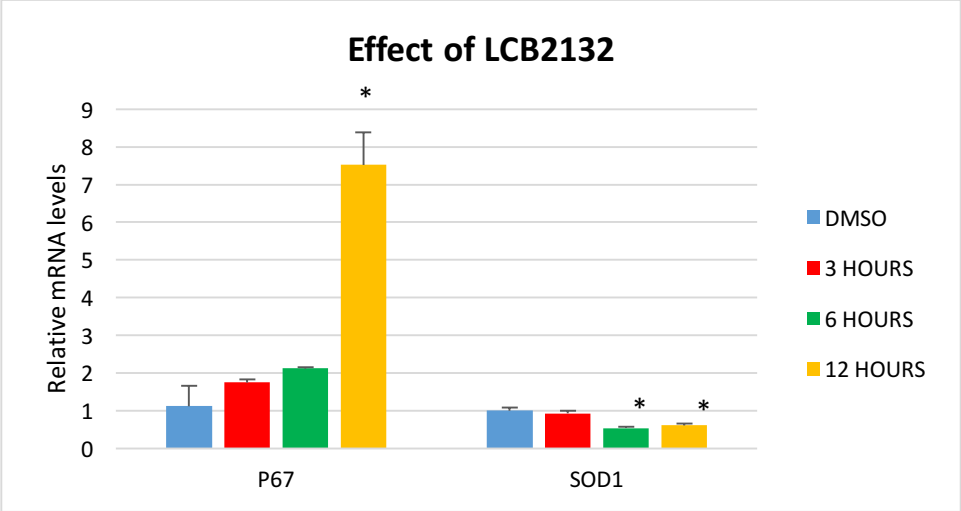
It was interesting to see whether LCB2151 and LCB2132 affect ROS production. The lipophilic moiety in these drugs could have an effect on a mitochondrial pathway, as previously suggested by Stuart *et al*. (2014) and Zachar *et al*. (2011). In order to determine the effect of these new compounds on mitochondrial stress, NADPH oxidase and SOD1 mRNA levels were measured from HepG2 cells treated with the concentration corresponding to the IC50 of LCB2151, LCB2132, Gemcitabine, or their respective vehicles, for 3, 6, or 12 hours. qPCRs were performed on cDNA reverse transcribed from extracted mRNA to determine the expression levels of certain oxidative stress marker genes (Figs. 15A, 15B and 15C). p67 mRNA level (corresponds to NADPH oxidase) was significantly up-regulated (>7 fold) after 12 hours of treatment with either LCB2151 or LCB2132. This increase in NADPH oxidase could suggest an intensified production of ROS in treated cancer cells. On the other hand, Gemcitabine's treatment showed no change

in expression of p67. Dalla Pozza *et al*. (2012) reported that Gemcitabine treatment increased the expression of the mitochondrial uncoupling protein 2 (UCP2), which acts as an antioxidant lowering ROS levels. They suggested this as a mechanism of resistance of cancer cells, since Gemcitabine had been proven to be able to increase ROS production. Figures 15A and 15B also show that mRNA levels of SOD1 were severely down-regulated in cells treated with LCB2151 and LCB2132, while Gemcitabine did not have a significant effect on it. These results suggest that our proprietary prodrugs induce significant changes in cancer cells, down-regulating antioxidants, while up-regulating oxidative proteins. In a future, it would be interesting to see the effect of our molecules in UCP2 expression, to determine if LCB2151 and LCB2132 is also bypassing this mechanism of resistance of cancer cells.

**A.**


Effect of LCB2151
Relative mRNA levels
9
8
7
6
5
4
3
2
1
0
*
*
P67
SOD1
DMSO
3 HOURS
6 HOURS
12 HOURS

**B.**


Effect of LCB2132
Relative mRNA levels
9
8
7
6
5
4
3
2
1
0
*
*
*
P67
SOD1
DMSO
3 HOURS
6 HOURS
12 HOURS

**C.**

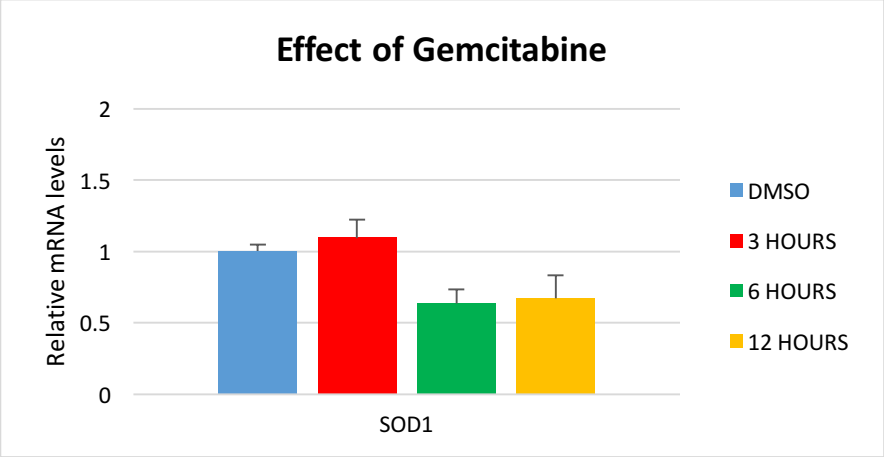



**Figure 15. Genetic changes in oxidative stress genes** mRNA levels of oxidative stress marker genes in HepG2 cell line treated with **A.** LCB2151, **B.** LCB2132, or **C.** Gemcitabine, for 3, 6, and 12 hours. Results were normalized to the housekeeping gene (RNPS1), and calibrated to the results with DMSO. * represents statistical significance compared to cells treated with vehicle, where $p < 0.05$ using t-test, where values are the means of triplicate samples from three independent experiments. LCB2151 and LCB2132 seems to be affecting cells' redox balance, while Gemcitabine does not have an effect on this pathway.

### 3.6. HPLC – MS Measurements of LCB2151 in cancer cells

As described before, LCB2151 is composed of two elements: a nucleoside analogue and a lipid. These are linked together by an amide bond. The design of these molecules was based on three hypotheses. Firstly, the molecule LCB2151 is active on its own. The second hypothesis is based on the cleavage of the lipid from the nucleobase. The released nucleoside or its phosphorylated conjugate could then be the active species. On the other hand, the last hypothesis is that the released lipid could also be the active component.

The HPLC-Mass spectrometry studies, realized by Denis Faubert's group at the IRCM, reveals firstly that LCB2151 is relatively well absorbed in or on the cell, 10 to 5 nano moles being present in the extracts at different time points. Interestingly, only pico moles (1000 less) of the released nucleoside analogue or the lipid were detected. Similarly, no mono-phosphate nucleoside of LCB2151 was detected. We still have however to verify the presence of di or tri-phosphates in the media, as the potential of the lipid on a given protein. These studies will require the preparation of radio-labelled LCB2151 analogues to ascertain these results.

**A.**

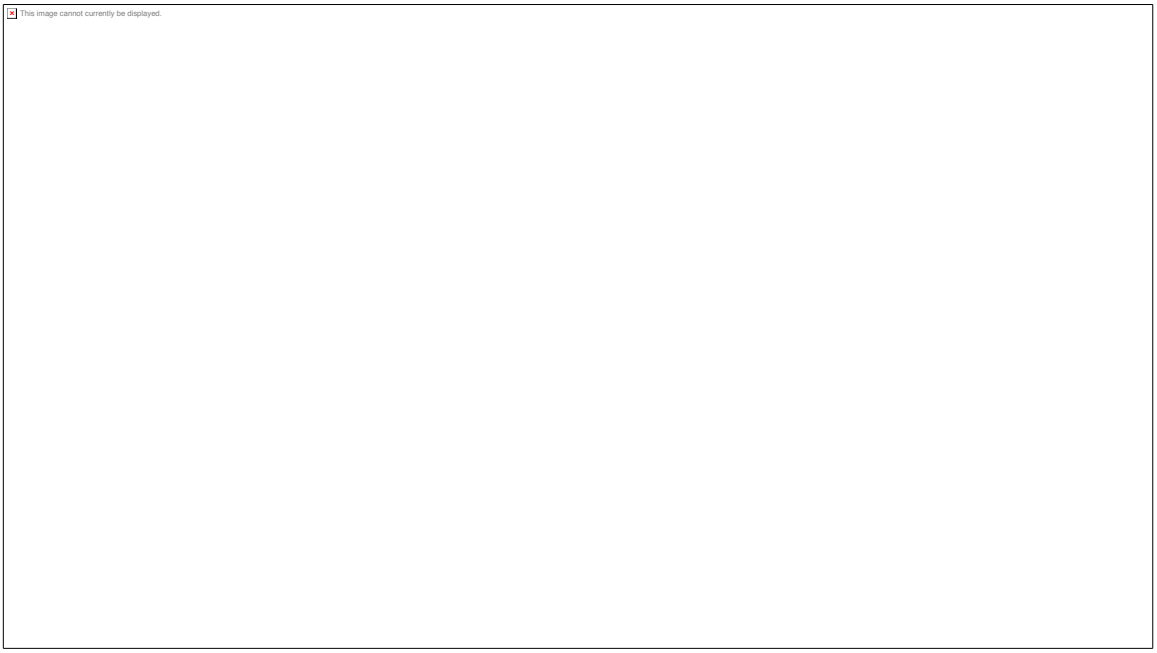



**Figure 16. HPLC-MS Results.** Measurement of the amount (nanomoles) of LCB2151 in the cell extract after addition of 80 nanomoles in the cell media in function of time. LCB2151 reaches its highest concentration inside the cell at 6 hours after treatment, and then it plateaus at 12 and 24 hours.

The exciting results presented in this study suggest that this new generation of nucleoside analogues could have a future in clinical studies. LCB2151 and LCB2132 showed high efficacy at killing cancer cells from three different human gastrointestinal cell lines: two pancreatic cancer cell lines (BxPC3 and Capan-2) and one hepatocellular carcinoma cell line (HepG2). It is important to emphasize that Capan-2 bears a mutated K-Ras gene, present in over 90% of pancreatic tumors, while BxPC3 bears a wild type (WT) K-Ras gene. LCB2151, the most potent compound, was chosen to study a possible synergy with the current standard of care for pancreatic cancer (Gemcitabine) and to determine its mechanism of action. Interestingly, LCB2151 was able to kill Gemcitabine-resistant cells in both HepG2 and Capan-2 cell lines. These results are important, considering that gemcitabine-resistant cancer cells are known to be more invasive and grow faster. On the other hand, when cancer cells were co-treated with LCB2151 and Gemcitabine, their effectiveness was hampered. This inhibitory effect could be explained by the up-regulation of genes involved in drug resistance by LCB2151, and Gemcitabine's dependence on nucleoside transporters to enter the cells. Regarding LCB2151's mechanism of action, we determined that its treatment causes a significant up-regulation of mRNA levels of pro-apoptotic genes, which was confirmed at the protein level. Furthermore, opposite to Gemcitabine, LCB2151 treatment did not show signs of p53 protein activation. Additionally, cancer cells did not present any changes in the cell cycle progression when treated with LCB2151, while Gemcitabine's treatment lowered the amount of cells in the S phase, and increased the cells in the G0/G1 phase. These results would suggest that LCB2151 and Gemcitabine trigger different pathways in the cancer cells. However, caspase 3 inhibition and immunocytochemistry experiments show that both compounds

cause apoptosis on cancer cells. LCB2151 however, promotes a significant up-regulation of NADPH oxidase and a down-regulation of Superoxide Dismutase mRNA levels. Gemcitabine on the other hand, does not have an effect on the expression of either of these genes. This effect of LCB2151 on mitochondrial stress could be beneficial in killing cancer cells through a nucleoside analogue mechanism, or through a mitochondrial mechanism. This would have to be determined with further experiments measuring Oxygen consumption and mitochondrial viability. This project presents exciting results that would benefit from an *in vivo* study in order to confirm LCB2151 efficiency and non-toxicity.

## 4. Discussion

The incidence of pancreatic cancer has increased over the last years, and its morbidity is still a major unsolved health problem (Kaltsas *et al*., 2014; Li *et al.*, 2004). Treatment options are limited, and their efficacy modest at best. The standard of care refers to the treatment that provides the best results for patients, and this will vary depending on the patient's overall health, the stage of the cancer and the treatment's side effects. Most of the pancreatic cancer patients are diagnosed when the cancer is at an advanced stage, so only in 20% of cases the cancer is resectable and surgery is an option (Kaltsas et al., 2014). After surgery, patients often get an adjuvant chemotherapy which consists of Gemcitabine, a nucleoside analogue used to treat various forms of cancer. Gemcitabine is now the standard of care, but it is not completely effective against pancreatic cancer, and in some cases, it results in drug resistance (Samulitis *et al.*, 2015). In the rest of cases where cancer is inoperable and advanced, the treatment depends on the overall health of the patient. For a long time, the first-line chemotherapy was Gemcitabine. However, FOLFIRINOX, a combination treatment that consists of Oxaliplatin, Irinotecan, Leucovorin, and Fluorouracil, has been shown to increase the overall survival of patients. Nevertheless, it causes major toxicity problems. For this reason, FOLFIRINOX is only administered when patients are in good health (Conroy *et al*., 2011a). Improvements in the detection, screening, and staging of patients could improve the outcome of the disease in many cases, but it will not be enough without a more effective drug (Li *et al*., 2004).

On the other hand, for hepatocellular carcinoma (HCC) patients, the only potentially curative treatment involves partial hepatectomy and liver transplantation. When surgery is not an option or the cancer is at an advanced stage, the current standard of care

is Sorafenib. As mentioned earlier, Sorafenib is a tyrosine kinase inhibitor that has shown to extend the life of patients for up to 5.5 months relative to no treatment (Alves *et al*., 2011; Llovet *et al.*, 2008).

The main problem of the current standard of care is the increasing incidence of patients with resistance to these treatments (Fryer *et al.*, 2011). The identification of the mechanisms of drug resistance to therapies like Gemcitabine is key to the development of new compounds (Samulitis *et al*., 2015). Various laboratories have been investigating the possible mechanisms of resistance to Gemcitabine in pancreatic cancer cells. NA need nucleoside transporters to enter the cancer cells. These have been identified as being the first rate-limiting proteins that the drugs encounter (Jordheim & Dumontet, 2007). Many investigations have been done regarding this important step, since the entrance of the drugs inside the cells is essential for their activation and their antineoplastic action. Mori *et al.* (2007) determined that hENT1 was significantly associated with Gemcitabine's sensitivity, while the other nucleoside transporters were not (Mori *et al*., 2007). Then, Farrell *et al.* (2009) developed a study with pancreatic cancer patients treated with Gemcitabine, and found that hENT1 expression correlates with overall disease-free survival. This study suggested that hENT1 expression could be used as a predictive marker of Gemcitabine's response in patients with pancreatic cancer. Furthermore, Perez-Torras *et al.* (2008) confirmed that adenoviral over-expression of hENT1 improved the therapeutic response to Gemcitabine. Once nucleoside analogues enter the cells, they need to get phosphorylated by an enzyme, deoxycytidine kinase (dCK). Studies have also shown that this is a crucial rate-limiting step in nucleoside analogue's activation. (Saiki *et al.*, 2012; Tang *et al*., 2011). Nucleoside analogues can also encounter enzymes that are able to deaminase them and

inactivate them inside or outside the cells. CDA up-regulation was found to be a limiting factor in nucleoside analogues' anticancer action (Yoshida *et al.*, 2010). Finally, multidrug resistance-associated proteins (MRP) are efflux pumps that have been proven to decrease the concentration of nucleoside analogues inside the cells and thus decrease their efficacy (Oguri *et al.*, 2006).

On the other hand, irregular activity in signaling pathways involved in cell cycle regulation and apoptosis have been shown to correlate with Gemcitabine resistance (Hamacher *et al.*, 2008). The Mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) is known to be overexpressed in cancer cells, promoting survival pathways that have been related to the chemoresistance acquired by cancer cells (Mhaidat *et al.*, 2009; Mirmohammadsadegh *et al.*, 2007). In the case of pancreatic cancer, Mitogen-Activated Protein Kinase Kinase 1 (MEK) expression was found to be correlated with cancer cells' sensitivity to Gemcitabine. In fact, Fryer *et al.* reported that inhibition of MEK in combination with Gemcitabine increased the growth inhibition of pancreatic cancer cells from 30% to 60% (Fryer *et al.*, 2011).

The identification of some of the mechanisms that cancer cells use to increase resistance to Gemcitabine was the driving force behind the Guindon laboratory's synthesis of a new generation of proprietary nucleoside analogues that can potentially bypass drug-induced resistance. More than 150 compounds were synthesized in the Guindon laboratory and were tested in the Nemer laboratory in two human pancreatic cancer cell lines and one human hepatocellular carcinoma cell line. The two most potent compounds were selected out of the screening study for further research. LCB2151 and LCB2132 proved to be the most cytotoxic molecules in the three cell lines (Fig. 3A and 3B). Furthermore, the $IC_{50}$

and $EC_{50}$ were established for each compound. In pharmacology, these values are important to determine the efficacy of a drug. $IC_{50}$ corresponds to the concentration at which the drug causes 50% growth inhibition, and $EC_{50}$ is the concentration that causes half of the maximal effect of the drug (Caldwell *et al.*, 2012). LCB2151 and LCB2132 had the lower $EC_{50}$ values among all the molecules tested. Since both compounds reached almost 100% growth inhibition in the three cell lines, their $IC_{50}$ and $EC_{50}$ were very similar.

Off target toxicity of anti-neoplastic drugs is a major issue when treating patients. Commonly used drugs, such as Gemcitabine, display high toxicity and have devastating effects on normal cells. Since pancreatic cancer patients have high levels of tumor-specific immune activity (Plate & Harris, 2000), a study by Davis *et al.* (2012) tested the effect of Gemcitabine in immune cells. They reported that treatment with Gemcitabine decreased the levels of memory T cells.

Since LCB2151 was efficient at killing cancer cells, it was important to evaluate it on non-cancerous cells. Selectivity index was determined using splenocytes, including all kinds of immune cells, extracted from a wild-type mouse spleen. The results showed that even at high concentrations, LCB2151 had no toxic effect on immune cells, as assessed by cell viability (Fig. 4). This finding is extremely encouraging and could open the door for eventual tests of combinatorial treatments with immunotherapy. Nevertheless, further testing of LCB2151 should be performed on fast dividing cells, such as fibroblasts, in order to confirm the selectivity index of this drug in various types of cells.

Given the low efficiency of Gemcitabine at killing cancer cells (Fig. 3D), and the development of resistance in the cells that bypass this treatment, it was interesting to test the ability of LCB2151 at killing these resistant cancer cells. A consecutive treatment was

tested, where HepG2 and Capan-2 cells were treated with Gemcitabine for 96 hours and the remaining cells were then treated with LCB2151 for 24 hours (Figs. 5 and 6). As expected, Gemcitabine had a limited effect on the cells, especially on Capan-2. Interestingly, LCB2151 was able to kill almost all of the remaining HepG2 cells (Fig.5), and up to 60% of the remaining Capan-2 cells in 48 hours treatment (Fig. 6). As mentioned earlier, cancer cells treated with Gemcitabine quickly develop resistance to the drug. In Figs. 5B, 5D, 6B and 6D, the results show that the remaining cells from Gemcitabine treatment are resistant to further treatment with Gemcitabine. The mechanisms by which cancer cells develop resistance to Gemcitabine include the down-regulation of nucleoside transporter, and the up-regulation of cytidine deaminase and multi-drug resistant proteins. Furthermore, it has been reported that Gemcitabine resistant cells develop an epithelial to mesenchymal transition phenotype (Shah *et al.*, 2007), and an increase in the invasiveness (Samulitis *et al*., 2015). It is important to emphasize that LCB2151 was able to potently eradicate these cancer cells that had already activated the mechanisms of drug resistance. The results obtained are extremely encouraging, and a future *in vivo* treatment model is necessary to further confirm the promising potential of our new prodrug. This kind of superb efficacy of a prodrug in Gemcitabine resistant cells has not been reported yet.

On the other hand, drug combination is often used in cancer treatment, mainly to increase the effect of the treatment through synergism, reducing the dose of the drugs and thus their toxicity (Chou, 2006). Considering Gemcitabine's limited efficacy and high toxicity, its synergism with LCB2151 was evaluated in HepG2 and Capan-2 cell lines. Increasing concentrations of Gemcitabine with LCB2151 were tested in both cell lines (Figs. 7 and 8). According to Chou (2006 and 2008), in order to determine synergy, it is

necessary to know the potency and shape of the dose-effect curve of each drug. Unexpectedly, the results showed an inhibitory relation between the two compounds. Figs 5 and 6 show that the effect of each drug by itself is higher than the effect of drugs when given in combination. This suggests that these two drugs induce different mechanisms of action that interfere with the activity of one another. LCB2151 could be increasing some of the mechanisms of resistance of cancer cells, such as the down-regulation of hENT, inhibiting the nucleoside analogues from entering the cells, or the up-regulation of multidrug resistance proteins MDR1 and 2, inducing the efflux of nucleoside analogues outside the cells.

In order to prove these theories, mRNA assays were carried out. Genetic changes in cancer marker genes have revealed that LCB2151 and LCB2132 induce an up-regulation of resistance mechanisms usually activated in response to other anti-cancer drugs (Fig. 9). For instance, the expression of hENT1 (SCL29A1 gene) has been previously proven to be crucial for the entry into cells of nucleoside analogues such as Gemcitabine. In a study by Farrell *et al.,* (2009), 229 patients with resectable pancreatic cancer were treated with 5-FU or Gemcitabine. It was found that the levels of hENT1 in pancreatic cancer patients could be used to personalize Gemcitabine therapy, determining who might be responsive to this treatment. Furthermore, the expression of multidrug resistant proteins MDR1 and MDR2 (ABCC1 and 2 genes) were also found to be limiting the efficacy of Gemcitabine. Similarly, Zhi *et al*. (2015) reported a significant decrease of Gemcitabine's $EC_{50}$ when three different pancreatic cancer cell lines where treated with siRNA to inhibit MDR1. These studies corroborate the results obtained in this project, where a significant up-regulation of MDR1 and 2, and a significant down-regulation of hENT1 show an increase

in the mechanisms of resistance by treated HepG2 cells (Fig. 9). Nevertheless, these mechanisms of resistance seem to have negligible effects on the efficacy of both LCB2151 and LCB2132. A future analysis of the protein levels will confirm these significant changes induced by our compounds. Additionally, it would be interesting to further determine the effect of these new prodrugs on the other factors of the mechanism of resistance of cancer cells like deoxycytidine kinase (dCK) and Cytidine deaminase (CDA). This could be done by looking at the expression of these genes through mRNA extraction and qPCRs, and confirming the results with whole cell extracts.

The results in Figs. 7 and 8 also show that Gemcitabine is lowering LCB2151's efficacy. Gemcitabine's mechanism of action could be inducing or decreasing the expression of proteins important for LCB2151's action. For example, LCB2151 could be promoting cell death by dramatically increasing the levels of ROS, while Gemcitabine's mechanism of action could be inhibiting this change, and blocking LCB2151's pathway. This will be discussed later in this discussion. Future experiments could be developed in order to determine the difference in gene expression when cancer cells are co-treated with Gemcitabine and LCB2151. An interesting experiment would be to develop radiolabeled analogues of these two drugs, in order to determine their presence inside or outside the cells through biodistribution studies as done previously by Wu *et al*. (2013). Finally, once the complete mechanism of action of LCB2151 is revealed, it will be easier to speculate the possible confrontations between the mechanisms of action of both drugs.

As previously mentioned, these compounds were synthesized with a lipophilic moiety attached to the nucleoside analogue. We hypothesize that this lipophilic moiety helps these prodrugs' cell entry without the need of active or abundant nucleoside

transporters. This hypothesis was confirmed when cancer cells from HepG2 and Capan-2 cell lines were treated with a combination of either LCB2151, LCB2132, or Gemcitabine with hENT1 inhibitor, NBMPR (Figs. 10 and 11). The potency of both LCB2151 and LCB2132 was independent of NBMPR treatment. On the other hand, the efficacy of Gemcitabine was abolished when it was given in combination of NBMPR, confirming its dependence to nucleoside transporters for cell entry.

The aforementioned results merited the determination of the mechanism of action of LCB2151. Genetic changes of pro-apoptotic and anti-apoptotic genes were studied to determine the mechanisms of cell death induced by this proprietary molecule. The mRNA levels of HepG2 cells treated with LCB2151 or LCB2132 showed a significant up-regulation of pro-apoptotic genes BBC3 and BIK (Fig. 12). Both genes are part of the BCL-2 family of proteins, involved in the activation of apoptosis. BBC3 and BIK neutralize anti-apoptotic proteins from the BCL-2 family, by binding to them through the BH3 domain, allowing BAK and BAX to engage in the formation of the mitochondrial outer membrane permeabilization and cytochrome c release (Chipuk & Green, 2008; Westphal *et al.*, 2011). The significant up-regulation of protein levels of BBC3, and the significant down-regulation of BCL-2 protein, would lead to an increased ratio of pro/anti-apoptotic proteins which is consistent with an initiation of an apoptotic response to LCB2151 (Fig. 13).

The tumor suppressor p53 protein has been widely studied due to its important functions as a regulator of apoptosis, cell cycle arrest, and senescence (Amaral, Xavier, Steer, & Rodrigues, 2010). Furthermore, it is well known that half the cancer patients have tumors harboring a mutant p53 gene (Brown, Lain, Verma, Fersht, & Lane, 2009). p53 is considered an important target in various cancer therapies, and thus the expression levels

of p53 and its downstream target p21 were assessed via qPCR (Fig. 11). The results showed no significant changes were induced by either LCB2151 or LCB2132 treatments. Furthermore, activated p53 was not detected in extracts from HepG2 cells treated with LCB2151 (Fig. 13). This result was not expected, given the significant up-regulation of BBC3, a well-known pro-apoptotic protein directly activated by p53 transcription factor (Han *et al*., 2001). Furthermore, Hill *et al*. reported that gemcitabine induced apoptosis by p53 activation and in a BBC3 (PUMA)-dependent manner (Hill *et al*., 2013). This result further confirms the difference in the mechanism of action of Gemcitabine and LCB2151.

According to mRNA and protein results, apoptosis is the likely mechanism by which LCB2151 was inducing cancer cell death. However, and despite the fact that necrosis was known to be a disorganized cell death mechanism, various recent studies have shown that in many cases necrosis can be programmed and thus might share certain factors and signals with apoptosis. One form of programmed necrosis termed necroptosis, is usually activated by the inhibition of caspase 8 by Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (Fulda, 2014; Hitomi *et al*., 2008; Linkermann *et al*., 2013; Vanden Berghe *et al*., 2014). Therefore, it was important to determine whether the cells treated with LCB2151 were dying through apoptosis and/or necroptosis. Given that apoptosis is caspase dependent, in which a cascade of initiator and effector caspases like caspase 3 are activated, HepG2 cells were treated with a combination of LCB2151 and the caspase 3 inhibitor, DEVD. Other cells were treated with a combination of LCB2151 and RIPK1 inhibitor, Necrostatin-1. Inhibiting the effector caspase (caspase 3), showed a significant shift of the response curve of LCB2151 (Fig. 16A). Meanwhile, the inhibition of RIPK-1 did not have an effect on the response curve of the drug. Autophagy was another

option of cell death induced by LCB2151. This type of cell death involves some of BCL-2 family proteins that participate in apoptosis (Hanahan & Weinberg, 2011). An immunofluorescence assay in which Microtubule-associated protein 1A/1B-light chain 3 (LC3) protein was stained, dismissed the possibility of autophagy (Fig. 16B). These results pooled together confirm that both LCB2151 and LCB2132 induce a stress response in cancer cells that culminates in apoptosis.

QPCR analysis shows a significant increase in the levels of NADPH oxidase (P67 gene) in cells treated with LCB2151 or LCB2132 (Fig. 17). This enzyme, usually up-regulated in tumors, is known to promote the formation of reactive oxygen species (ROS) that enhances tumor angiogenesis and the stabilization and activation of hypoxia-inducible factor 1 (HIF-1), which can also promote neovascularization through secretion of VEGF (Ushio-Fukai & Nakamura, 2008). Nevertheless, the LCB2151 and LCB2132 induced increase was even higher than the P67 elevated levels frequently seen in cancer cells. This might be enhancing the production of ROS beyond the capacity of cancer cells to cope with and thus helping in the permeabilization of the mitochondrial membrane and ultimately causing cell death (Reinehr *et al.*, 2005). On the contrary, treatment with Gemcitabine did not show expression of NADPH oxidase. As mentioned earlier, Gemcitabine up-regulates the expression of UCP2, which acts as an antioxidant and decreases the production of ROS. This mechanism could explain the inhibitory effect between Gemcitabine and LCB2151 mentioned before. Furthermore, LCB2151 and LCB2132 also caused a significant down-regulation of mRNA levels of super oxide dismutase 1 (SOD1). The increased activity of NADPH renders cancer cells dependent on super oxide dismutase (SOD) for survival. Donate *et al.*, (2008) and Juarez *et al*. (2006) reported an anti-proliferative and pro-

apoptotic effects of SOD1 inhibition and Copper levels depletion. Therefore, these prodrug-induced changes in the levels of the above stress markers seem to be key to the induction of apoptosis by LCB2151 and LCB2132. Further mitochondrial stress experiments would be useful to determine this hypothesis. Oxygen consumption would be measured in cancer cells treated with LCB2151 or Gemcitabine to detect changes in their metabolism. This could be done using the XF Extracellular Flux Analyzer (Seahorse Bioscience).

Additional studies are still needed in order to further elucidate the mechanism(s) of action of these novel nucleoside analogues. For instance, it is important to determine whether these molecules interfere with DNA synthesis by DNA incorporation. Mass spectrometry results showed that LCB2151 is a stable compound that does not get cleaved into a nucleoside analogue and a lipid moiety. However, further experiments using this technique are need to determine if LCB2151 gets tri-phosphorylated to become an active compound. Furthermore, a cancer mouse model is an indispensable venue in determining the effect of these drugs *in vivo*. In a previous pilot *in vivo* study done by a postdoc in the lab (results not shown), BxPC3 tumor xenografts were injected intraperitoneally (*i.p.*) in Nod-SCID mice (n=3 per group). Then they were treated with an i.p. injection of either 20 mg/Kg of LCB2151 (5 injections every other day), 20 mg/kg of Gemcitabine, or DMSO. In 30% of the mice treated with LCB2151, tumor growth was arrested, and in 20% of these mice tumors regressed or even disappeared. Meanwhile, all of the mice treated with Gemcitabine died after the third *i.p.* injection due to general organ failure. A bigger *in vivo* study is ultimately required to accurately determine the most effective treatment regimen with minimal off target toxicity. These experiments will be performed by another member

of the Nemer Lab who will continue the project and will help determining the biological profiles of LCB2151.

In 2013, cancer became the second leading cause of death (behind cardiovascular diseases), with over 8 million deaths worldwide (Global Burden of Disease Cancer Collaboration *et al*., 2015). Scientists are therefore trying to find new avenues of therapies to overcome this medical hurdle. 150 molecules were tested in this project, from which 2 were chosen as the most potent ones against pancreatic and liver cancer cell lines. This research project showed how a new generation of nucleoside analogues could have a position among the new anticancer treatments used in clinical trials. LCB2151 was chosen for its high efficacy to determine its mechanism of action. We found that this new molecule has the ability to kill Gemcitabine-resistant pancreatic and liver cancer cells. This exciting discovery could easily change the future of cancer treatment, since Gemcitabine-resistant cells are widely known to be resistant to other treatments available in the market. We further determined some of the mechanism of action of this compound. LCB2151 was found to induce the expression of pro-apoptotic genes, and it was later confirmed through caspase 3 inhibition, that in fact this molecule induces an apoptotic response in cancer cells. Additionally, LCB2151 causes an important induction in the oxidative protein NADPH oxidase and it down-regulates the expression of the antioxidant protein Superoxide dismutase 1. This makes us believe that LCB2151 has a major role in the cancer cells' redox balance that could influence an apoptotic trigger. More experiments are being planned in order to further elucidate this molecule's mechanism(s) of action. Furthermore, *in vivo* studies would be an important next step to confirm the efficacy of LCB2151 in mice bearing tumors.

Currently, gastrointestinal cancers do not have a curative treatment, besides tumor resection. Additionally, the incidence of pancreatic and liver cancers has increased over the last decade, and it might continue to rise. The results obtained so far are promising, and can potentially pave the way for the development of chemotherapeutic agents with enhanced efficiency against one of the deadliest cancers.

References

Abbas, T., & Dutta, A. (2009). P21 in cancer: Intricate networks and multiple activities. *Nature Reviews.Cancer, 9*(6), 400-414. doi:10.1038/nrc2657 [doi]

Alderton, G. K., & Bordon, Y. (2012). Tumour immunotherapy--leukocytes take up the fight. *Nature Reviews.Immunology, 12*(4), 237.

Aleksandrova, K., Boeing, H., Nothlings, U., Jenab, M., Fedirko, V., Kaaks, R., . . . Pischon, T. (2014). Inflammatory and metabolic biomarkers and risk of liver and biliary tract cancer. *Hepatology (Baltimore, Md.), 60*(3), 858-871. doi:10.1002/hep.27016 [doi]

Alian, O. M., Azmi, A. S., & Mohammad, R. M. (2012). Network insights on oxaliplatin anti-cancer mechanisms. *Clinical and Translational Medicine, 1*, 26-1326-1-26. doi:2001-1326-1-26 [pii]

Alves, R. C., Alves, D., Guz, B., Matos, C., Viana, M., Harriz, M., . . . Polletti, P. (2011). Advanced hepatocellular carcinoma. review of targeted molecular drugs. *Annals of Hepatology, 10*(1), 21-27. doi:931872 [pii]

Amaral, J. D., Xavier, J. M., Steer, C. J., & Rodrigues, C. M. (2010). The role of p53 in apoptosis. *Discovery Medicine, 9*(45), 145-152.

Aries, A., Paradis, P., Lefebvre, C., Schwartz, R. J., & Nemer, M. (2004). Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proceedings of the*

*National Academy of Sciences of the United States of America, 101*(18), 6975-6980. doi:10.1073/pnas.0401833101 [doi]

Azrak, R. G., Cao, S., Slocum, H. K., Toth, K., Durrani, F. A., Yin, M. B., . . . Rustum, Y. M. (2004). Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 10*(3), 1121-1129.

Bardeesy, N., & DePinho, R. A. (2002). Pancreatic cancer biology and genetics. *Nature Reviews.Cancer, 2*(12), 897-909. doi:10.1038/nrc949 [doi]

Bardenheuer, W., Lehmberg, K., Rattmann, I., Brueckner, A., Schneider, A., Sorg, U. R., . . . Flasshove, M. (2005). Resistance to cytarabine and gemcitabine and in vitro selection of transduced cells after retroviral expression of cytidine deaminase in human hematopoietic progenitor cells. *Leukemia, 19*(12), 2281-2288. doi:2403977 [pii]

Bender, D. M., Bao, J., Dantzig, A. H., Diseroad, W. D., Law, K. L., Magnus, N. A., . . . McCarthy, J. R. (2009). Synthesis, crystallization, and biological evaluation of an orally active prodrug of gemcitabine. *Journal of Medicinal Chemistry, 52*(22), 6958-6961. doi:10.1021/jm901181h [doi]

Bengala, C., Guarneri, V., Giovannetti, E., Lencioni, M., Fontana, E., Mey, V., . . . Conte, P. F. (2005). Prolonged fixed dose rate infusion of gemcitabine with autologous haemopoietic support in advanced pancreatic adenocarcinoma. *British Journal of Cancer, 93*(1), 35-40. doi:6602673 [pii]

Bepler, G., Kusmartseva, I., Sharma, S., Gautam, A., Cantor, A., Sharma, A., & Simon, G. (2006). RRM1 modulated in vitro and in vivo efficacy of gemcitabine and platinum in non-small-cell lung cancer. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 24*(29), 4731-4737. doi:JCO.2006.06.1101 [pii]

Bergmann, U., Funatomi, H., Yokoyama, M., Beger, H. G., & Korc, M. (1995). Insulin-like growth factor I overexpression in human pancreatic cancer: Evidence for autocrine and paracrine roles. *Cancer Research, 55*(10), 2007-2011.

Boivin, A. J., Gourdeau, H., & Momparler, R. L. (2004). Action of troxacitabine on cells transduced with human cytidine deaminase cDNA. *Cancer Investigation, 22*(1), 25-29.

Brown, C. J., Lain, S., Verma, C. S., Fersht, A. R., & Lane, D. P. (2009). Awakening guardian angels: Drugging the p53 pathway. *Nature Reviews.Cancer, 9*(12), 862-873. doi:10.1038/nrc2763 [doi]

Bruix, J., Sherman, M., & American Association for the Study of Liver Diseases. (2011). Management of hepatocellular carcinoma: An update. *Hepatology (Baltimore, Md.), 53*(3), 1020-1022. doi:10.1002/hep.24199 [doi]

Cairns, R. A., Harris, I. S., & Mak, T. W. (2011). Regulation of cancer cell metabolism. *Nature Reviews.Cancer, 11*(2), 85-95. doi:10.1038/nrc2981; 10.1038/nrc2981

Caldwell, G. W., Yan, Z., Lang, W., & Masucci, J. A. (2012). The IC(50) concept revisited. *Current Topics in Medicinal Chemistry, 12*(11), 1282-1290. doi:CTMC-EPub-20120507-007 [pii]

Caliceti, P., & Veronese, F. M. (2003). Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Advanced Drug Delivery Reviews, 55*(10), 1261-1277. doi:S0169409X0300108X [pii]

Cappella, P., Tomasoni, D., Faretta, M., Lupi, M., Montalenti, F., Viale, F., . . . Ubezio, P. (2001). Cell cycle effects of gemcitabine. *International Journal of Cancer.Journal International Du Cancer, 93*(3), 401-408. doi:10.1002/ijc.1351 [pii]

Cattel, L., Ceruti, M., & Dosio, F. (2003). From conventional to stealth liposomes: A new frontier in cancer chemotherapy. *Tumori, 89*(3), 237-249.

Cerqueira, N. M., Fernandes, P. A., & Ramos, M. J. (2007). Understanding ribonucleotide reductase inactivation by gemcitabine. *Chemistry (Weinheim an Der Bergstrasse, Germany), 13*(30), 8507-8515. doi:10.1002/chem.200700260 [doi]

Chan, K. S., Koh, C. G., & Li, H. Y. (2012). Mitosis-targeted anti-cancer therapies: Where they stand. *Cell Death & Disease, 3*, e411. doi:10.1038/cddis.2012.148 [doi]

Chang, M. C., Wong, J. M., & Chang, Y. T. (2014). Screening and early detection of pancreatic cancer in high risk population. *World Journal of Gastroenterology : WJG, 20*(9), 2358-2364. doi:10.3748/wjg.v20.i9.2358; 10.3748/wjg.v20.i9.2358

Chapdelaine, D., Cardinal-David, B., Prevost, M., Gagnon, M., Thumin, I., & Guindon, Y. (2009). A stereoselective approach to nucleosides and 4'-thioanalogues from acyclic precursors. *Journal of the American Chemical Society, 131*(47), 17242-17245. doi:10.1021/ja905452f; 10.1021/ja905452f

Chipuk, J. E., & Green, D. R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends in Cell Biology, 18*(4), 157-164. doi:10.1016/j.tcb.2008.01.007 [doi]

Chou, T. C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews, 58*(3), 621-681. doi:58/3/621 [pii]

Chou, T. C. (2008). Preclinical versus clinical drug combination studies. *Leukemia & Lymphoma, 49*(11), 2059-2080. doi:10.1080/10428190802353591 [doi]

Chuang, K. H., Wang, H. E., Chen, F. M., Tzou, S. C., Cheng, C. M., Chang, Y. C., . . . Cheng, T. L. (2010). Endocytosis of PEGylated agents enhances cancer imaging and anticancer efficacy. *Molecular Cancer Therapeutics, 9*(6), 1903-1912. doi:10.1158/1535-7163.MCT-09-0899 [doi]

Clark, T., Maximin, S., Meier, J., Pokharel, S., & Bhargava, P. (2015). Hepatocellular carcinoma: Review of epidemiology, screening, imaging diagnosis, response assessment, and treatment. *Current Problems in Diagnostic Radiology,* doi:S0363-0188(15)00059-6 [pii]

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., Becouarn, Y., . . . PRODIGE Intergroup. (2011a). FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *The New England Journal of Medicine, 364*(19), 1817-1825. doi:10.1056/NEJMoa1011923 [doi]

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., Becouarn, Y., . . . PRODIGE Intergroup. (2011b). FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *The New England Journal of Medicine, 364*(19), 1817-1825. doi:10.1056/NEJMoa1011923 [doi]

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nature Reviews.Molecular Cell Biology, 15*(1), 49-63. doi:10.1038/nrm3722 [doi]

Dalla Pozza, E., Fiorini, C., Dando, I., Menegazzi, M., Sgarbossa, A., Costanzo, C., . . . Donadelli, M. (2012). Role of mitochondrial uncoupling protein 2 in cancer cell resistance to gemcitabine. *Biochimica Et Biophysica Acta, 1823*(10), 1856-1863. doi:10.1016/j.bbamcr.2012.06.007 [doi]

Davis, M., Conlon, K., Bohac, G. C., Barcenas, J., Leslie, W., Watkins, L., . . . Plate, J. M. (2012). Effect of pemetrexed on innate immune killer cells and adaptive immune T cells in subjects with adenocarcinoma of the pancreas. *Journal of Immunotherapy (Hagerstown, Md.: 1997), 35*(8), 629-640. doi:10.1097/CJI.0b013e31826c8a4f [doi]

Deer, E. L., Gonzalez-Hernandez, J., Coursen, J. D., Shea, J. E., Ngatia, J., Scaife, C. L., . . . Mulvihill, S. J. (2010). Phenotype and genotype of pancreatic cancer cell lines.

*Pancreas, 39*(4), 425-435. doi:10.1097/MPA.0b013e3181c15963; 10.1097/MPA.0b013e3181c15963

Doll, R., Peto, R., Wheatley, K., Gray, R., & Sutherland, I. (1994). Mortality in relation to smoking: 40 years' observations on male british doctors. *BMJ (Clinical Research Ed.), 309*(6959), 901-911.

Donate, F., Juarez, J. C., Burnett, M. E., Manuia, M. M., Guan, X., Shaw, D. E., . . . Mazar, A. P. (2008). Identification of biomarkers for the antiangiogenic and antitumour activity of the superoxide dismutase 1 (SOD1) inhibitor tetrathiomolybdate (ATN-224). *British Journal of Cancer, 98*(4), 776-783. doi:10.1038/sj.bjc.6604226 [doi]

Ducreux, M., Mitry, E., Ould-Kaci, M., Boige, V., Seitz, J. F., Bugat, R., . . . Rougier, P. (2004). Randomized phase II study evaluating oxaliplatin alone, oxaliplatin combined with infusional 5-FU, and infusional 5-FU alone in advanced pancreatic carcinoma patients. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO, 15*(3), 467-473.

Duplessis, M., Waltz, M. E., Bencheqroun, M., Cardinal-David, B., & Guindon, Y. (2009). Stereoselective quaternary center construction via atom-transfer radical cyclization using silicon tethers on acyclic precursors. *Organic Letters, 11*(14), 3148-3151. doi:10.1021/ol901126y [doi]

Duxbury, M. S., Ito, H., Zinner, M. J., Ashley, S. W., & Whang, E. E. (2004). RNA interference targeting the M2 subunit of ribonucleotide reductase enhances

pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene, 23*(8), 1539-1548. doi:10.1038/sj.onc.1207272 [doi]

Eberle, M. A., Pfutzer, R., Pogue-Geile, K. L., Bronner, M. P., Crispin, D., Kimmey, M. B., . . . Brentnall, T. A. (2002). A new susceptibility locus for autosomal dominant pancreatic cancer maps to chromosome 4q32-34. *American Journal of Human Genetics, 70*(4), 1044-1048. doi:S0002-9297(07)60314-0 [pii]

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516. doi:779478428 [pii]

Eser, S., Schnieke, A., Schneider, G., & Saur, D. (2014). Oncogenic KRAS signalling in pancreatic cancer. *British Journal of Cancer, 111*(5), 817-822. doi:10.1038/bjc.2014.215 [doi]

Fan, H., Huang, A., Villegas, C., & Wright, J. A. (1997). The R1 component of mammalian ribonucleotide reductase has malignancy-suppressing activity as demonstrated by gene transfer experiments. *Proceedings of the National Academy of Sciences of the United States of America, 94*(24), 13181-13186.

Fan, H., Villegas, C., Huang, A., & Wright, J. A. (1998). The mammalian ribonucleotide reductase R2 component cooperates with a variety of oncogenes in mechanisms of cellular transformation. *Cancer Research, 58*(8), 1650-1653.

Farrell, J. J., Elsaleh, H., Garcia, M., Lai, R., Ammar, A., Regine, W. F., . . . Mackey, J. R. (2009). Human equilibrative nucleoside transporter 1 levels predict response to

gemcitabine in patients with pancreatic cancer. *Gastroenterology, 136*(1), 187-195. doi:10.1053/j.gastro.2008.09.067 [doi]

Flejou, J. F. (2011). WHO classification of digestive tumors: The fourth edition. [Classification OMS 2010 des tumeurs digestives: la quatrieme edition] *Annales De Pathologie, 31*(5 Suppl), S27-31. doi:10.1016/j.annpat.2011.08.001 [doi]

Forner, A., Reig, M. E., de Lope, C. R., & Bruix, J. (2010). Current strategy for staging and treatment: The BCLC update and future prospects. *Seminars in Liver Disease, 30*(1), 61-74. doi:10.1055/s-0030-1247133 [doi]

Fryer, R. A., Barlett, B., Galustian, C., & Dalgleish, A. G. (2011). Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitisation by the iMiD lenalidomide. *Anticancer Research, 31*(11), 3747-3756.

Fulda, S. (2014). Therapeutic exploitation of necroptosis for cancer therapy. *Seminars in Cell & Developmental Biology, 35*, 51-56. doi:10.1016/j.semcdb.2014.07.002 [doi]

Gaddikeri, S., McNeeley, M. F., Wang, C. L., Bhargava, P., Dighe, M. K., Yeh, M. M., . . . Lalwani, N. (2014). Hepatocellular carcinoma in the noncirrhotic liver. *AJR.American Journal of Roentgenology, 203*(1), W34-47. doi:10.2214/AJR.13.11511 [doi]

Galmarini, C. M., Mackey, J. R., & Dumontet, C. (2002). Nucleoside analogues and nucleobases in cancer treatment. *The Lancet.Oncology, 3*(7), 415-424.

Gilbert, J. A., Salavaggione, O. E., Ji, Y., Pelleymounter, L. L., Eckloff, B. W., Wieben, E. D., . . . Weinshilboum, R. M. (2006). Gemcitabine pharmacogenomics: Cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 12*(6), 1794-1803. doi:12/6/1794 [pii]

Giovannetti, E., Del Tacca, M., Mey, V., Funel, N., Nannizzi, S., Ricci, S., . . . Danesi, R. (2006). Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Research, 66*(7), 3928-3935. doi:66/7/3928 [pii]

Global Burden of Disease Cancer Collaboration, Fitzmaurice, C., Dicker, D., Pain, A., Hamavid, H., Moradi-Lakeh, M., . . . Naghavi, M. (2015). The global burden of cancer 2013. *JAMA Oncology, 1*(4), 505-527. doi:10.1001/jamaoncol.2015.0735 [doi]

Hamacher, R., Schmid, R. M., Saur, D., & Schneider, G. (2008). Apoptotic pathways in pancreatic ductal adenocarcinoma. *Molecular Cancer, 7*, 64-4598-7-64. doi:10.1186/1476-4598-7-64 [doi]

Han, J., Flemington, C., Houghton, A. B., Gu, Z., Zambetti, G. P., Lutz, R. J., . . . Chittenden, T. (2001). Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proceedings of the National Academy of Sciences of the United States of America, 98*(20), 11318-11323. doi:10.1073/pnas.201208798 [doi]

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013 [doi]

Harlan, L. C., Parsons, H. M., Wiggins, C. L., Stevens, J. L., & Patt, Y. Z. (2015). Treatment of hepatocellular carcinoma in the community: Disparities in standard therapy. *Liver Cancer, 4*(1), 70-83. doi:10.1159/000367729 [doi]

Harrap, K. R. (1985). Preclinical studies identifying carboplatin as a viable cisplatin alternative. *Cancer Treatment Reviews, 12 Suppl A*, 21-33.

Harris, J. M., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nature Reviews.Drug Discovery, 2*(3), 214-221. doi:10.1038/nrd1033 [doi]

He, W., Zhao, H., Chan, W., Lopez, D., Shroff, R. T., & Giordano, S. H. (2015). Underuse of surgical resection among elderly patients with early-stage pancreatic cancer. *Surgery,* doi:S0039-6060(15)00360-8 [pii]

Heinemann, V., Hertel, L. W., Grindey, G. B., & Plunkett, W. (1988). Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine. *Cancer Research, 48*(14), 4024-4031.

Hill, R., Rabb, M., Madureira, P. A., Clements, D., Gujar, S. A., Waisman, D. M., . . . Lee, P. W. (2013). Gemcitabine-mediated tumour regression and p53-dependent gene expression: Implications for colon and pancreatic cancer therapy. *Cell Death & Disease, 4*, e791. doi:10.1038/cddis.2013.307 [doi]

Hitomi, J., Christofferson, D. E., Ng, A., Yao, J., Degterev, A., Xavier, R. J., & Yuan, J. (2008). Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell, 135*(7), 1311-1323. doi:10.1016/j.cell.2008.10.044 [doi]

Hodge, L. S., Taub, M. E., & Tracy, T. S. (2011). The deaminated metabolite of gemcitabine, 2',2'-difluorodeoxyuridine, modulates the rate of gemcitabine transport and intracellular phosphorylation via deoxycytidine kinase. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 39*(11), 2013-2016. doi:10.1124/dmd.111.040790 [doi]

Hortobagyi, G. N. (1997). Anthracyclines in the treatment of cancer. an overview. *Drugs, 54 Suppl 4*, 1-7.

Horwitz, S. B. (1994). Taxol (paclitaxel): Mechanisms of action. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO, 5 Suppl 6*, S3-6.

Huang, D., Zhang, Y., & Chen, X. (2003). Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquid chromatography. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences, 784*(1), 101-109.

Hui, Y. F., & Reitz, J. (1997). Gemcitabine: A cytidine analogue active against solid tumors. *American Journal of Health-System Pharmacy : AJHP : Official Journal of the American Society of Health-System Pharmacists, 54*(2), 162-70; quiz 197-8.

Jiang, X. (2005). Random mutagenesis of the B'A' core domain of yeast DNA topoisomerase II and large-scale screens of mutants resistant to the anticancer drug etoposide. *Biochemical and Biophysical Research Communications, 327*(2), 597-603. doi:S0006-291X(04)02847-5 [pii]

Johansson, N. G., & Eriksson, S. (1996). Structure-activity relationships for phosphorylation of nucleoside analogs to monophosphates by nucleoside kinases. *Acta Biochimica Polonica, 43*(1), 143-160.

Jordan, M. A., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nature Reviews.Cancer, 4*(4), 253-265. doi:10.1038/nrc1317 [doi]

Jordheim, L. P., & Dumontet, C. (2007). Review of recent studies on resistance to cytotoxic deoxynucleoside analogues. *Biochimica Et Biophysica Acta, 1776*(2), 138-159. doi:S0304-419X(07)00024-8 [pii]

Jordheim, L. P., Durantel, D., Zoulim, F., & Dumontet, C. (2013a). Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nature Reviews.Drug Discovery, 12*(6), 447-464. doi:10.1038/nrd4010; 10.1038/nrd4010

Jordheim, L. P., Durantel, D., Zoulim, F., & Dumontet, C. (2013b). Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nature Reviews.Drug Discovery, 12*(6), 447-464. doi:10.1038/nrd4010 [doi]

Juarez, J. C., Betancourt, O.,Jr, Pirie-Shepherd, S. R., Guan, X., Price, M. L., Shaw, D. E., . . . Donate, F. (2006). Copper binding by tetrathiomolybdate attenuates angiogenesis and tumor cell proliferation through the inhibition of superoxide dismutase 1. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 12*(16), 4974-4982. doi:12/16/4974 [pii]

Kaltsas, S., Syrigos, K. N., & Saif, M. W. (2014). Pancreatic cancer in 2014. *JOP : Journal of the Pancreas, 15*(2), 84-86. doi:10.6092/1590-8577/2403

Kelland, L. (2007). The resurgence of platinum-based cancer chemotherapy. *Nature Reviews.Cancer, 7*(8), 573-584. doi:nrc2167 [pii]

Kim, R., Tan, A., Lai, K. K., Jiang, J., Wang, Y., Rybicki, L. A., & Liu, X. (2011). Prognostic roles of human equilibrative transporter 1 (hENT-1) and ribonucleoside reductase subunit M1 (RRM1) in resected pancreatic cancer. *Cancer, 117*(14), 3126-3134. doi:10.1002/cncr.25883 [doi]

Kindler, H. L., Richards, D. A., Garbo, L. E., Garon, E. B., Stephenson, J. J.,Jr, Rocha-Lima, C. M., . . . Fuchs, C. S. (2012). A randomized, placebo-controlled phase 2 study of ganitumab (AMG 479) or conatumumab (AMG 655) in combination with gemcitabine in patients with metastatic pancreatic cancer. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO, 23*(11), 2834-2842. doi:10.1093/annonc/mds142 [doi]

Kyriazis, A. A., Kyriazis, A. P., Sternberg, C. N., Sloane, N. H., & Loveless, J. D. (1986). Morphological, biological, biochemical, and karyotypic characteristics of

human pancreatic ductal adenocarcinoma capan-2 in tissue culture and the nude mouse. *Cancer Research, 46*(11), 5810-5815.

Laverty, H., Benson, C., Cartwright, E., Cross, M., Garland, C., Hammond, T., . . . Valentin, J. (2011). How can we improve our understanding of cardiovascular safety liabilities to develop safer medicines? *British Journal of Pharmacology, 163*(4), 675-693. doi:10.1111/j.1476-5381.2011.01255.x [doi]

Li, D., Xie, K., Wolff, R., & Abbruzzese, J. L. (2004). Pancreatic cancer. *Lancet (London, England), 363*(9414), 1049-1057. doi:10.1016/S0140-6736(04)15841-8 [doi]

Li, L., & Wang, H. (2015). Heterogeneity of liver cancer and personalized therapy. *Cancer Letters,* doi:S0304-3835(15)00473-5 [pii]

Linkermann, A., Brasen, J. H., Darding, M., Jin, M. K., Sanz, A. B., Heller, J. O., . . . Krautwald, S. (2013). Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America, 110*(29), 12024-12029. doi:10.1073/pnas.1305538110 [doi]

Liu, L. F. (1989). DNA topoisomerase poisons as antitumor drugs. *Annual Review of Biochemistry, 58*, 351-375. doi:10.1146/annurev.bi.58.070189.002031 [doi]

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., . . . SHARP Investigators Study Group. (2008). Sorafenib in advanced hepatocellular carcinoma.

*The New England Journal of Medicine, 359*(4), 378-390. doi:10.1056/NEJMoa0708857; 10.1056/NEJMoa0708857

Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-fluorouracil: Mechanisms of action and clinical strategies. *Nature Reviews.Cancer, 3*(5), 330-338. doi:10.1038/nrc1074 [doi]

Lowenfels, A. B., & Maisonneuve, P. (2004). Epidemiology and prevention of pancreatic cancer. *Japanese Journal of Clinical Oncology, 34*(5), 238-244.

Lynch, H. T., Smyrk, T., Kern, S. E., Hruban, R. H., Lightdale, C. J., Lemon, S. J., . . . Ghadirian, P. (1996). Familial pancreatic cancer: A review. *Seminars in Oncology, 23*(2), 251-275.

Mack, T. M., Yu, M. C., Hanisch, R., & Henderson, B. E. (1986). Pancreas cancer and smoking, beverage consumption, and past medical history. *Journal of the National Cancer Institute, 76*(1), 49-60.

Maharsy, W., Aries, A., Mansour, O., Komati, H., & Nemer, M. (2014). Ageing is a risk factor in imatinib mesylate cardiotoxicity. *European Journal of Heart Failure, 16*(4), 367-376. doi:10.1002/ejhf.58 [doi]

Mans, D. R., Grivicich, I., Peters, G. J., & Schwartsmann, G. (1999). Sequence-dependent growth inhibition and DNA damage formation by the irinotecan-5-fluorouracil combination in human colon carcinoma cell lines. *European Journal of*

*Cancer (Oxford, England : 1990), 35*(13), 1851-1861. doi:S0959-8049(99)00222-1 [pii]

Mhaidat, N. M., Alali, F. Q., Matalqah, S. M., Matalka, I. I., Jaradat, S. A., Al-Sawalha, N. A., & Thorne, R. F. (2009). Inhibition of MEK sensitizes paclitaxel-induced apoptosis of human colorectal cancer cells by downregulation of GRP78. *Anti-Cancer Drugs, 20*(7), 601-606. doi:10.1097/CAD.0b013e32832e3120 [doi]

Mirmohammadsadegh, A., Mota, R., Gustrau, A., Hassan, M., Nambiar, S., Marini, A., . . . Hengge, U. R. (2007). ERK1/2 is highly phosphorylated in melanoma metastases and protects melanoma cells from cisplatin-mediated apoptosis. *The Journal of Investigative Dermatology, 127*(9), 2207-2215. doi:5700870 [pii]

Moore, M. J., Goldstein, D., Hamm, J., Figer, A., Hecht, J. R., Gallinger, S., . . . National Cancer Institute of Canada Clinical Trials Group. (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: A phase III trial of the national cancer institute of canada clinical trials group. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 25*(15), 1960-1966. doi:JCO.2006.07.9525 [pii]

Mori, R., Ishikawa, T., Ichikawa, Y., Taniguchi, K., Matsuyama, R., Ueda, M., . . . Shimada, H. (2007). Human equilibrative nucleoside transporter 1 is associated with the chemosensitivity of gemcitabine in human pancreatic adenocarcinoma and biliary tract carcinoma cells. *Oncology Reports, 17*(5), 1201-1205.

Moysan, E., Bastiat, G., & Benoit, J. P. (2013). Gemcitabine versus modified gemcitabine: A review of several promising chemical modifications. *Molecular Pharmaceutics, 10*(2), 430-444. doi:10.1021/mp300370t [doi]

Nakano, Y., Tanno, S., Koizumi, K., Nishikawa, T., Nakamura, K., Minoguchi, M., . . . Kohgo, Y. (2007). Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. *British Journal of Cancer, 96*(3), 457-463. doi:6603559 [pii]

Nordlund, P., & Reichard, P. (2006). Ribonucleotide reductases. *Annual Review of Biochemistry, 75*, 681-706. doi:10.1146/annurev.biochem.75.103004.142443 [doi]

Oguri, T., Achiwa, H., Sato, S., Bessho, Y., Takano, Y., Miyazaki, M., . . . Ueda, R. (2006). The determinants of sensitivity and acquired resistance to gemcitabine differ in non-small cell lung cancer: A role of ABCC5 in gemcitabine sensitivity. *Molecular Cancer Therapeutics, 5*(7), 1800-1806. doi:5/7/1800 [pii]

Park, E. J., Lee, J. H., Yu, G. Y., He, G., Ali, S. R., Holzer, R. G., . . . Karin, M. (2010). Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell, 140*(2), 197-208. doi:10.1016/j.cell.2009.12.052 [doi]

Pasut, G., & Veronese, F. M. (2012). State of the art in PEGylation: The great versatility achieved after forty years of research. *Journal of Controlled Release : Official Journal of the Controlled Release Society, 161*(2), 461-472. doi:10.1016/j.jconrel.2011.10.037 [doi]

Patel, S. R., Gandhi, V., Jenkins, J., Papadopolous, N., Burgess, M. A., Plager, C., . . . Benjamin, R. S. (2001). Phase II clinical investigation of gemcitabine in advanced soft tissue sarcomas and window evaluation of dose rate on gemcitabine triphosphate accumulation. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 19*(15), 3483-3489.

Paul, M. K., & Mukhopadhyay, A. K. (2004). Tyrosine kinase - role and significance in cancer. *International Journal of Medical Sciences, 1*(2), 101-115.

Perez-Torras, S., Garcia-Manteiga, J., Mercade, E., Casado, F. J., Carbo, N., Pastor-Anglada, M., & Mazo, A. (2008). Adenoviral-mediated overexpression of human equilibrative nucleoside transporter 1 (hENT1) enhances gemcitabine response in human pancreatic cancer. *Biochemical Pharmacology, 76*(3), 322-329. doi:10.1016/j.bcp.2008.05.011 [doi]

Philip, P. A., Benedetti, J., Corless, C. L., Wong, R., O'Reilly, E. M., Flynn, P. J., . . . Blanke, C. D. (2010). Phase III study comparing gemcitabine plus cetuximab versus gemcitabine in patients with advanced pancreatic adenocarcinoma: Southwest oncology group-directed intergroup trial S0205. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 28*(22), 3605-3610. doi:10.1200/JCO.2009.25.7550 [doi]

Plate, J. M., & Harris, J. E. (2000). Immune cell functions in pancreatic cancer. *Critical Reviews in Immunology, 20*(5), 375-392.

Reinehr, R., Becker, S., Eberle, A., Grether-Beck, S., & Haussinger, D. (2005). Involvement of NADPH oxidase isoforms and src family kinases in CD95-dependent hepatocyte apoptosis. *The Journal of Biological Chemistry, 280*(29), 27179-27194. doi:M414361200 [pii]

Rofougaran, R., Vodnala, M., & Hofer, A. (2006). Enzymatically active mammalian ribonucleotide reductase exists primarily as an alpha6beta2 octamer. *The Journal of Biological Chemistry, 281*(38), 27705-27711. doi:M605573200 [pii]

ROSENBERG, B., VANCAMP, L., & KRIGAS, T. (1965). Inhibition of cell division in escherichia coli by electrolysis products from a platinum electrode. *Nature, 205*, 698-699.

Sabini, E., Hazra, S., Konrad, M., Burley, S. K., & Lavie, A. (2007). Structural basis for activation of the therapeutic L-nucleoside analogs 3TC and troxacitabine by human deoxycytidine kinase. *Nucleic Acids Research, 35*(1), 186-192. doi:gkl1038 [pii]

Saiki, Y., Yoshino, Y., Fujimura, H., Manabe, T., Kudo, Y., Shimada, M., . . . Horii, A. (2012). DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells. *Biochemical and Biophysical Research Communications, 421*(1), 98-104. doi:10.1016/j.bbrc.2012.03.122 [doi]

Samulitis, B. K., Pond, K. W., Pond, E., Cress, A. E., Patel, H., Wisner, L., . . . Landowski, T. H. (2015). Gemcitabine resistant pancreatic cancer cell lines acquire an invasive phenotype with collateral hypersensitivity to histone deacetylase

inhibitors. *Cancer Biology & Therapy, 16*(1), 43-51. doi:10.4161/15384047.2014.986967 [doi]

Saran, A., & Chatterjee, C. L. (1980). Molecular orbital studies on the structure of nucleoside analogs. III. conformation of 3-deazapyrimidine nucleosides. *Biochimica Et Biophysica Acta, 607*(3), 490-502.

Shah, A. N., Summy, J. M., Zhang, J., Park, S. I., Parikh, N. U., & Gallick, G. E. (2007). Development and characterization of gemcitabine-resistant pancreatic tumor cells. *Annals of Surgical Oncology, 14*(12), 3629-3637. doi:10.1245/s10434-007-9583-5 [doi]

Siddik, Z. H. (2003). Cisplatin: Mode of cytotoxic action and molecular basis of resistance. *Oncogene, 22*(47), 7265-7279. doi:10.1038/sj.onc.1206933 [doi]

Sloat, B. R., Sandoval, M. A., Li, D., Chung, W. G., Lansakara-P, D. S., Proteau, P. J., . . . Cui, Z. (2011). In vitro and in vivo anti-tumor activities of a gemcitabine derivative carried by nanoparticles. *International Journal of Pharmaceutics, 409*(1-2), 278-288. doi:10.1016/j.ijpharm.2011.02.037 [doi]

Stuart, S. D., Schauble, A., Gupta, S., Kennedy, A. D., Keppler, B. R., Bingham, P. M., & Zachar, Z. (2014). A strategically designed small molecule attacks alpha-ketoglutarate dehydrogenase in tumor cells through a redox process. *Cancer & Metabolism, 2*(1), 4-3002-2-4. doi:10.1186/2049-3002-2-4; 10.1186/2049-3002-2-4

Szebeni, J. (2005). Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity. *Toxicology, 216*(2-3), 106-121. doi:S0300-483X(05)00352-5 [pii]

Tambutet, G., Becerril-Jimenez, F., Dostie, S., Simard, R., Prevost, M., Mochirian, P., & Guindon, Y. (2014). Dual-face nucleoside scaffold featuring a stereogenic all-carbon quaternary center. intramolecular silicon tethered group-transfer reaction. *Organic Letters, 16*(21), 5698-5701. doi:10.1021/ol502777r [doi]

Tang, K., Zhang, Z., Bai, Z., Ma, X., Guo, W., & Wang, Y. (2011). Enhancement of gemcitabine sensitivity in pancreatic cancer by co-regulation of dCK and p8 expression. *Oncology Reports, 25*(4), 963-970. doi:10.3892/or.2011.1139 [doi]

Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and autophagy. *Methods in Molecular Biology (Clifton, N.J.), 445*, 77-88. doi:10.1007/978-1-59745-157-4_4 [doi]

Trevisani, F., Frigerio, M., Santi, V., Grignaschi, A., & Bernardi, M. (2010). Hepatocellular carcinoma in non-cirrhotic liver: A reappraisal. *Digestive and Liver Disease : Official Journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 42*(5), 341-347. doi:10.1016/j.dld.2009.09.002 [doi]

Ueno, H., Okusaka, T., Funakoshi, A., Ishii, H., Yamao, K., Ishikawa, O., . . . Saitoh, S. (2007). A phase II study of weekly irinotecan as first-line therapy for patients with metastatic pancreatic cancer. *Cancer Chemotherapy and Pharmacology, 59*(4), 447-454. doi:10.1007/s00280-006-0283-9 [doi]

Ushio-Fukai, M., & Nakamura, Y. (2008). Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Letters, 266*(1), 37-52. doi:10.1016/j.canlet.2008.02.044 [doi]

Vandana, M., & Sahoo, S. K. (2010). Long circulation and cytotoxicity of PEGylated gemcitabine and its potential for the treatment of pancreatic cancer. *Biomaterials, 31*(35), 9340-9356. doi:10.1016/j.biomaterials.2010.08.010 [doi]

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: The expanding network of non-apoptotic cell death pathways. *Nature Reviews.Molecular Cell Biology, 15*(2), 135-147. doi:10.1038/nrm3737 [doi]

Visser, F., Vickers, M. F., Ng, A. M., Baldwin, S. A., Young, J. D., & Cass, C. E. (2002). Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. *The Journal of Biological Chemistry, 277*(1), 395-401. doi:10.1074/jbc.M105324200 [doi]

Visser, F., Zhang, J., Raborn, R. T., Baldwin, S. A., Young, J. D., & Cass, C. E. (2005). Residue 33 of human equilibrative nucleoside transporter 2 is a functionally important component of both the dipyridamole and nucleoside binding sites. *Molecular Pharmacology, 67*(4), 1291-1298. doi:mol.104.005884 [pii]

Wang, J., & Yi, J. (2008). Cancer cell killing via ROS: To increase or decrease, that is the question. *Cancer Biology & Therapy, 7*(12), 1875-1884. doi:7067 [pii]

Westphal, D., Dewson, G., Czabotar, P. E., & Kluck, R. M. (2011). Molecular biology of bax and bak activation and action. *Biochimica Et Biophysica Acta, 1813*(4), 521-531. doi:10.1016/j.bbamcr.2010.12.019; 10.1016/j.bbamcr.2010.12.019

White, E., Mehnert, J. M., & Chan, C. S. (2015). Autophagy, metabolism, and cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 21*(22), 5037-5046. doi:10.1158/1078-0432.CCR-15-0490 [doi]

Wilhelm, S. M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., . . . Trail, P. A. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Research, 64*(19), 7099-7109. doi:64/19/7099 [pii]

Wohlhueter, R. M., McIvor, R. S., & Plagemann, P. G. (1980). Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic acid into cultured mammalian cells. *Journal of Cellular Physiology, 104*(3), 309-319. doi:10.1002/jcp.1041040305 [doi]

Wu, C. Y., Chou, L. S., Chan, P. C., Ho, C. H., Lin, M. H., Shen, C. C., . . . Wang, H. E. (2013). Monitoring tumor response with radiolabeled nucleoside analogs in a hepatoma-bearing mouse model early after doxisome((R)) treatment. *Molecular Imaging and Biology : MIB : The Official Publication of the Academy of Molecular Imaging, 15*(3), 326-335. doi:10.1007/s11307-012-0604-9 [doi]

Yoshida, T., Endo, Y., Obata, T., Kosugi, Y., Sakamoto, K., & Sasaki, T. (2010). Influence of cytidine deaminase on antitumor activity of 2'-deoxycytidine analogs in vitro and in vivo. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 38*(10), 1814-1819. doi:10.1124/dmd.110.034397 [doi]

Yue, L., Saikawa, Y., Ota, K., Tanaka, M., Nishimura, R., Uehara, T., . . . Koizumi, S. (2003). A functional single-nucleotide polymorphism in the human cytidine deaminase gene contributing to ara-C sensitivity. *Pharmacogenetics, 13*(1), 29-38.

Zachar, Z., Marecek, J., Maturo, C., Gupta, S., Stuart, S. D., Howell, K., . . . Bingham, P. M. (2011). Non-redox-active lipoate derivates disrupt cancer cell mitochondrial metabolism and are potent anticancer agents in vivo. *Journal of Molecular Medicine (Berlin, Germany), 89*(11), 1137-1148. doi:10.1007/s00109-011-0785-8; 10.1007/s00109-011-0785-8

Zhi, X., Chen, W., Xue, F., Liang, C., Chen, B. W., Zhou, Y., . . . Liang, T. (2015). OSI-027 inhibits pancreatic ductal adenocarcinoma cell proliferation and enhances the therapeutic effect of gemcitabine both in vitro and in vivo. *Oncotarget, 6*(28), 26230-26241. doi:10.18632/oncotarget.4579 [doi]

Zhou, B., Su, L., Hu, S., Hu, W., Yip, M. L., Wu, J., . . . Yen, Y. (2013). A small-molecule blocking ribonucleotide reductase holoenzyme formation inhibits cancer cell growth and overcomes drug resistance. *Cancer Research, 73*(21), 6484-6493. doi:10.1158/0008-5472.CAN-13-1094 [doi]