UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
Neuroscienze, Biomedicina e Movimento

SCUOLA DI DOTTORATO DI Scienze della Vita e della Salute

DOTTORATO DI RICERCA IN
Medicina Biomolecolare
30° ciclo/ 2014-2017

IN VITRO STUDIES ON CSC-TARGETED THERAPY IN PANCREATIC ADENOCARCINOMA WITH NANOPARTICLE FORMULATIONS

S.S.D. BIO/10

Coordinatore: Prof.ssa Lucia De Franceschi
Tutor: Prof.ssa Marta Palmieri
Co-tutor: Dott.ssa Rosa Angela Cardone

Dottorando: Dott.ssa Stefania Forciniti

In vitro studies on CSC-targeted therapy in pancreatic adenocarcinoma with nanoparticle formulations.
Stefania Forciniti
Tesi di Dottorato
Verona, 12 gennaio 2018

TABLE OF CONTENTS

1. ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease and one of the major causes of death among cancer patients. Diagnosis of PDAC during the early stages of cancer is difficult because no effective screening for detection of early stage tumors is yet available. PDAC is sustained by a distinct subset of quiescent cells, called cancer stem cells (CSCs) that are responsible for resistance to standard therapy, metastatic potential and disease relapse following treatments. The current therapy for PDAC preferentially targets the more differentiated cancer cell population, leaving CSCs as a source that supports the tumour, causing recurrence. For this reason, targeting pancreatic CSCs could contribute to the clinical development of more efficacious drugs treatment, aimed to permanently remove the tumor and prevent recurrence. In this study, we investigated two therapeutic approaches for the treatment of PDAC. First of all, we studied a pro-drug approach using gemcitabine conjugated with fatty acid chains, C12 GEM and C18 GEM, by testing their cytotoxic activity on Panc1 cell line and the derived CSCs. Both cell lines were more sensitive to the treatment with the lipophilic pro-drugs than GEM, but only Panc1 CSCs showed a high sensitivity to C18 GEM treatment. Furthermore, the two cell lines exhibited different intracellular uptake mechanisms of the drugs that involved mainly membrane nucleoside transporters in Panc1 parental cells or fatty acid translocase CD36 for C18 GEM uptake in both cell lines. Furthermore, we have highlighted a peculiar feature of CSCs regarding the apoptotic response to treatment. In Panc1 parental cells, the treatments induced a PARP-dependent apoptosis, while in Panc1 CSCs they involved a mechanism of caspase-independent apoptosis mediated by AIF. The second therapeutic approach concerned a targeted drug delivery system using PEGylated liposomes containing disulfiram (DSF) or diethyldithiocarbamate-copper ($Cu(DDC)_2$) and liposomes selective for pancreatic CSCs expressing CD44 coated with HA. We evaluated the effect on cell proliferation of the various DSF formulations using pancreatic CSCs derived from cell lines or patients. The encapsulation of $Cu(DDC)_2$ complex in liposomes increased its anti-proliferative activity on our cell models. This method represents a good strategy to make the $Cu(DDC)_2$ complexes suitable for PDAC patients. In this study, we propose two

valid and alternative therapeutic approaches with a promising potential to achieve efficient and encouraging results in PDAC treatment.

2. SOMMARIO

L'adenocarcinoma pancreatico duttale (PDAC) è una malattia letale e rappresenta una delle principali cause di morte per cancro. La diagnosi precoce del tumore è difficile perché screening efficaci che lo diagnostichino nei primi stadi non sono ancora disponibili. Il PDAC è sostenuto da una sottopopolazione di cellule quiescenti, chiamate cellule staminali tumorali (CSCs) che sono responsabili della resistenza alle terapie convenzionali, delle metastasi e delle recidive in seguito ai trattamenti. L'attuale terapia per il trattamento del PDAC colpisce preferenzialmente la popolazione di cellule tumorali più differenziate, lasciando le CSCs come sorgente che supporta lo sviluppo del tumore, causando recidive della malattia. Per questo motivo, è importante scoprire nuovi trattamenti più efficaci e specifici per le cellule staminali tumorali pancreatiche, che rimuovano definitivamente il tumore e prevengano le recidive. In questo lavoro abbiamo studiato due approcci terapeutici per il trattamento del PDAC. Il primo approccio è stato quello delle pro-drugs, nel quale abbiamo utilizzato la gemcitabina (GEM) coniugata con catene di acidi grassi di diversa lunghezza, C12 GEM e C18 GEM. La loro attività citotossica è stata saggiata sulla linea cellulare Panc1 e sulle CSCs derivate da questa linea. Entrambe le linee cellulari sono risultate più sensibili al trattamento con le pro-drugs lipofile rispetto alla GEM, ma solo le CSCs hanno mostrato una forte sensibilità alla C18 GEM. Inoltre, le due linee cellulari hanno mostrato possedere differenti meccanismi di uptake intracellulari per le droghe, che implicano principalmente i trasportatori nucleosidici di membrana nelle Panc1 parentali, o la trasclocasi degli acidi grassi CD36 per la C18 GEM in entrambe le linee cellulari. Inoltre, abbiamo evidenziato una caratteristica peculiare delle CSCs che riguarda la risposta apoptotica al trattamento. Nelle Panc1 parentali, i trattamenti inducono un'apoptosi PARP-dipendente, mentre nelle Panc1 CSCs viene innescato un meccanismo di apoptosi caspasi-indipendente mediato da AIF. Il secondo approccio studiato riguarda un sistema specifico di somministrazione delle droghe che fa uso dei liposomi decorati con PEG e contenenti disulfiram (DSF) o dietilditiocarbammato-rame ($Cu(DDC)_2$), e liposomi selettivi per CSCs pancreatiche esprimenti CD44 decorati con acido ialuronico. Abbiamo valutato, quindi, l'effetto delle varie formulazioni di DSF sulla proliferazione cellulare di

CSCs pancreatiche derivate da linee cellulari o da pazienti. L'incapsulamento del complesso $Cu(DDC)_2$ nei liposomi aumenta la sua attività anti-proliferativa nei nostri modelli cellulari. Questo metodo rappresenta una buona strategia per rendere i complessi $Cu(DDC)_2$ adatti per la somministrazione ai pazienti. In questo studio noi proponiamo due validi ed alternativi approcci terapeutici con un promettente potenziale per raggiungere risultati efficaci ed incoraggianti nel trattamento del PDAC.

3. INTRODUCTION

Pancreatic cancer is caused by the abnormal and uncontrolled growth of cells in the pancreas, a large gland in the digestive and endocrine systems. There are different types of pancreatic cancer divided into two main groups: exocrine tumours that start in the exocrine cells, where enzymes which help food digestion are made; endocrine tumours, also called neuroendocrine tumours, that start in the endocrine cells, which produce and release insulin and other hormones (Fig.1). Most pancreatic cancers are exocrine and more than 8 out of 10 are ductal adenocarcinomas (1).

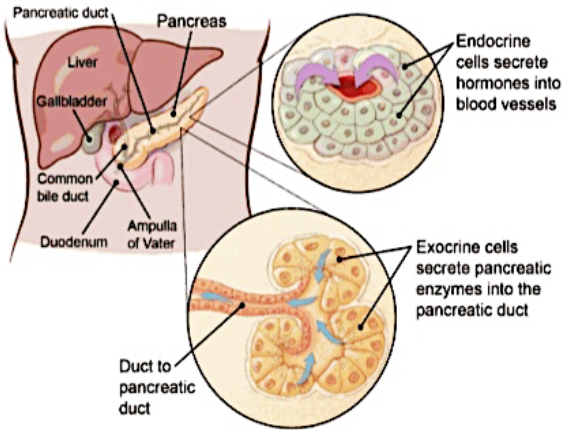


Fig.1: Anatomical representation of the pancreas consisting of an endocrine and an exocrine part. Endocrine cells secrete hormones into blood vessels; exocrine cells secrete pancreatic enzymes into the pancreatic duct. Pancreatic cancer may arise from these cells and disrupt any of their functions.

Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest epithelial malignancies and develops from cells lining small tubes called ducts. These carry the digestive juices, which contain enzymes, into the main pancreatic duct and then into the duodenum.

3.1 Pancreatic ductal adenocarcinoma pathogenesis

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-related deaths. The incidence and death rates continue to increase and PDAC is predicted to become the second most frequent cause of cancer-related death by 2030 (2), making this disease a major priority in public health care. The prognosis of PDAC is poor, with an overall 5-years survival rate of less than 5% after diagnosis, which is often made when metastatic events have occurred. More than 85% of patients who undergo surgical resection of small pancreatic tumour with clear surgical margins and no evidence of metastasis, die from metastasis within 5 years (3, 4). PDAC evolves from precursor lesions that, in the context of their genetic features, define the genetic progression model of pancreatic carcinogenesis (5). Early disease histology manifests as several distinct types of precursor lesions: the microscopic pancreatic intraepithelial neoplasia (PanIN), followed by the macroscopic cysts such as the intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). The non-invasive PanIN lesions were classified into three grades according to the extent of cytological and architectural atypia (Fig. 2): PanIN-1A and PanIN-1B show low-grade of dysplasia; PanIN 2 presents an additional loss of polarity, cell extension and hyperchromasia with papillary formation; and PanIN3 represents advanced lesions with severe nuclear atypia, luminal necrosis and manifests epithelial cell budding into the ductal lumen (6, 7). Most recently, this classification has been replaced by a two-tier low-grade (PanIN-1 and PanIN-2) and high-grade (PanIN-3) classification, which recognizes that the latter lesions are those with the greatest biological potential for progression and these lesions should be studied for early detection (8). Low-grade lesions are frequently observed in normal adult pancreas or patients with chronic pancreatitis and are associated with a low risk of developing PDAC. High-grade PanIN3 lesions, instead, are almost exclusively found in patients with invasive PDAC (9, 10).

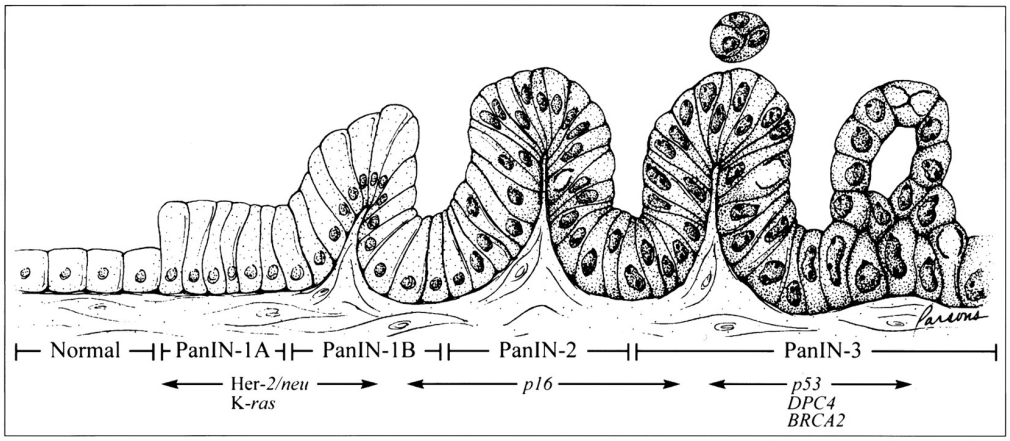


Fig. 2: Progression model of PDAC. From left to right: Normal duct epithelium progresses to cancer through defined precursors (PanINs). The inactivation of p53, DPC4, and BRCA2 occurs in the late stage; the inactivation of p16 gene at an intermediate stage; the overexpression of HER-2/neu and point mutations in the K-ras gene occur in early stage. Adapted from (6).

Since most cases of PDAC become clinically evident at advanced stages, the identification of high-risk precursor lesions has provided an essential framework to define the genomic features that drive cancer and develop effective screening and targeted therapies for earlier stage of disease (11).

3.2 Genetic characteristics of PDAC

Studies of next generation sequencing and computational biology have transformed our understanding on genetic alterations associated with the genesis and progression of PDAC, highlighting several mutations, epigenetic alterations, gene expression changes, and chromosomal rearrangements (12). Recent studies of whole-exome sequencing have reaffirmed the signature mutations of human pancreatic cancers, including oncogenic mutations of KRAS and the frequent inactivation of TP53, SMAD4 and CDKN2A tumour suppressors (13,14). These analyses also identified additional novel recurrent mutations in PDAC that converge on some pathways and processes, including Notch, Hedgehog, β-catenin, chromatin remodelling, and DNA repair pathways (15). Over 90% of PDACs are driven by early KRAS mutations, which lead to constitutive activation

of the molecule (16). Activated KRAS signals through a phosphorylation cascade of RAF, MEK and ERK or PI3K/AKT/mTOR leading to the transcription of proliferation genes. Pancreatic cancer lacking KRAS mutations shows activation of RAS through an upstream signalling receptor, such as EGFR, and in a small fraction of patients oncogenic activation of the downstream B-RAF molecule is detected (17). Histologically, PDAC is characterized by a dense stroma content including fibroblasts, hyaluronic acid (HA), collagen and other extracellular matrix proteins, inflammatory cells and cancer stem cells. There are conflicting opinions whether the stroma supports malignancy or acts as a protective barrier. However, increasing evidence has proved that the tumour stroma impairs drug delivery and supports an immunosuppressive tumour microenvironment playing a key role in the development of PDAC (18).

3.3 Cancer stem cell concept

Cancer stem cells (CSCs) have been intensively studied for decades. The first concept was that CSCs constituted a subset of cells in malignant cell population with an exclusive ability to create endless copy of themselves through self-renewal (19, 20). This concept offers a possible explanation for disease relapse following therapy, even with effective treatments that induce initial tumour regression. CSCs are often more resistant to treatment than the more differentiated tumor cells (21, 22, 23). As a consequence, the tumour is apparently eliminated after therapy, but it grows back because the rare CSC population has survived. This points out the urgent need to discover CSCs-targeted therapies to prevent recurrence. The acquired resistance to therapy is driven, in part, by intratumoural heterogeneity, that is the phenotypic diversity of cancer cells within a single tumour mass (24). At least two models have emerged trying to explain the heterogeneity and the cancer stem cell evolution. The first one concerns the expansion of an initially mutated or epigenetically altered progenitor cell that gains a proliferative advantage over its normal counterparts (25) (Fig 3). The initial clone can produce distinct subclones controlled by the immune system or surrounding environment. In the first phase of cancer evolution (phase I), tumour stem cells are pre-malignant. If one or more subclones undergo sufficient changes

to acquire malignant potential, the tumour develops. At phase II, the neoplastic stem cells become cancer stem cells. However, the other neoplastic subclones and their stem cells may remain and continue to generate additional pre-malignant or malignant subclones.

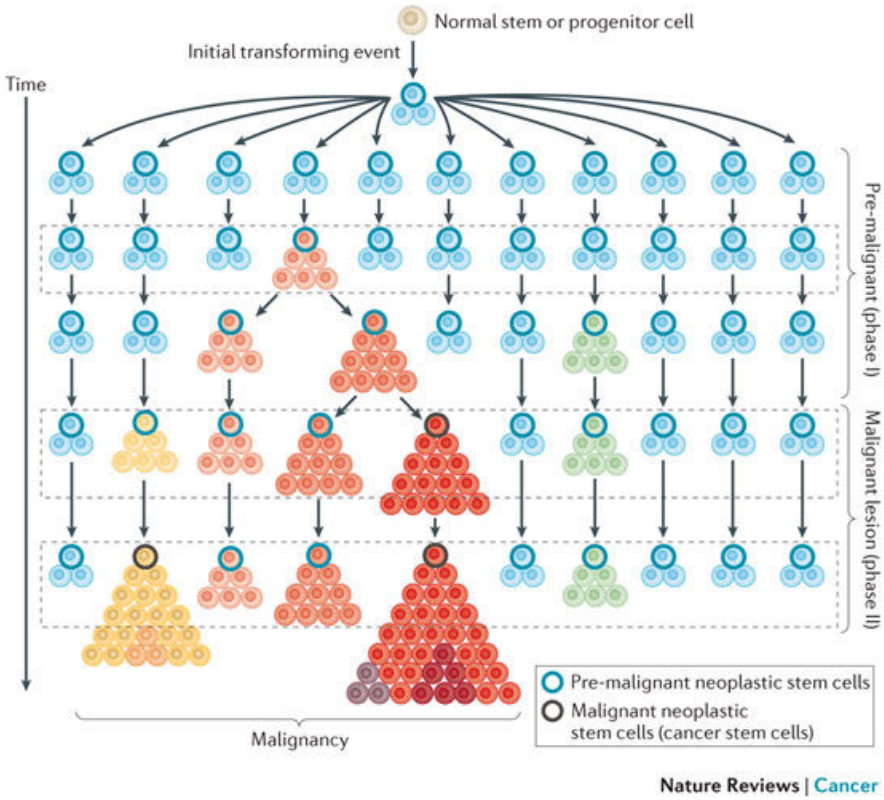
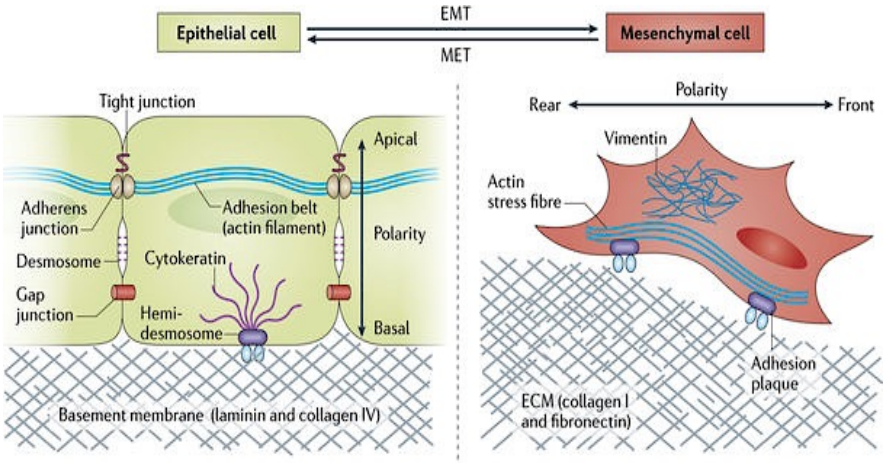


Fig 3: Model of cancer stem cell evolution. The normal stem or progenitor cell produces different subclones. In phase I the stem cells are pre-malignant. The accumulation of mutations in one or more subcolones gives rise to the tumour. At phase II neoplastic cells become cancer stem cells. Adapted from (25).

The second proposed model concerns the concept of CSCs as a subpopulation of neoplastic cells in the bulk of the tumour that serves as a critical driver of tumor progression (25) and undergoes a biological program termed epithelial-to-mesenchymal transition (EMT) (26). This program gives phenotypic changes to tumour cells through epigenetic modifications, thereby carcionoma cells lose many of their epithelial characteristics such as the epithelial cell junctions and apical-basal polarity, and acquire mesenchymal features, exhibiting an elongated,

fibroblast-like morphology and increased migration and invasion (Fig. 4) (27, 28). Moreover, the induction of EMT program in epithelial tumour cells increases their tumour-initiating ability (29) and confers them resistance to many therapeutic agents (30, 31).



Tight junctions, adherens junctions, gap junctions, and desmosomes	**Cell–cell junctions**	Minimal
• Actin filaments are aligned with adherens junctions (adhesion belt) • Cytokeratin intermediate filaments	**Cytoskeleton**	• Actin stress fibres • Vimentin intermediate filaments
Via integrin $\alpha_6\beta_4$ at hemidesmosomes; linked to cytokeratins	**Interactions with ECM**	Via β_1-containing or β_3-containing integrins at adhesion plaques; linked to actin stress fibres
Apical–basal polarity	**Polarity**	Front–rear polarity

Fig. 4: Changes associated with the activation of the epithelial-to-mesenchymal transition (EMT) program. Adapted from (31).

3.4 Pancreatic CSCs

An increasing number of studies indicate that several tumors, including PDAC, contain a subset of tumorigenic CSCs, which drive tumor initiation, metastasis and resistance to radio-and chemotherapy (32-35). Pancreatic CSCs have been first described in 2007 by Li et al. (36). The authors showed that pancreatic cancer cells with the $CD44^+/CD24^+/ESA^+$ phenotype (0.2–0.8%) possessed a 100-fold increased tumorigenic potential compared with non-tumorigenic markers-negative cancer cells; only a small fraction of $CD44^+/CD24^+/ESA^+$ cells was sufficient to give rise to tumours histologically indistinguishable from the primary human

tumour of derivation. In PDAC, as in other solid tumours, CSCs possess the exclusive ability to recapitulate the parental tumour upon transplantation into immunodeficient mice (37). Although CSCs bear cell-intrinsic stemness features, it has been shown that these cells are also affected by the surrounding microenvironment which contributes to their aggressiveness, metastatic activity, and drug resistance (38,39). For this reason, it is important to study CSCs in the context of their niche and to develop models that recapitulate the heterogeneity of primary tumours and the surrounding environment. Increasing efforts have been made to design in vitro cultures and in vivo xenograft models from resected tumours (40, 41). Patient-derived xenograft (PDX) models have been obtained from PDAC and have been shown to replicate the characteristics of the primary tumour including genetic features and cellular heterogeneity (42). In the past years, pancreatic CSCs have been identified by a variety of biomarkers, such as CD44, EPCAM, CD133, ALDH1 and hepatocyte growth factor receptor CMET (43). Although the number of CSC biomarkers is still increasing, their expression is variable because it is affected by culture conditions and response to treatment, and is not exclusively linked to a CSC phenotype (44). Recently, an intrinsic autofluorescent phenotype of PDAC CSCs has been identified and used to isolate and characterize these cells (45). It has been demonstrated that the autofluorescence is derived from riboflavin actively sequestered in cytoplasmic vesicles by an ATP-dependent process. Interestingly, only in digested tumours and early passage in vitro cultures from these tumours, the autofluorescent population was detected, but not in cell lines such as Panc1. This specific autofluorescent phenotype allowed the identification and purification of PDAC CSCs by fluorescence-activated cell sorting and confocal microscopy, without the use of antibodies and independently of expression of cell surface markers.

3.5 Treatment and chemoresistance in pancreatic cancer

Although different therapeutic strategies for treating pancreatic cancer are available, the chemotherapy remains the first choice, especially for advanced tumour stages. Numerous efforts have been made to improve the treatment in PDAC (46), but unfortunately, the therapeutic response is still largely ineffective and not durable (47). The failure is due to many factors, including extrinsic (48) or intrinsic (49) resistance to conventional approaches of chemotherapy. For a long time, the standard of care for PDAC has been the deoxycytidine (dCyd) nucleoside analog, gemcitabine (GEM) (50). The metabolite GEM 3-phosphate interferes with tumor growth through incorporation into DNA or alternatively, GEM diphosphate can interfere with DNA synthesis and thus tumor growth through inhibition of ribonucleotide reductase. Like other anticancer agents, GEM induces reactive oxygen species (ROS) generation (51), cell cycle blocking in S phase (52) and apoptosis of human pancreatic carcinoma cells correlated with Bcl-2 content (53). Despite its effective anticancer activity, GEM suffers from various drawbacks, such as a rapid deamination to the inactive metabolite 2',2'-difluorodeoxyuridine by cytidine deaminase, resulting in a short in vivo half-life, and then it must be administered at very high dose. Indeed, this therapy only confers a marginal survival advantage to patients, showing efficacy in less than 20% of them (54). Furthermore, a large number of patients is resistant to these therapies mainly because the dense tumour stroma functions as a barrier and extrinsic resistance (55). Small improvements in short-term survival have been achieved by the addition of erlotinib (56) or capecitabine (57) to GEM, but the benefit is in the order of weeks. Recently, more encouraging results have emerged with combined treatment of GEM/*nab*-paclitaxel, an albumin-based formulation of the chemotherapeutic drug paclitaxel, (58) and the *FOLFIRINOX*, a potent chemotherapy regimen made up of four drugs, oxaliplatin, irinotecan, fluorouracil, and leucovorin (59). Although these treatments improve median survival of patients, they are highly toxic and don't allow a long-term survival. As a consequence, it is crucial to understand the mechanisms of resistance for the design of drug combinations in order to achieve successful therapies.

3.6 The prodrug approach: a successful tool for improving drug stability

The prodrug approach is a strategy of molecular modification widely used to optimize the physical, chemical and pharmacological properties of drugs in order to improve their solubility and pharmacokinetic features. A prodrug is an active or slightly active compound containing the native drug that undergoes an *in vivo* transformation through chemical or enzymatic cleavage, enabling the release of the active molecule at specific sites (Fig. 5) (60).

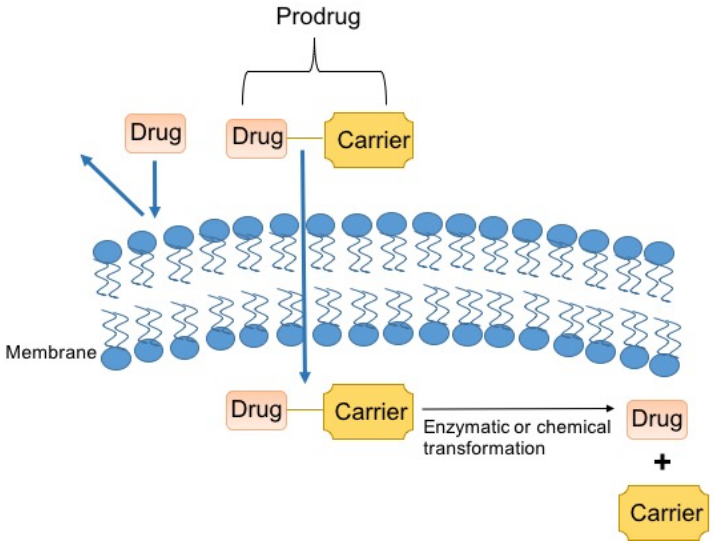


Fig. 5: Bioactivation of prodrugs by enzymatic or chemical transformations.

Prodrugs are classified into two classes, carrier-linked prodrugs and bioprecursors. Carrier-linked prodrugs can be divided in bipartite prodrugs, in which the carrier is directly linked to the parental drug, and tripartite prodrugs, in which the carrier is linked to the drug through a spacer. Carriers are generally attached by chemical groups such as amide, carbamate, ester, carbonate, phosphate and others. Mutual prodrugs, instead, are a type of carrier-linked prodrug in which both compounds are active and each acts as the carrier to the other. The bioprecursors are inactive compounds that are rapidly converted to an active drug after metabolic reactions (61). Some properties, including poor aqueous solubility, chemical instability, low half-life, fast metabolism or problems related to drug formulation and delivery are resolved using the prodrug approach

(62, 63, 64). For example, a series of lipophilic prodrugs were synthesized by linking the 4-amino group of GEM with valeroyl, lauroyl or stearoyl linear acyl derivatives to increase the stability and the bioavailability of the drug (Fig. 6).

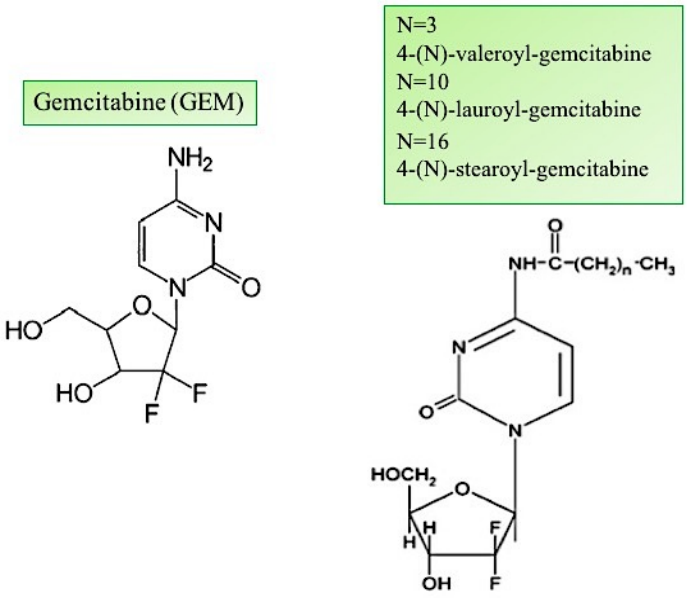


Fig 6: Gemcitabine and 4-(N)-acyl-gemcitabine prodrug structure.

This increase in stability occurs because the acyl moiety chemically protects the amidic group in the N-4 position of cytosine that is rapidly metabolized in plasma. Studies of differential scanning calorimetry have shown that the lipophilic prodrugs are more able than pure GEM to interact with lipid bilayers, thus modulating its transport and release inside the cellular compartments (65).

3.7 Targeted drug delivery system for pancreatic cancer

The selective delivery of therapeutic agents to their cellular targets represents the main objective of the current investigations for effective treatment of pancreatic cancer. Several approaches have been utilized, such as liposomes, polymers, and micelles carrying anti-cancer drugs with the aim of passive targeting through enhanced permeation and retention effects (66). The most known carrier for drug delivery system is the liposome, a single lipid bilayer vesicle which, because of its structure, can encapsulate a wide range of molecules with hydrophilic,

amphiphilic or lipophilic characteristics. Furthermore, its phospholipidic framework ensures complete biocompatibility (67). However, lipid-based carriers are quickly cleared from the bloodstream by the reticuloendothelial system. To overcome this issue, different chemical modifications of carriers with synthetic polymers have been frequently carried out (68). One of the most successful modification is the coating with polyethylene glycol (PEG), a non-toxic, non-immunogenic, non-antigenic and highly water soluble polymer. The use of PEG has several advantages: it can "mask" the agent from the immune system, reducing immunogenicity and antigenicity, and increase its hydrodynamic size, prolonging the circulatory time. Furthermore, PEGylation can also provide water solubility to hydrophobic drugs (69). An example is doxorubicin in PEG-coated liposomes, which is widely used in clinical practice to treat solid tumours, including breast, pancreatic and prostate cancer (70). Over the years, the research in liposome field has been oriented towards the development of systems capable of specifically recognizing the target cells. This strategy involves the use of a targeting molecule able to drive the nanosystem directly on the target cells by recognition of specific molecules expressed on cell surface (Fig. 7) (71).

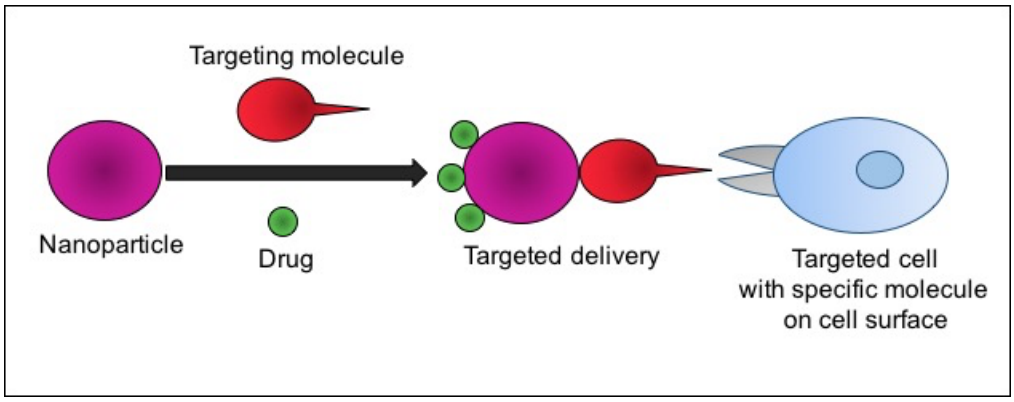


Fig. 7: Schematic representation of active targeting strategy of drug delivery.

According to this strategy, different liposomes are decorated with peptides, aptamers, antibody or small molecules (72, 73). It has been demonstrated that pancreatic cancer cells overexpress CD44, the hyaluronic acid (HA) receptor (74). For this reason, HA is employed as a good targeting agent to obtain liposomes

able to selective target and destroy pancreatic cancer cells. Furthermore, HA is a non-toxic, non-immunogenic and biodegradable polymer, features that render it an ideal candidate as targeting agent (75).

3.8 Disulfiram and derivatives as promising anticancer drugs

Disufiram (DSF) (Antabuse®), an irreversible inhibitor of aldehyde dehydrogenase, is a FDA approved drug for the treatment of alcoholism (76). It has been shown that DSF acts as antitumoral drug (77, 78) and represents a possible anti-CSC agent in breast cancer and glioblastoma (79, 80). The anticancer activity of DSF has not yet been fully elucidated. Indeed, various mechanisms have been proposed, such as the inhibition of aldehyde dehydrogenase, proteasome, NF- κB, DNA methyltransferase and multidrug resistance p-glycoprotein activities (81). In the bloodstream, DSF is converted into two molecules of diethyldithiocarbamate (DDC) (Fig 8 A).

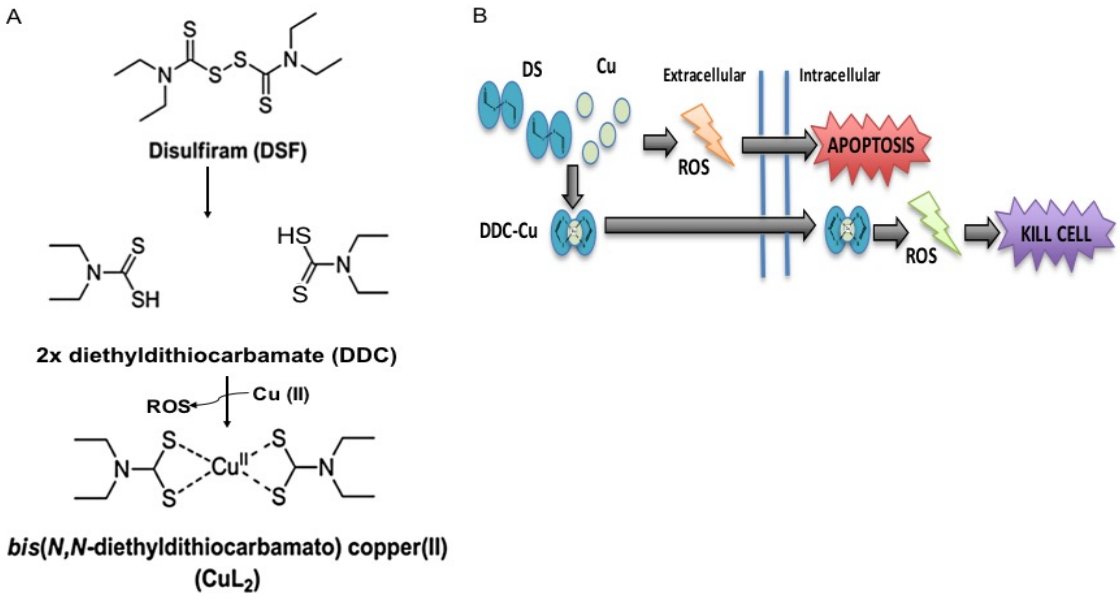


Fig. 8: Anticancer mechanism of disulfiram (DSF). A) Formation of a new chemical $Cu(DDC)_2$ from DSF; B) Phases of anticancer model of DSF and DDC.

The complex of DDC with a metal ion, usually copper or zinc, is mainly responsible for the anticancer activity of DSF (82). Two phases have been

proposed in the anticancer model of DSF and DDC (Fig. 8 B) (83). First, the chelation of DDC and copper generates extracellular radical oxygen species (ROS) inducing cancer cell apoptosis. Second, the chelation-generated compound, $Cu(DDC)_2$, can easily penetrate into cancer cells, trigger the generation of intracellular ROS and induce cancer cell apoptosis. Despite its powerful anticancer activity, the development of DSF-based cancer therapy is prevented by different factors, such us poor solubility of DSF in biological fluid and its rapid degradation in the bloodstream (76). It is therefore necessary to overcome these limitations to develop effective anticancer therapies.

3.9 Endocytic route and interaction modalities of molecules with cell membrane

The release of drugs in cells occurs following the crossing of the plasma membrane barrier. Plasma membrane presents high complexity and has a critical function in the cellular adhesion, communication, and division, and endocytosis plays an important role in the regulation of these critical functions (84).

The uptake route of some molecules depends on their physical characteristics including particle size, shape and surface charge, and also on the type of cell.

Usually, small and non-polar molecules use a passive mechanism, such as diffusion or facilitated diffusion, to enter into the cell without the use of energy. On the other hand, macromolecules use other mechanisms of transport such as endocytosis, in which the macromolecule that need to be internalized is surrounded by an area of plasma membrane, which forms a vesicle inside the cell containing the ingested material (85).

3.9.1 Main pathways of endocytosis

Endocytosis pathways can be divided into three categories namely:

- Macropinocytosis is the invagination of the cell membrane to form a vesicle filled with a large volume of extracellular fluids and molecules.

The intracellular fate of the vesicles is cell dependent, and in the cytosol vesicles fuse with endosomes and lysosomes (86);

- Phagocytosis is the internalization of solid particles such as debris or apoptotic cells to form an internal compartment known as phagosome, involving the uptake of large membrane areas (87);
- Receptor-mediated endocytosis, also called clathrin-mediated endocytosis is the most frequent endocytic pathway and is normally used by the cells for the specific uptake of certain substances (hormones, metabolites, other proteins) (88). Clathrin-mediated endocytosis involves the internalization of fragments of the cytoplasmic membrane in form of vesicles containing all of their contents and coated with polymerized clathrin (Fig. 9). In particular, clathrin and cargo molecules are assembled into clathrin-coated pits on the plasma membrane with adaptor proteins, such as AP-2, that links clathrin with transmembrane receptors.

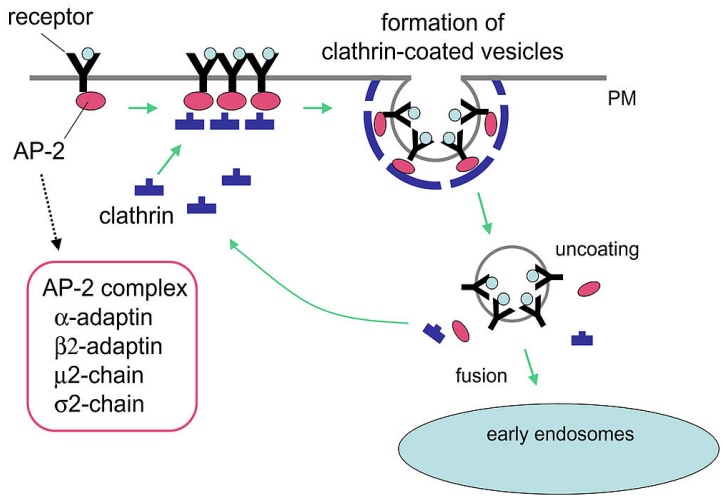


Fig. 9: Mechanism of clathrin-dependent endocytosis. The adaptor protein AP-2 links clathrin with receptor, forming the clathrin-coated vesicles. Clathrin is released and vesicles are transported to early endosomes.

In conclusion, the mature clathrin-coated vesicles are formed and then actively

uncoated and transported to early endosomes, or recycled to the plasma membrane surface (89).

The vesicles can also be targeted to mature endosomes and later to compartments such as lysosomes. Different molecular biological tools and pharmacological agents have been used to selectively inhibit clathrin-mediated endocytosis (90). Molecular approaches include the use of RNA interference technology and the downregulation of the endogenous proteins involved in clathrin-mediated endocytosis (91). Pharmacological and chemical agents, such as chlorpromazine (CPM), are more advantageous than molecular approaches, because pharmacological inhibition prevent the entry into the cell and is not dependent on transfection efficiency. CPM is a cationic amphipathic drug that is believed to inhibit clathrin-coated pit formation through a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles (92). However, a major drawback of pharmacological agents is the non-specific effect, influencing other related processes (93).

3.9.2 Lipid-raft mediated endocytosis

The lipid-rafts are an assembly of specific lipids, usually glycosphingolipids and cholesterol, into a glycolipoprotein domain within the membrane bilayer (Fig.10 A). Some proteins, for example caveolin-1, are essential in the membrane raft formation and function and can be found as constitutive components of rafts. However, caveolin-1 is not the only protein involved in raft formation, but others may display the scaffolding functions in caveolin-independent rafts, such as Reggies/flotillins (94). Lipid rafts are more ordered and tightly packed than the surrounding membrane, but float in the lipid bilayer (95). These specialized domains have been intensely studied for years (96). The highly dynamic raft domains are essential in signalling processes and are also sorting platforms for the traffic and recruitment of proteins (97) (Fig. 10 B). This recruitment in rafts takes place through interactions between the lipids within the raft and the transmembrane domain of integral proteins (lipid-protein interaction) or the lipid moiety of protein attached to the membrane by a lipid modification (lipid-lipid interaction). On the other hand, the recruitment of cytosolic proteins by protein-

protein interactions through modular domain (e.g. SH2 domain), can take place in both raft and non-raft membranes (98).

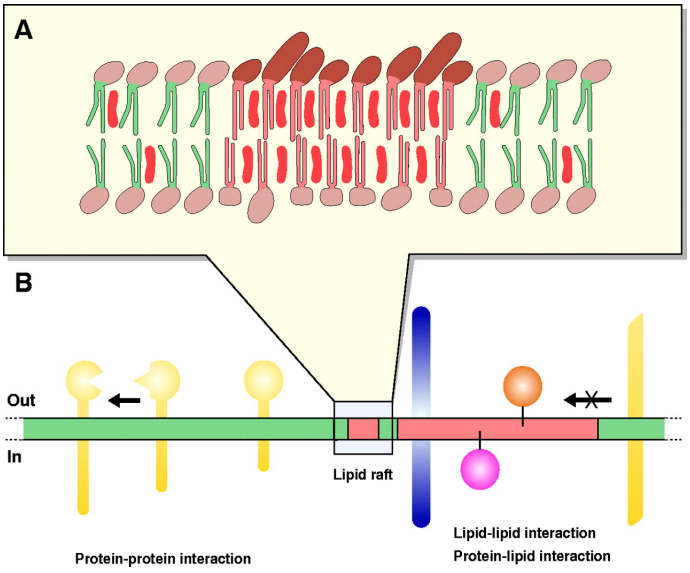


Fig.10: Model of lipid-raft structure and function in biological membranes. A) Lipid rafts are microdomains formed by sphingolipids (dark-brown structures) and cholesterol (red bean-shaped structures) immersed in a phospholipid bylayer (light-brown structures); B) Selective recruitment of proteins in rafts. This process occurs through lipid-protein interaction or lipid-lipid interaction. The recruitment of cytosolic proteins by protein-protein interactions can occur in both raft and non-raft membranes. Proteins included in rafts are in blue (integral membrane proteins), light brown (GPI-anchored proteins) or pink (acylated proteins such as Src family kinases or Ras); Proteins excluded from rafts are in yellow. Adapted from (98).

It is now widely accepted that lipid rafts possess an internalizing capacity activated by some ligands through multiple endocytic mechanisms (99). These mechanisms include three distinct raft-associated routes that are dependent or independent of dynamin (100). Dynamin-dependent endocytosis can involve or not the caveolin-1 while the third raft-associate route, which is both dynamin and caveolin independent, requires the fotillin-1 and CDC42. The lipid-raft domains are also used by lipophilic nanoparticle substances to enter directly into the plasma membrane through mechanisms of lipid particle fusion and lipid mixing,

and subsequently trafficked to the cytosol (101). Different chemical agents, such as methyl-β-cyclodextrins (MβCD), can modify the lipid rafts content in the membrane, extracting cholesterol from these domains (102). MβCD is a cyclic heptasaccharide consisting of external hydrophilic structure and internal hydrophobic cavities, and remove cholesterol from lipid domain due to its high affinity towards it (103). The process of selective cholesterol removal is dependent on MβCD concentration. At high concentrations, MβCD also removes phospholipids.

3.9.3 Membrane nucleoside trasporter-mediated uptake

The uptake of natural nucleosides and various nucleoside-derived drugs is mediated by membrane transport proteins, belonging to two families of transporters, concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) (104). Both families consist of three or four members, respectively, which differ in their energy requirements and the substrate selectivity. The expression of these transporters can be regulated, and their tissue distribution is not uniform in the various tissues (Table 1). The vast majority of CNT and ENT transporters is localized in the apical and basolateral membranes, where they can play physiological roles, such as the modulation of extracellular and intracellular adenosine concentrations (105). All three CNTs transport uridine but exhibit different preferences for other molecules. CNT1 exhibits selectivity for pyrimidine nucleosides, CNT2 for purine nucleosides, and CNT3 for both purine and pyrimidine nucleosides (106, 107). Moreover, ENT1, ENT2 and ENT3 possess similar selectivity for purine and pyrimidine nucleosides, while ENT4 is uniquely selective for adenosine, but also transports organic cations (108).

Protein name	*Transport type*	*Predominant tissue/ subcellular distribution*
CNT 1	Concentrative (sodium/nucleoside 1:1)	Liver, kidney, small intestine. Localization: primarily plasma membrane
CNT 2	Concentrative (sodium/nucleoside 1:1)	Heart, skeletal muscle, liver, kidney, intestine, pancreas, placenta and brain. Localization: Primarily plasma membrane.
CNT 3	Concentrative (sodium/nucleoside 2:1 or proton/nucleoside 1:1)	Mammary gland, pancreas, bone marrow, trachea and intestine. Localization: primarily in plasma membrane, but intracellular in some cell types and splice variants.
ENT 1	Facilitated diffusion	Widely expressed, primarily in the plasma membrane but also in nuclear and mitochondrial membranes.
ENT 2	Facilitated diffusion	Skeletal muscle Localization: primarily plasma membrane but also in nuclear membranes.
ENT 3	Facilitated diffusion	Widely expressed, intracellular (late endosomal/lysosomal and mitochondrial membranes).
ENT 4	Unclear, possibly proton linked	Heart, brain, and skeletal muscle. Localization: Primarily plasma membrane

Table 1: List of CNT and ENT nucleoside transporters, their transport type and predominant tissue/ subcellular distribution.

Multiple factors, including cell cycle phases and cytokines, regulate the expression of nucleoside transporters in human and other mammalian cells (109). Moreover, these transporters have also clinical significance. Nucleoside transporters are responsible for the cellular uptake of several nucleoside-derived drugs used in cancer chemotherapy, such as GEM (110, 111). Thus, the nucleoside transporter content of cells can affect the response to treatment. ENT1 is considered to be predominantly involved in GEM transport (112). The relationship between ENT1 and GEM has been widely studied and, during the last decade, several pre-clinical studies confirmed that the overexpression of ENT1 might serve as a predictive biomarker for the efficacy of GEM (113, 114). In particular, Giovannetti et al. demonstrated that in PDAC patients treated with GEM, the high expression of ENT1 was associated with a significant improvement in overall survival (115). Several pharmacological agents can be used to block the activity of nucleoside transporters, such as dipyridamole that can

inhibit the antiproliferative effect of GEM in various cancer cell lines (116).

3.9.4 Fatty acid translocase/CD36-mediated uptake and regulation of fatty acid transport

Some evidence shows that long-chain fatty acids are actively transported across cell membrane by specialized proteins instead of passive diffusion (117). The proteins involved in the fatty acids transport and trafficking include the fatty acid transport proteins (FATPs) or fatty acid binding proteins (FABPs) that are expressed most abundantly in tissues involved in active lipid metabolism (118). The CD36 or fatty acid translocase protein mediates the uptake of fatty acids in a variety of cell types and its increased expression is correlated with elevated uptake of fatty acids (119, 120). In particular, CD36 is a heavily glycosylated integral membrane protein with high affinity for long chain fatty acids. The role of CD36 in cellular fatty acid uptake was identified in 1993 (121) and is now supported by strong evidence generated in CD36 deficient humans (122). Polymorphisms in the CD36 gene have been linked with alterations in plasma lipid levels, such as abnormal serum fatty acids and low-density lipoproteins (LDL), and with increased susceptibility to the metabolic syndrome (123). In the context of tumours, CD36 has previously been described in the regulation of endothelial cell function in multiple types of cancer including breast, melanoma, and glioblastoma (124). For example, in breast cancer the repression of CD36 is involved in the regulation of pro-tumorigenic phenotypes, including angiogenesis, cell-extracellular matrix interactions and adipocyte differentation. It has also been demonstrated that CD36 recognizes a variety of ligands in the tumour microenvironment. For example, it is responsible for the inhibition of vascular growth in glioblastoma through the interaction with vasculostatin, inducing the endothelial cell apoptosis (125). Interestingly, some studies on glioblastoma provide evidence that CD36 was particularly enriched in CSCs and was able to functionally distinguish cells with self-renew capability. Furthermore, CSCs selectively used the scavenger receptor CD36 to promote their maintenance (126). Given the observation that CD36 expression was associated with self-renewal and CSC marker expression, its inhibition resulted in concomitant loss of self-renewal

and tumour initiation capacity. The fatty acids uptake mediated by CD36 is inhibited in a variety of cell types using the membrane-impermeable sulfosuccinimidyl oleate (SSO) (127). This molecule reacts rapidly to form stable bonds primarily with amino groups in lysine side chains and the negative charge of sulfonate group contribute to restrict the membrane permeability (128).

3.10 Apoptosis and molecular targeting therapy in cancer

Apoptosis is the programmed cell death with distinct biochemical and genetic pathways that play a critical role in the development and homeostasis of normal tissues (129). Apoptosis is triggered by proteases, called caspases which are typically activated in the early stages of apoptosis. These enzymes cleave key cellular components that are required for the normal cellular functions including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes (130). During the apoptotic process, cells display distinctive features. Generally, the cell begins to shrink following the cleavage of lamin and actin filaments, and the breakdown of chromatin leads to nuclear condensation. After these changes, in order to promote the phagocytosis by macrophages, apoptotic cells undergo plasma membrane modifications. One of these modifications is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The last stages of apoptosis are characterized by the formation of membrane blebs and small vesicles, called apoptotic bodies (131). The mechanisms of apoptosis are highly complex and evolutionarily conserved (132). There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Fig. 11). The extrinsic pathway that requires an external stimulation, occurs via a death receptor family member located on the plasma membrane, such as different tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, FAS or TNF receptor 1. After ligand binding, the death receptors activate caspases that lead to a cleavage of substrates and a rapid cell death (133, 134, 135). The intrinsic apoptotic pathway, which is often deregulated in cancer, is activated by a wide range of stimuli, such as DNA damage and endoplasmic reticulum stress. Cell stresses lead to one crucial event that is the mitochondrial outer membrane permeabilization (MOMP) triggered by

BAX and BAK activity, which are directly activated by the BH3-domain of BCL-2 (136). Following mitochondrial permeabilization, cytochrome C and other mitochondrial proteins such as SMAC are released into the cytosol. Cytocrome C interacts with apoptotic protease activating factor 1 (APAF1), triggering the apoptosome assembly, which activates caspase-9. Active caspase-9, in turn, activates caspase-3 and caspase-7, leading to apoptosis (137, 138). Mitochondrial release of SMAC leads to apoptosis by blocking the X-linked inhibitor of apoptosis protein (XIAP) and thus preventing its binding to caspases. This allows normal caspase activity to proceed (139). In addition to cytocrome C and SMAC, mitochondria also release other molecules, such as apoptosis inducing factor (AIF) that may promote caspase-independent cell death (140). AIF is a flavoprotein located in mitochondria with NADH oxidase activity. When apoptosis is induced, AIF traslocates from mitochondria to the cytosol as well as to the nucleus. It has been demonstrated that when added to purified nuclei, AIF induces partial chromatin condensation as well as the DNA fragmentation in a caspase-independent fashion (141).

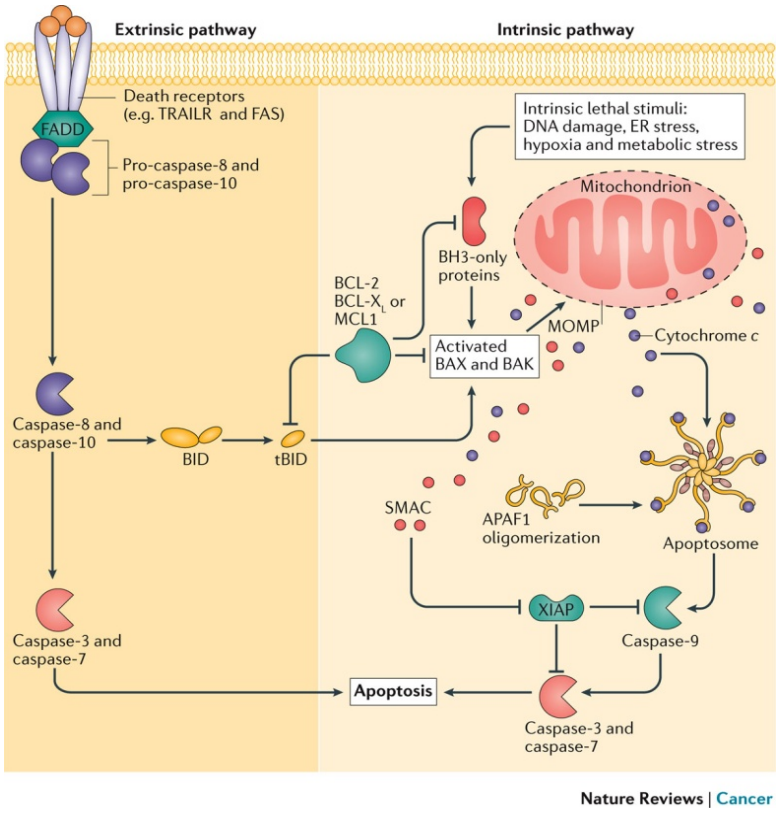


Fig. 11: Extrinsic and intrinsic apoptotic signalling pathway. Adapted from (132).

There is an additional apoptotic pathway that involves T-cell cytotoxicity and granzyme A or B. Granzyme A can induce a caspase- independent cell death pathway through single strand DNA damage (142), whereas granzyme B pathway involves the activation of initiator caspase, which in turn will activate the executioner caspase-3 (143). Defect in apoptosis can promote tumorigenesis, allowing the neoplastic cells to survive, subverting the need for exogenous survival factors and providing protection from oxidative stress and hypoxia. These events lead to the accumulation of genetic alterations that deregulate cell proliferation and promote angiogenesis and invasiveness during tumour progression. Thus, the evasion of apoptosis is an evident hallmark of cancer (144). It has been demonstrated that the inhibition of cell death, in combination with mitogenic oncogenes, can promote cancer in mouse models (145, 146). Moreover, many oncogenic pathways can inhibit apoptosis, whereas tumour suppressors, such as p53 can induce apoptosis (147). It is frequently observed the positive correlation between apoptotic sensitivity and therapeutic efficacy in cancer cells (148). However, although the inhibition of apoptosis contributes to promote cancer, tumor cells are not often inherently resistant to apoptosis. Indeed, paradoxically, high levels of apoptosis have been shown to correlate with poorer prognosis in some cancers, whereas high levels of anti-apoptotic proteins correlate with better prognosis (149, 150). The ability of the therapy to induce apoptosis by targeting the overexpressed anti-apoptotic proteins or by stimulating the pro-apoptotic molecules expression, is crucial for the success of each therapeutic strategy.

3.11 The role of autophagy in cancer disease

Autophagy is an intracellular catabolic process by which damaged material, macromolecules and organelles, are degraded by lysosomes or vacuoles and then recycled. This process involves the formation of double-membrane vesicles known as autophagosomes that engulf proteins and organelles for delivery to the lysosome. The resulting structure is an autophagolysosome and its content is degraded by lysosomal hydrolases (Fig. 12) (151). Autophagy is controlled by

highly regulated events and it occurs at basal level in all cells or is induced by diverse signals and cellular stresses (152).

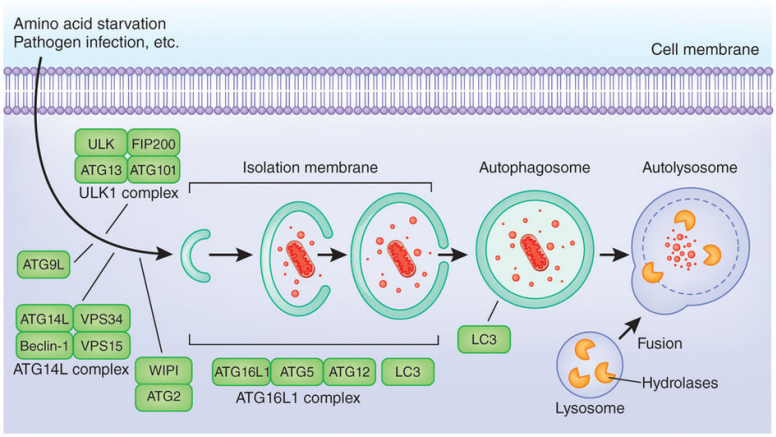


Fig. 12: Schematic representation of the various steps of autophagy.

The formation and turnover of the autophagosome requires the activation of some autophagy related genes (ATGs) and is divided into distinct stages; the initiation begins with the activation of ATG complex that activates a class III PI3K complex comprising VPS34, ATG14 and other proteins, all of which are scaffolded by the tumour suppressor Beclin 1 (153). The expansion of the autophagosome membrane involves the ATG5-ATG12 complex in conjugation with ATG16, and members of LC3 family are conjugated to the phosphatidylethanolamine (PE) and recruited to the membrane. ATG4B with ATG7 conjugates LC3I and PE to form LC3II. This lipidic form of LC3 is used as an autophagosome marker (154). Finally, the autophagosome fuses with the lysosome and the content is degraded and molecular precursors are recycled. The role of autophagy in cancer is controversial and it is likely dependent on the tumour type and cellular context (155, 156). Numerous evidences suggest that autophagy prevents cancer development (157). Conversely, in an established cancer, increased autophagic flux often induces tumour cell growth and survival (158). For this reason, interventions to both stimulate and inhibit autophagy have been proposed as cancer therapies. Despite this potential confusion on the role of autophagy in cancer, the vast majority of studies is focused on inhibiting autophagy and evaluating its effect on clinical outcome (159, 160). Animal

models with a specific expression of some oncogenes have been shown to develop tumours that regress after genetic or pharmacological inhibition of autophagy. Similarly, in vitro studies, genetically engineered mouse models (GEMMs) and patient-derived xenograft (PDX) mouse models have shown the increased antitumour effects when anticancer drugs are combined with genetic or pharmacological inhibitors of autophagy (161, 162, 163). The most used autophagic inhibitor is chloroquine that deacidifies the lysosome and blocks the fusion of autophagosomes with lysosomes, thus preventing cargo degradation (164). Other studies have reported the potential role of autophagy in the ability of cancers to develop resistance to chemotherapy. Moreover, the inhibition of autophagy could reverse acquired resistance to drugs (165).

3.12 Cancer as a cell cycle defect

Cell cycle, the process by which cells progress and divide, is at the centre of the tumour. In normal cells, the cell cycle is controlled by different signalling pathways by which a cell grows, replicates its DNA and divides. There are four phases in the cell cycle (166) (Fig. 13): G1 phase in which the cell grows and prepares itself to synthesize DNA; S or synthesis phase, in which the cell synthesizes DNA; G2 phase consists in the preparation for cell division which occurs in the M phase or mitosis. The various steps in the cell cycle are tightly regulated by arrest at G1 or G2 checkpoints and multiple molecular pathways, including cyclin dependent kinases (CDKs) and their regulatory inhibitors. These enzymes are activated by forming complexes with cyclins, a group of regulatory proteins only present for a short period in the cell cycle (167). Inhibitors of CDKs, such as $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ induce the arrest of cell proliferation by negatively regulating cell cycle checkpoints (168). In normal conditions, the regulatory proteins act by controlling cell growth and inducing the death of damaged cells. In cancer, genetic mutations cause the malfunction or disruption of the cell cycle control mechanisms, resulting in uncontrolled cell proliferation and then in the tumour development (169).

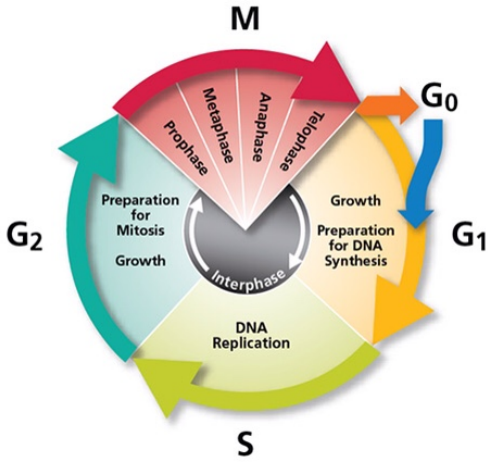


Fig. 13: Phases of cell cycle: G_0 phase- Quiescent state; G_1 phase-preparation for DNA synthesis; S phase- DNA replication; G_2 phase- preparation for mitosis; M- mytosis.

Two types of gene play an important role in the cell cycle and in cancer development: oncogenes (e.g. Ras, c-Myc) and tumour suppressor genes (e.g. p53 and Rb) (170). For example, p53 has been described as "the guardian of the genome" (171), since its major role is to prevent the genome of mutated cells from proliferation. Mutations of tumour suppressor genes, which control the cell cycle checkpoints, allow damaged cells to progress through the cell cycle, giving rise to the tumour. The development of cell cycle-based cancer therapies is widely studied, in order to inhibit the growth of cancer cells. In general, DNA damaging drugs such as GEM induce cell cycle arrest in S or G_2 phases in a manner regulated by ChK1 kinase (172). The arrest allows the repair of DNA before the cell progresses through the cell cycle.

4. AIM OF THE STUDY

PDAC is the fourth leading cause of cancer-related deaths due to disease presentation at an advanced stage, early metastasis and generally a very limited response to radio and chemotherapy. The current therapeutic strategies are often unsatisfactory. For a long time, the first-line therapy has been GEM that confers only a marginal survival advantage to patients. Small successes in medial survival have been achieved with combined treatment of GEM/nab-paclitaxel and the FOLFIRINOX. However, these therapies are highly toxic and a long-term survival is still a rare case. The absence of effective treatments for PDAC prompts researchers to supplement the current therapies and investigate urgently new therapeutic strategies. An increasing number of studies indicate that PDAC is sustained by a distinct population of quiescent cells, which maintain a mesenchymal phenotype and express specific surface markers of CSCs. The functional properties of CSCs are self-renewal, anchorage-independent growth, long-term proliferative capacity, chemo- and radio-resistance. These cells are also essential for metastatic behaviour and are responsible for the disease relapse following treatment, even with effective drugs that induce initial tumour regression (e.g. nab-paclitaxel). In this context, a major impact on tumour progression is only possible with the use of combined treatments against both differentiated cells, that represent the bulk of the tumour, and CSCs. In this work, we investigate novel treatment strategies in order to identify new ways for targeting CSCs. Given their particular resistance to traditional chemotherapy, the specific killing of CSCs represents one of the most important challenges to eradicate the tumour and prevent recurrence.

Based on the above considerations, the main objectives of my PhD project can be summarized as follows:

- Evaluate the antitumor activity of lipophilic prodrugs obtained by conjugating GEM with the fatty acid chains, (4-(N)-lauroyl-GEM, C12

GEM) and (4-(N)-stearoyl-GEM, C18 GEM), on pancreatic cancer cell line and the derived CSCs;

- Elucidate the cell inhibition mechanisms induced by the drugs, such as apoptosis, autophagy and cell cycle analysis, and the involved intracellular uptake mechanisms;
- Evaluate the effectiveness of a valid approach of drug delivery system which include PEGylated liposomes containing DSF or $Cu(DDC)_2$ and liposomes selective for pancreatic CSCs, using HA as targeting agent. The antitumor activity of the various DSF formulations has been investigated on CSCs derived from PDAC cell lines or patients.

5. MATERIALS AND METHODS

5.1 Cell lines and primary human pancreatic cancer cells

The human Panc1 PDAC cell line was grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and 50 µg/ml gentamicin sulfate (Gibco, Life Technologies). Adherent cells were maintained in standard conditions for a few passages at 37° C with 5% CO_2. Panc1 CSCs were generated as previously described (173) and cultured in CSC medium, i.e. DMEM/F-12 without glucose (US biological Life Sciences) supplemented with 1g/l glucose, B27 (Gibco, Life Technologies), 1 µg/ml Fungizone (Gibco, Life Technologies), 1% penicillin/streptomycin (Gibco, Life Technologies), 5 µg/ml heparin (Sigma), 20 ng/ml EGF (epidermal growth factor, Peprotech), and 20 ng/ml FGF (fibroblast growth factor, Peprotech). Human PDAC tissues were obtained with written informed consent from all patients and expanded in vivo as patient-derived xenografts (PDX) (174). PDXs- derived tumours (A6L, 12556) were minced, enzymatically digested with collagenase (STEMCELL Technologies) for 90 minutes at 37 °C, and after centrifugation for 5 minutes at 1200 rpm, cell pellets were resuspended and cultured in RPMI (Invitrogen) supplemented with 10% FBS and 50 U/mL penicillin– streptomycin. PDX-derived tumours were used in culture until passage 10. Circulating tumour cells (CTC)- derived cultures (C75, C76, C102) were established from blood of PDAC patients. Briefly, blood sample was incubated with a cocktail of antibody-coated beads (CELLection Pan Mouse IgG Dynabeads, Invitrogen) for 20 minutes, and run through the IsoFlux machine (Fluxion). Cells are positively isolated from the sample using an immunomagnetic capture device IsoFlux®. Isolated cells were then released from the beads by incubating for 20 minutes with Release Buffer and cultured in RPMI supplemented with 10% FBS and 50 U/mL penicillin– streptomycin.

5.2 Drugs preparation

All compounds tested on our cell models were provided from University of Turin (in collaboration with Prof. S. Arpicco). GEM was dissolved in distilled water at the final concentration of 3mM and stored at -80°C. C12 GEM and C18 GEM

were dissolved in ethanol at the final concentration of 2.2mM and 1.9mM respectively, and stored at -20°C.

DSF was dissolved in ethanol, DSF conjugated with zinc ($Zn(DDC)_2$), or copper ($Cu(DDC)_2$), or iron ($Fe(DDC)_2$) were dissolved in DMSO and stored at -20°C. The liposomes were prepared by a lipid film hydration-extrusion method (DSF-5%PEG liposome) or using ion gradient method ($Cu(DDC)_2$-5%PEG liposome, $Cu(DDC)_2$-5% HA 4800 liposome, $Cu(DDC)_2$-5% HA 17000 liposome, $Cu(DDC)_2$-2%PEG-3%HA 4800 liposome, $Cu(DDC)_2$-2%PEG-3%HA 17000 liposome). Lipid and drugs (DSF or $CuCl_2$) were dissolved in chloroform and evaporated by rotary evaporator until the formation of a thin layer lipid film. After hydration, liposomes were then extruded (Extruder, Lipex, Vancouver, Canada) under nitrogen, through 220 nm polycarbonate filters (Costar, Corning Incorporated, NY) using pressure of 10 bar. Liposomal preparations were purified from unencapsulated DSF or $CuCl_2$ through chromatography on a Sepharose CL-4B columns, eluting with HEPES buffer for DSF or SHE buffer for $CuCl_2$. To obtain liposomes containing $Cu(DDC)_2$ complex, a solution of the active metabolite of DSF (DDC) (0.25 mg/50 µl MilliQ® water) was added to liposomes containing $CuCl_2$ and incubated for 25 minutes at room temperature. A drastic colour change, from light blue to brown, indicated the formation of $Cu(DDC)_2$ complex inside the liposomes.

5.3 Cell viability Assay

Panc1 and Panc1 CSCs were plated in 96-well cell culture plates. Viable cells were counted by Trypan Blue dye exclusion and $7x10^3$ cells were seeded in each well and incubated at 37˚C with 5% CO_2. After 24 hours, both cell lines were treated as following: 50µM GEM, 50µM C12 GEM and 50µM C18 GEM for 72 hours. To determine their mechanism of intracellular transport, cells were treated also with different membrane entry inhibitors: dipyridamole (Sigma aldrich, Milan), methyl-β-cyclodextrin (MβCD, Sigma Aldrich, Milan), chlorpromazine (CPM, Sigma Aldrich, Milan), sulfo-N-succinimidyl oleate (SSO, Cayman). Moreover, the antitumor activity of other compounds was tested. Panc1 and Panc1

CSCs were treated for 24 hours with a range of concentrations from 0.05 to 100 μM of the following compounds: DSF, $Zn(DDC)_2$, $Cu(DDC)_2$ or $Fe(DDC)_2$, or liposomes (DSF-5%PEG liposome, $Cu(DDC)_2$-5%PEG liposome, $Cu(DDC)_2$-5% HA 4800 liposome, $Cu(DDC)_2$-5% HA 17000 liposome, $Cu(DDC)_2$-2%PEG-3%HA 4800 liposome, $Cu(DDC)_2$-2%PEG-3%HA 17000 liposome). At the end of the treatments, cell viability and the inhibitory activity were evaluated by Resazurin Cell Viability Assay Kit (Immunological Science). This kit detects cell viability by converting resazurin, a non-fluorescent dye, to resorufin, a highly red fluorescent dye, in response to chemical reduction of growth medium due to cell growth. Sixty μl of resazurin solution (10μl of resazurin and 50μl of fresh medium) were added in each well. After about 1 hour, the fluorescent signal was monitored using 535 nm excitation wavelength and 590 nm emission wavelength. The fluorescent signal generated from the assay is proportional to the number of living cells in the well. Three independent experiments were performed for each condition and cell viability was reported as the percentage relative to control.

5.4 Immunoblot analysis for apoptosis

Cells were plated in 60 mm culture plates ($5x10^5$ cells/plate) and treated as described above with GEM and prodrugs of GEM. After 48 hours, cells were collected, washed in 1X PBS, and resuspended in RIPA buffer, pH 8.0 (150 mM NaCl, pH 8.0; 50mM Tris-HCl; 1% Igepal; 0.5% Na-Doc; and 0.1% SDS), 1mM PMSF, 1mM Na3VO4, 1mM NaF, 2.5mM EDTA, and 1X protease inhibitor cocktail (Calbiochem; Merck Millipore) for 30 min on ice. The lysate was centrifuged at 2,300 × g for 10 min at 4°C and the supernatant was used for proteins quantification. Protein concentration was measured with the Bradford Protein Assay Reagent (Thermo Fisher Scientific) using bovine serum albumin as a standard. Thirty micrograms of protein extracts were electrophoresed through a 12% SDS-polyacrylamide gel and electroblotted onto PVDF membranes (Merck Millipore). Membranes were then incubated for 1 h at room temperature with blocking solution, i.e., 5% low-fat milk in TBST (100 mM Tris, pH 7.5, 0.9% NaCl, and 0.1% Tween-20), and incubated overnight at 4°C with the polyclonal rabbit clived-PARP primary antibody (1:1000 in blocking solution, Cell

signaling). Horseradish peroxidase conjugated polyclonal IgG (1:2000 in blocking solution, Cell signaling) was used as secondary antibody. Immunodetection was carried out using chemiluminescent HRP substrates (Merck Millipore) and recorded with Amersham Hyperfilm ECL (GE Healthcare). To quantify clived-PARP expression, bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units using the public domain NIH Image software (http://rsb.info.nih.gov/nihimage/), and normalized on alpha-tubulin signal used as control.

5.5 Cell cycle analysis

Cell cycle distribution was analyzed using propidium iodide (PI)-stained cells. Briefly, 5×10^5 cells were plated in 60 mm culture plates and treated as described above with GEM and prodrugs of GEM. After 48 hours, cells were washed with PBS, were collected, and incubated with 0.1% sodium citrate dihydrate, 0.1% Triton X-100, 200 μg/ml RNase A and 50 μg/ml propidium iodide (Roche Molecular Biochemicals). The analysis was performed through flow cytometry (BD FACSCanto, BD Biosciences). Approximately 10,000 gated events were acquired for each sample and analyzed using FlowJo software (TreeStar, Inc.). Dead cells and debris were excluded based upon forward scatter and side scatter measurements.

5.6 Labeling of autophagic vacuoles with MDC

To quantify the induction of autophagic process, 5×10^5 cells were plated in 60 mm culture plates and treated as described above with GEM and prodrugs of GEM. Following the treatment, cells were incubated with 50 μM of monodansylcadaverine (MDC) (Sigma aldrich, Milan) in PBS at 37°C for 15 minutes. After incubation, cells were washed with PBS, trypsinized and analyzed by flow cytometry (BD FACSCanto, BD Biosciences). Approximately 10,000 gated events were acquired for each sample and analyzed using FlowJo software (TreeStar, Inc.). Dead cells and debris were excluded based upon forward scatter and side scatter measurements.

5.7 Annexin V and Apoptosis Inducing Factor analysis

Cells were seeded in a 24-well plate on glass cover-slips at a density of 4×10^4/well. After 24 h, cells were treated with 50μM of GEM, C12GEM or C18GEM for 48 hours. Cells were then rinsed in PBS and fixed in 4% (w/v) paraformaldehyde. After blocking in 5% bovine serum albumin (BSA) and 0,05% of triton-X-100, cells were incubated with Annexin V (Molecular probes) 1:40 or apoptosis inducing factor (AIF) antibody (Bethyl Laboratories) 1:100 overnight. Alexa Fluor conjugated antibodies (Life Technologies) were used as secondary antibodies and nuclei were stained with Dapi. Cover-slips were mounted over slides in AF1 medium (Dako). Cell images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Leica Microsystem, Manheim, Germany) at 63× magnification and analysed using Image J software. For figure preparation images were processed using Adobe Photoshop.

5.8 Cytotoxicity assay on primary human pancreatic cancer cells

PDXs- derived tumours (A6L, 12556) and circulating tumour cells (CTC)-derived cultures (C75, C76, C102) cells were plated in 96 well cell culture plates. Viable cells were counted by Trypan Blue dye exclusion and $3x10^3$ cells were seeded in each well and cultured as spheres with DMEM: F12 (Invitrogen, Karlsruhe, Germany) supplemented with B27 (GIBCO, Karlsruhe, Germany) and bFGF (PeproTech EC, London, UK) in anchorage independent conditions for 3 days (First generation spheres). First generation tumor spheres were harvested using a 40 μm cell strainer, dissociated into single cells by trypsinization, and then re-cultured for additional 7 days (second generation spheres). After 3 days, spheres were treated with 0.1 μM of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG or Lipo$Cu(DDC)_2$-2%PEG-3%HA 17000 for 24 hours. Cell viability was evaluated using Resazurin Cell Viability Assay Kit as described in section 3.3. Three independent experiments were performed for each condition and cell viability was reported as the percentage relative to control.

5.9 Sphere formation assay

Pancreatic cancer spheres of first and second generation were obtained by culturing primary pancreatic cancer cells as described in section 3.8. Ten thousand cells were seeded in each well in 24-well cell culture plate and incubated for 3 days. Then, first and second generation spheres were treated with 0.1 μM of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG or Lipo$Cu(DDC)_2$-2%PEG-3%HA 17000 for 4 days. After treatment, a CASY Cell Counter (Roche Applied Sciences, Mannheim, Germany) was used to quantify spheres > 40 microns. Each condition was performed in triplicate.

5.10 Statistical analysis

ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 (GraphPad Software) and used for multiple-group comparison. Student's t-test was used for individual group comparison. P-values < 0.05, 0.01, 0.001 were indicated as *, **, ***, respectively

RESULTS

6. Identification of CSC specific therapy with lipophilic pro-drug based strategy

6.1 Chemo-sensitivity of Panc1 CSCs to GEM and lipophilic pro-drug treatments

It is known that CSCs represent a sub-population of quiescent cells within the tumor that can sustain its malignant behavior giving rise to more differentiated cancer cells and contributing to relapse. However, their high resistance to traditional chemotherapy and radiotherapy imposes the development of alternative CSCs-targeted therapeutic approaches. Panc1 and Panc1 CSCs were plated in 96-well cell culture plates. After 24 hours, cells were treated with 50µM GEM and the lipophilic pro-drugs C12 GEM and C18 GEM. Cell viability was measured with Resazurin Assay after 72 hours (Fig. 14).

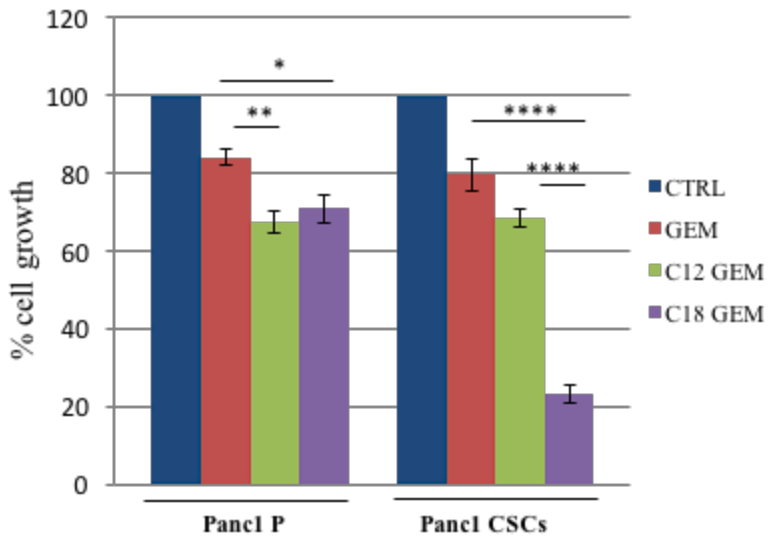


Fig.14: Comparison of GEM, C12 GEM or C18 GEM antiproliferative activity on Panc1 and Panc1 CSCs after 72 hours of treatment; values are the mean (±SD) of four independent experiments, p value <0.05 is considered significant.

The in vivo antitumor activity of the lipophilic produgs against a variety of tumours is generally superior to that of the native drug (175, 176).

We observed that the lipophilic pro-drugs were more efficacious than GEM in both cell lines, but only Panc1 CSCs showed a high sensitivity to C18 GEM treatment with a strong reduction of cell growth.
This result indicates that C18 GEM is more effective than C12 GEM and GEM alone on Panc1 CSCs and then, it could be a potential candidate for a specific CSC-targeted therapy.

6.2 Intracellular uptake mechanisms of GEM and lipophilic pro-drugs in Panc1 parental cell line and Panc1 CSCs

To explain the greater sensitivity of Panc1 CSCs to C18 GEM treatment than parental cell line, we investigated the mechanism of intracellular transport of the drugs using different membrane entry inhibitors. In the literature, a wide range of nucleoside-derived antitumour drugs, such as gemcitabine, are described to enter into the cells through the membrane nucleoside transporters (177). However, for the lipophilic prodrugs of GEM, the mechanism of intracellular transport is not completely clear.
Panc1 and Panc1 CSCs were treated with 50μM of GEM, C12 GEM and C18 GEM plus increasing amounts of the following membrane entry inhibitors: methyl-β-cyclodextrin (MβCD), an inhibitor of lipid raft formation by cholesterol depletion; chlorpromazine (CPM), an inhibitor of clathrin- mediated uptake; dipyridamole, a non-specific inhibitor of membrane nucleoside transporters and sulfo-N-succinimidyl oleate (SSO), an irreversible inhibitor of the fatty acids translocase CD36. Cell viability was measured with Resazurin assay after 72 hours of treatment and the condition with each inhibitor alone was tested to exclude their toxicity. We found that in Panc1 (fig. 15 A), but not in Panc1 CSCs (fig. 15 B), the lipophilic formulations of GEM and GEM alone were dependent on nucleoside transporters for entering into the cells, as suggested by the increase of cell viability after combined treatment with drugs and dipyridamole.

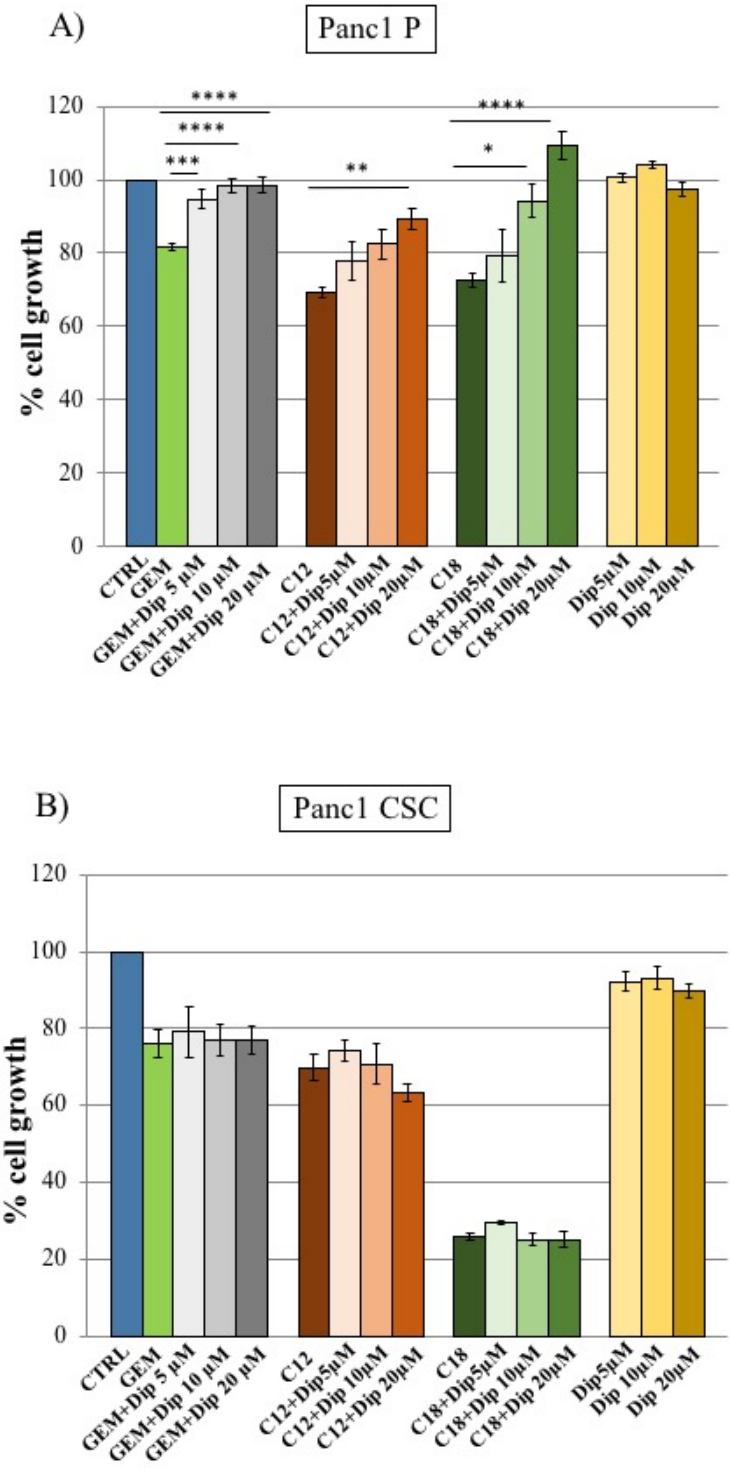


Fig. 15: Effect of the nucleoside transporter inhibitor dipyridamole on the antiproliferative activity of GEM, C12 GEM and C18 GEM in Panc1 cells (A) and Panc1 CSCs (B). Values are the means (±SD) of three independent experiments; p value <0.05 is considered significant.

Afterward, we investigated the transport mechanism mediated by CD36, the translocase involved in the fatty acids uptake. As shown in fig. 16, in Panc1 parental cell line (A) and Panc1 CSCs (B), only C18 GEM was in part dependent on CD36 for entering into the cells. Indeed, the SSO inhibitor didn't influence the effect of GEM or C12GEM on cell growth and determined its partial rescue only after C18 GEM treatment.

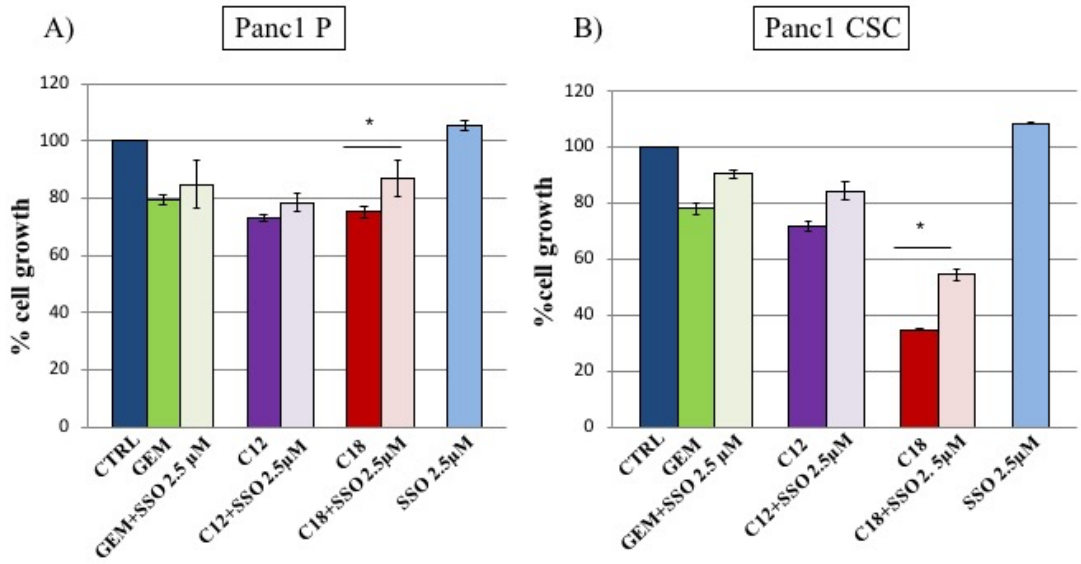


Fig. 16: Effect of the CD36 inhibitor SSO on the antiproliferative activity of GEM, C12 GEM and C18 GEM in Panc1 cells (A) and Panc1 CSCs (B). Values are the means (±SD) of three independent experiments; p value <0.05 is considered significant.

6.2.1 Investigation of clathrin and lipid rafts-mediated endocytosis

In contrast to these results, the investigation of the role of clathrin and lipid rafts-mediated endocytosis, has shown that in both Panc1 parental cell line and Panc1 CSCs, the inhibitory activity of CPM (fig. 17 A and 17 B) and MβCD (fig. 18 A and 18 B) didn't influence the effect of all the drugs on cell growth, suggesting that the lipophilic formulations and GEM alone were not dependent on clathrin and on lipid rafts for entering into the cells.

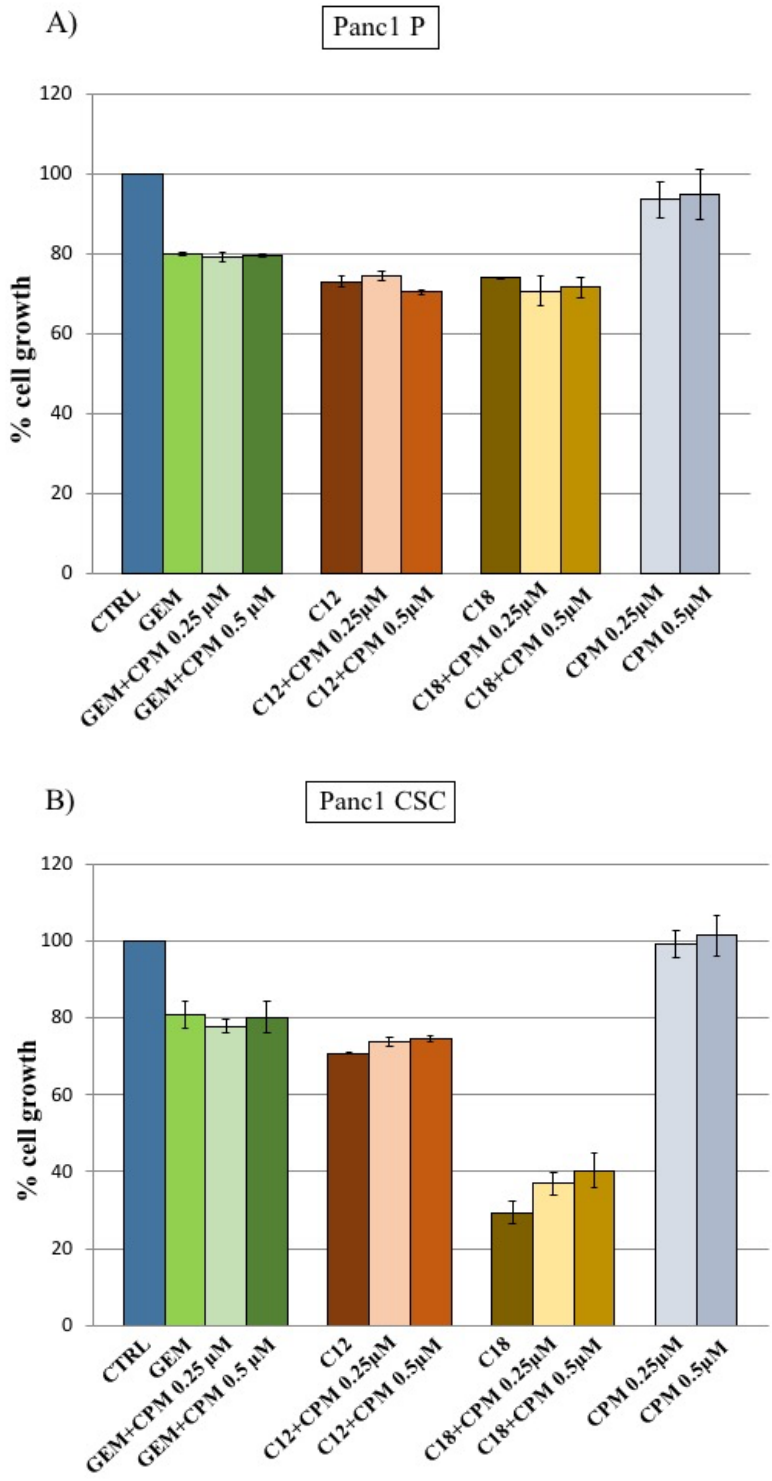


Fig. 17: Effect of the clathrin-mediated endocytosis inhibitor chlorpromazine (CPM) on the antiproliferative activity of GEM, C12 GEM and C18 GEM in Panc1 cells (A) and Panc1 CSCs (B). Values are the means (±SD) of two independent experiments.

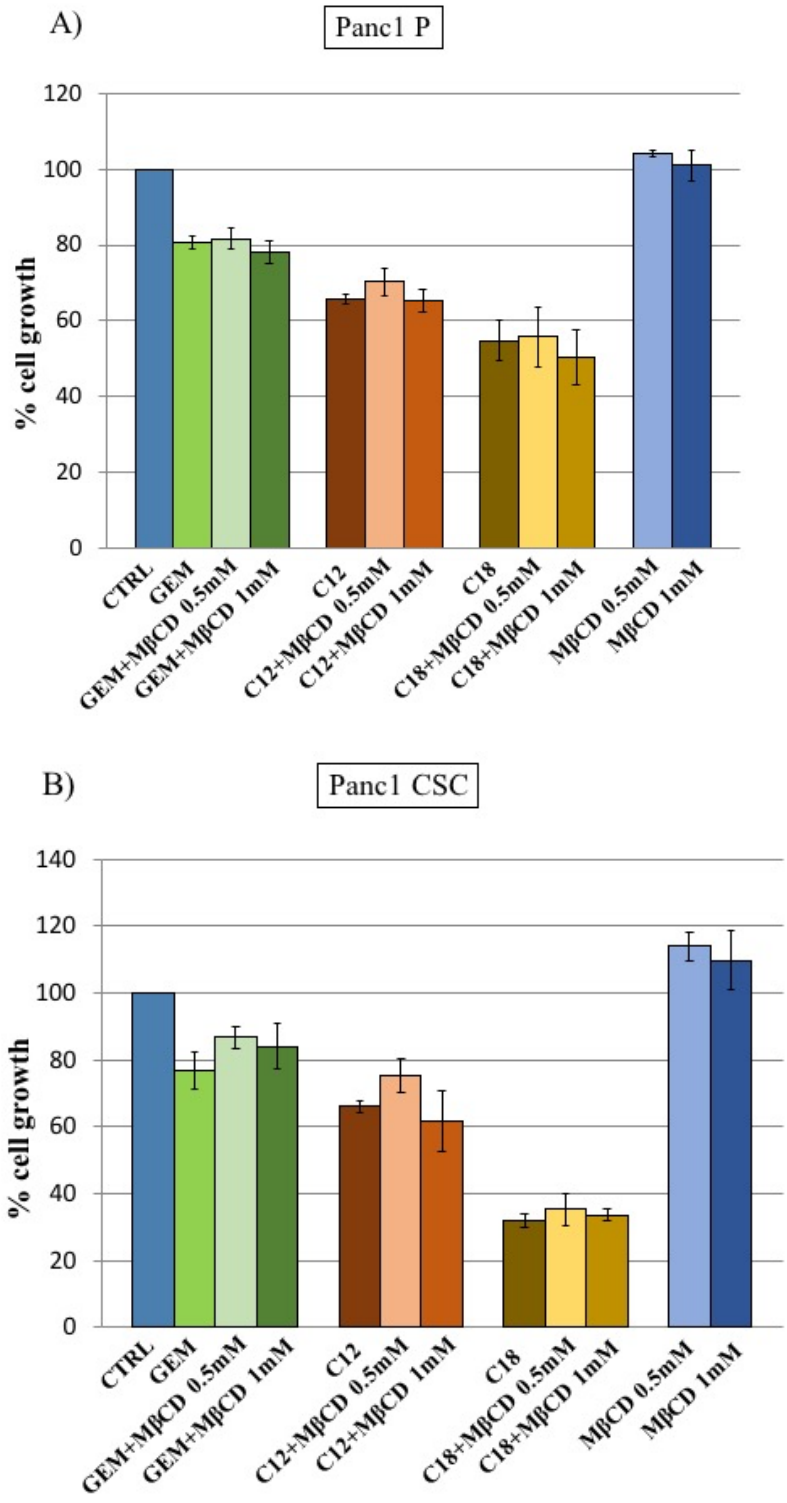


Fig. 18: Effect of the lipid raft-mediated endocytosis inhibitor methyl-β-cyclodextrin (MβCD) on the antiproliferative activity of GEM, C12 GEM and C18 GEM in Panc1 cells (A) and Panc1 CSCs (B). Values are the means (±SD) of three independent experiments.

Taken together the data described above suggest that the transport mechanisms of the drugs into the cells are different between Panc1 parental cell line and Panc1 CSCs. This difference suggests that CSCs possess different membrane characteristics that affect the entry of the drugs and thus their anticancer activity. In Panc1, the uptake of GEM and both lipophilic pro-drugs is strongly dependent on membrane nucleoside transporters and partially on CD36 translocase only for C18 GEM. On the contrary, in Panc1 CSCs the uptake of C18 GEM is at least partially dependent on CD36 translocase and, together with GEM and C12 GEM, based on other mechanisms that need further clarification.

6.3 Evaluation of cell inhibition mechanisms by GEM and lipophilic pro-drugs in Panc1 parental cell line and Panc1 CSCs

6.3.1 Cell cycle is not affected by GEM and lipophilic formulations treatment

To investigate the possible effects of GEM, C12 GEM and C18 GEM treatment on the cell cycle, we analyzed the G_1-S-G_2 phase distribution in the cell population by propidium iodide staining. Panc1 and Panc1 CSCs were treated with 50 μM of GEM and lipophilic formulations and were analyzed by flow cytometry. We observed that the treatment for 48 hours with all drugs didn't affect the cell cycle in either Panc1 (fig. 19 A) or Panc1 CSCs (fig. 19 B).

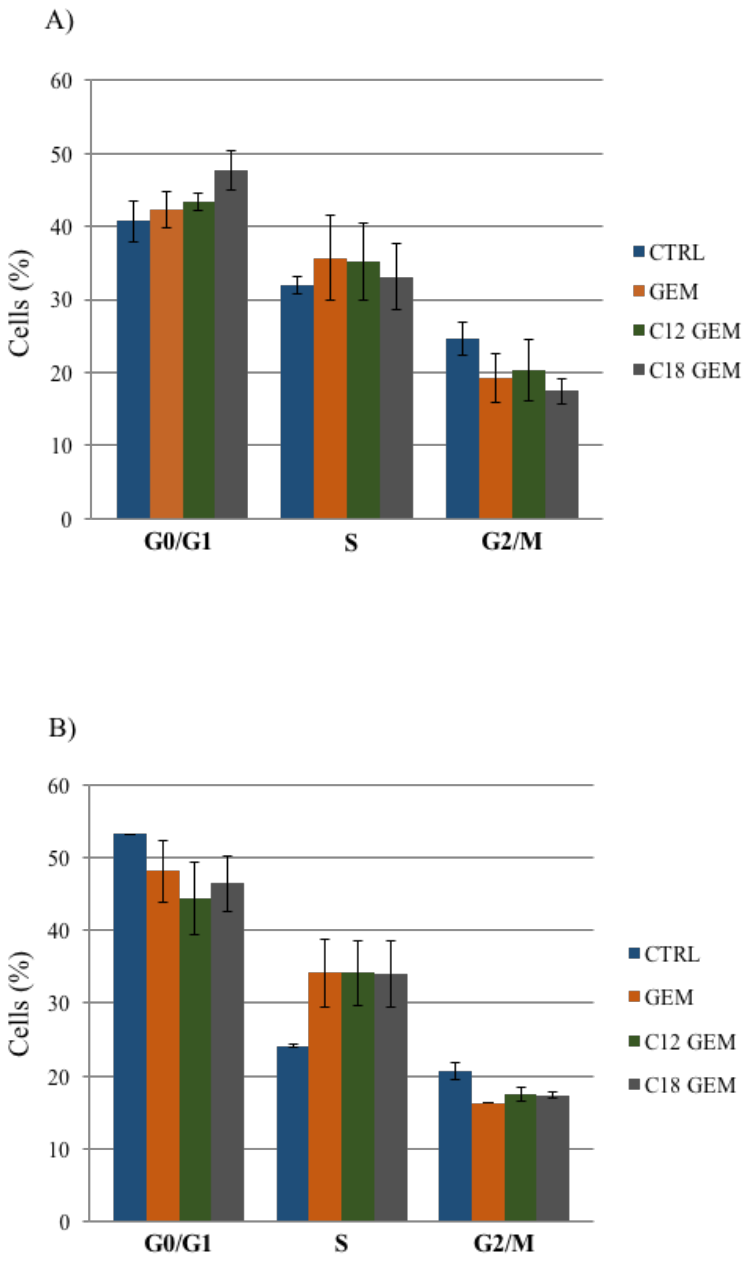


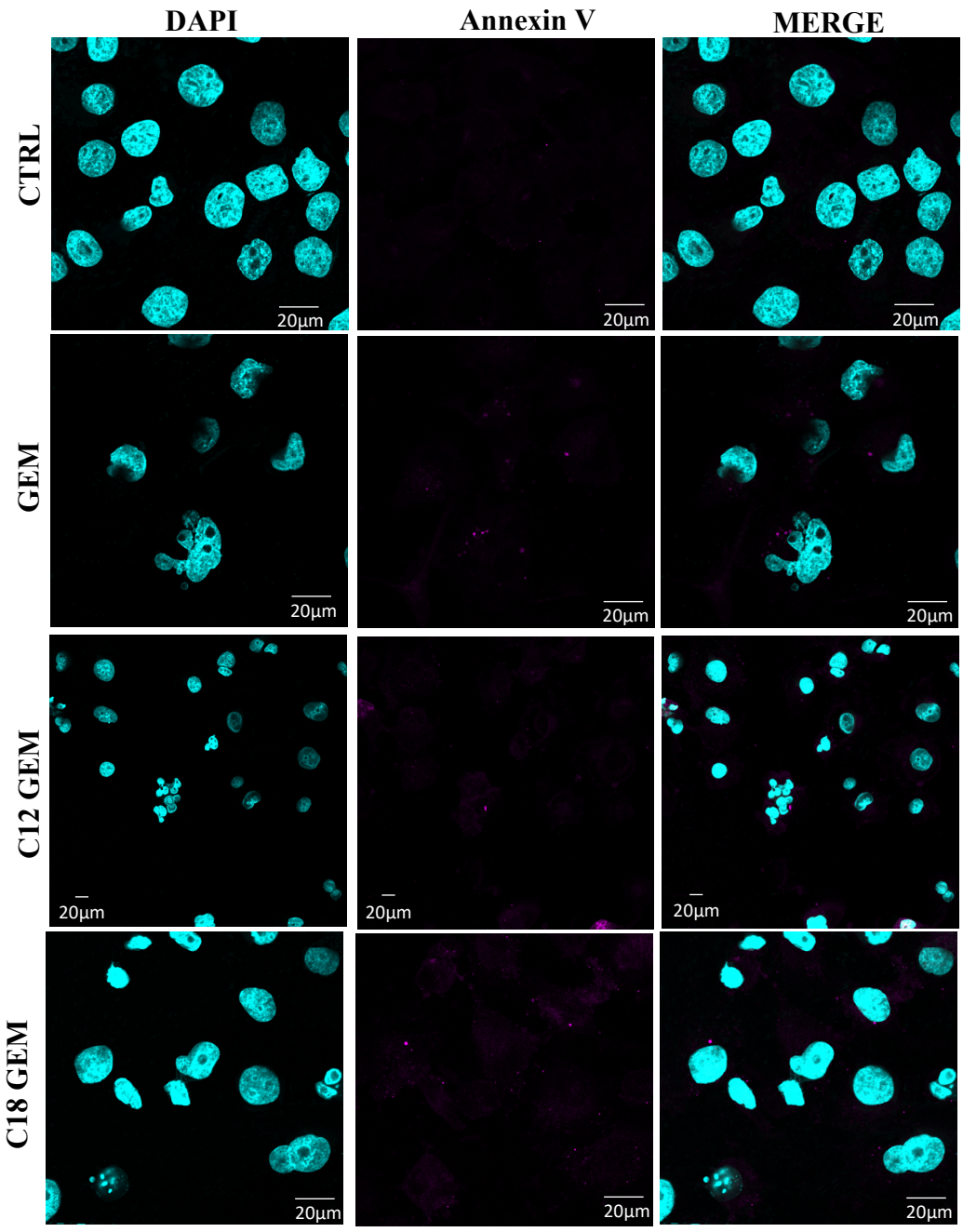
Fig. 19: Effect of GEM, C12 GEM and C18 GEM on cell cycle performed by flow cytometry in Panc1 cells (A) and Panc1 CSCs (B). Values are the mean of three independent experiments.

6.3.2 Analysis of different mechanisms of apoptosis in Panc1 and Panc1 CSCs

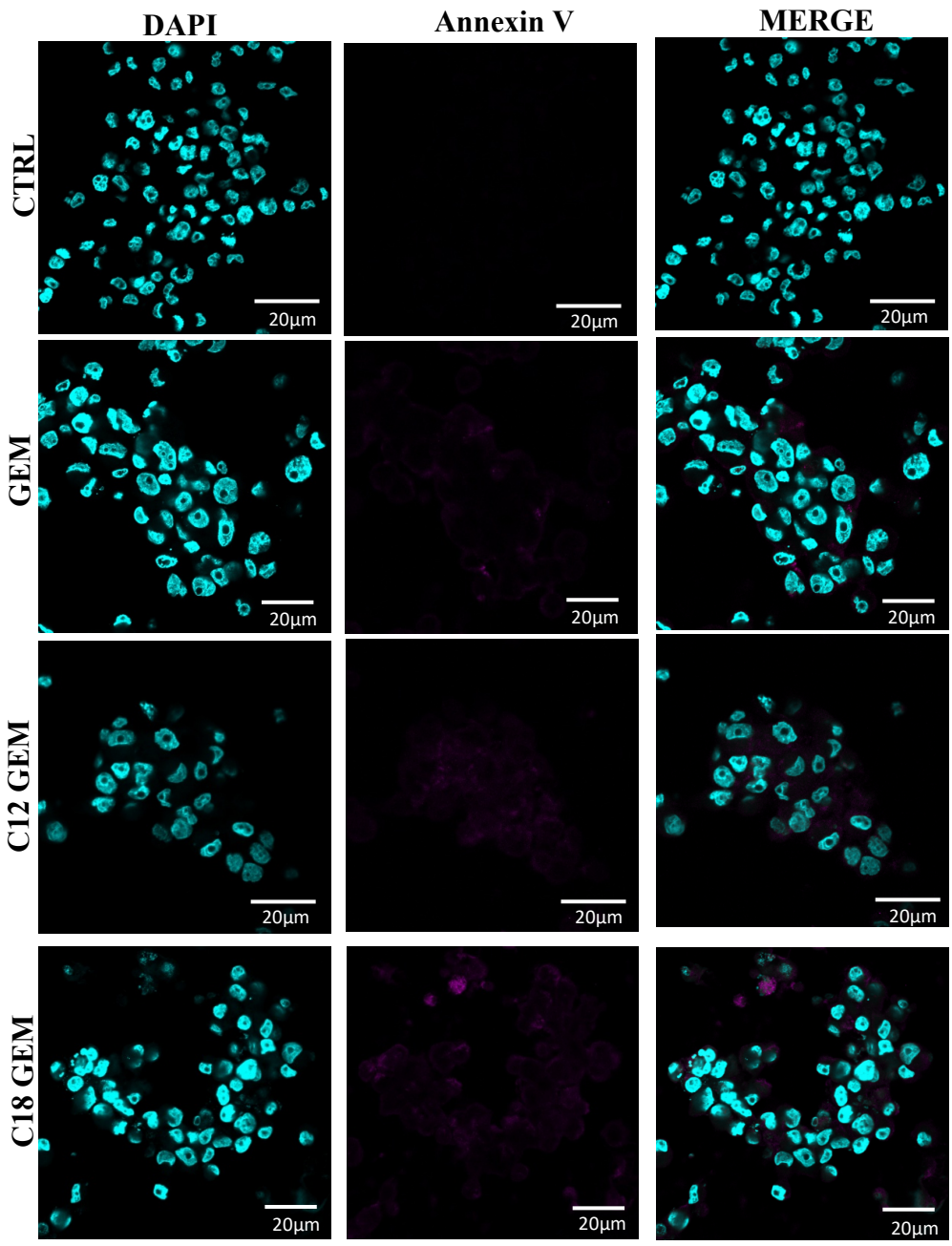
It is known that the cytotoxic effect of GEM occours through induction of programmed cell death, which correlates with Bcl-2 content (178). The apoptotic response to treatment was first investigated through Annexin V staining that is used for detecting translocated phosphatidylserine, a hallmark of apoptosis.

Panc1 and Panc1 CSCs were plated in a 24-well plates or on glass cover-slips and treated with 50μM of GEM, C12 GEM and C18 GEM for 48 hours. After treatments, cells were incubated with Annexin V as described in Material and Methods and images were captured using a confocal laser-scanning fluorescence microscope. As shown in fig. 20, the annexin signal was increased with the treatments in Panc1 (A) and Panc1 CSCs (B), as indicated by the increase of fluorescence intensity, which was strongest in both cell lines after C12 GEM and C18 GEM treatment (C). As it can be seen in the histogram summarizing all the data (C), the effect was much stronger in the CSCs.

A



B



C

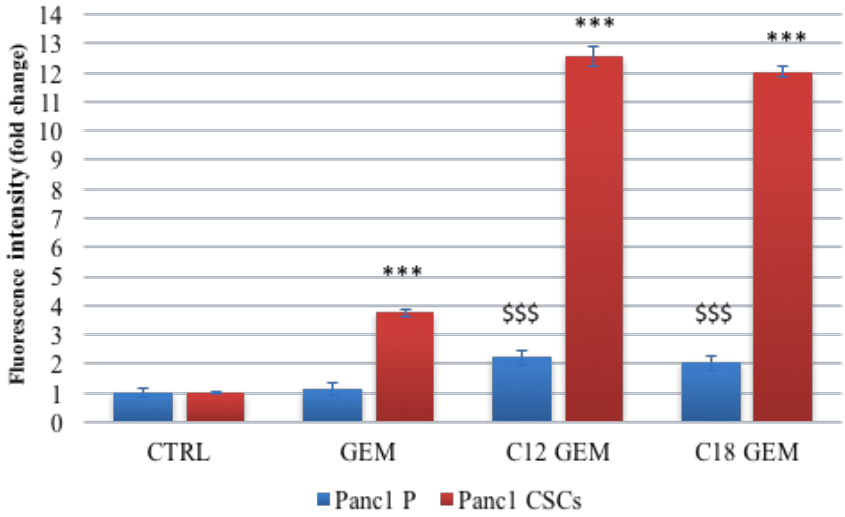
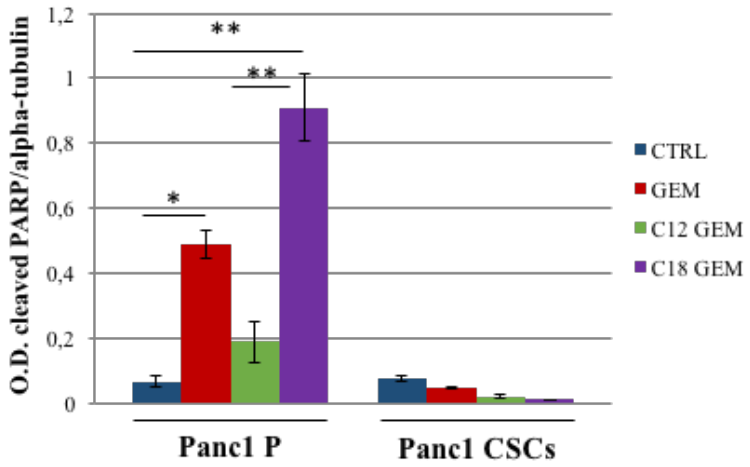


Fig. 20: Confocal microscopy images of Panc1 parental (A) and Panc1 CSCs (B) treated for 48 hours with GEM, C12 GEM or C18 GEM and incubated with Annexin V antibody and Dapi. Cyan channel shows nuclei labeled with Dapi, magenta channel shows the positivity to annexin, and the merge represents cellular association of annexin; C) Histogram of the fluorescence intensity of Panc1 parental cells and Panc1 CSCs treated for 48 hours with GEM, C12 GEM or C18 GEM and incubated with Annexin V antibody. Single cells of three random fields were analysed through Leica LAS software. Values are the means (±SEM) of ten ROI values reported as a fold change relative to the control. Statistical analysis: *CTRL vs treated in Panc1 CSCs; $ CTRL vs treated in Panc1 P.

We next evaluated the molecular mechanism of apoptosis through the analysis of cleaved-PARP expression that is increased following the activation of caspase 3 during apoptosis. Interestingly, we found a different expression of cleaved-PARP between Panc1 and Panc1 CSCs (Fig. 21 A and 21 B). In Panc1 cells, we found an increase of cleaved-PARP expression depending on treatments, especially for C18 GEM, while in Panc1 CSCs, in all conditions, we found a very low expression of clived-PARP that was slightly reduced with treatment.

A)



B)

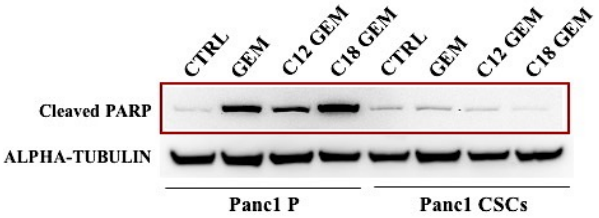


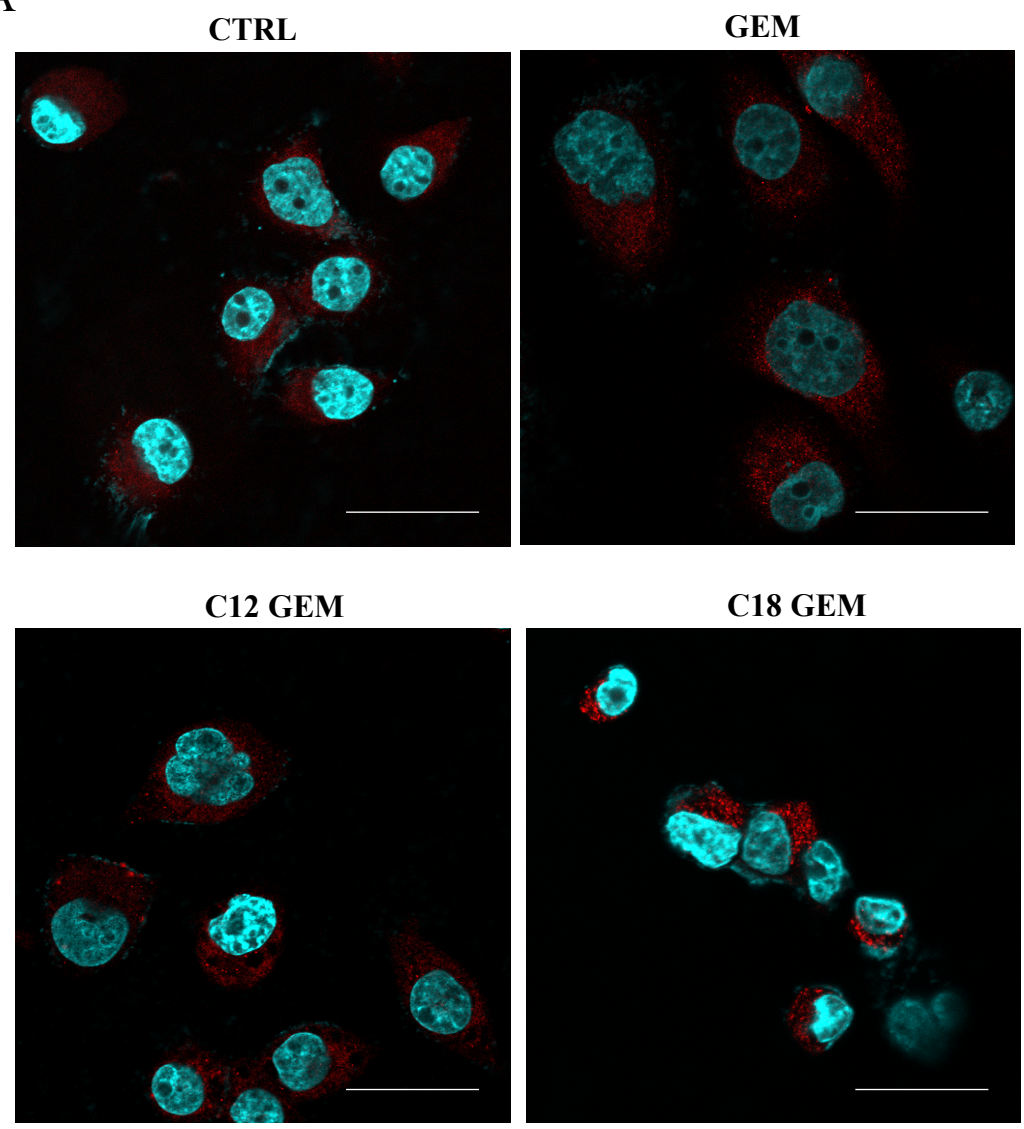
Fig. 21: Effect of GEM, C12 GEM and C18 GEM on apoptosis analyzed through cleaved-PARP expression. A) Quantitative evaluation of clived-PARP expression levels in Panc1 and Panc1 CSCs. The bands of Western blot analysis were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units. The value of alpha-tubulin was used as a normalizing factor. Values are the mean of two independent experiments. P< 0.01 (**) CTRL versus treated and C12 GEM versus C18 GEM. B) Representative Western blot analysis of cleaved-PARP expression in Panc1 and Panc1 CSCs treated with GEM, C12 GEM and C18 GEM. Alpha-tubulin was used as control loading.

These results suggest a different mechanism of apoptosis in Panc1 and Panc1 CSCs induced by the drugs that could be caspase-independent in CSCs. To confirm this hypothesis, we investigated the involvement in the apoptosis of the mitochondrial protein AIF, following its translocation from the mitochondria to

the nucleus. There is no evidence in literature that explains this preferential AIF-mediated pathway for CSCs.

In Fig. 22, we have reported representative images of Panc1 (A) and Panc1 CSCs (B) treated with 50 μM of GEM, C12 GEM and C18 GEM for 48 hours, and incubated with AIF antibody.

A



B

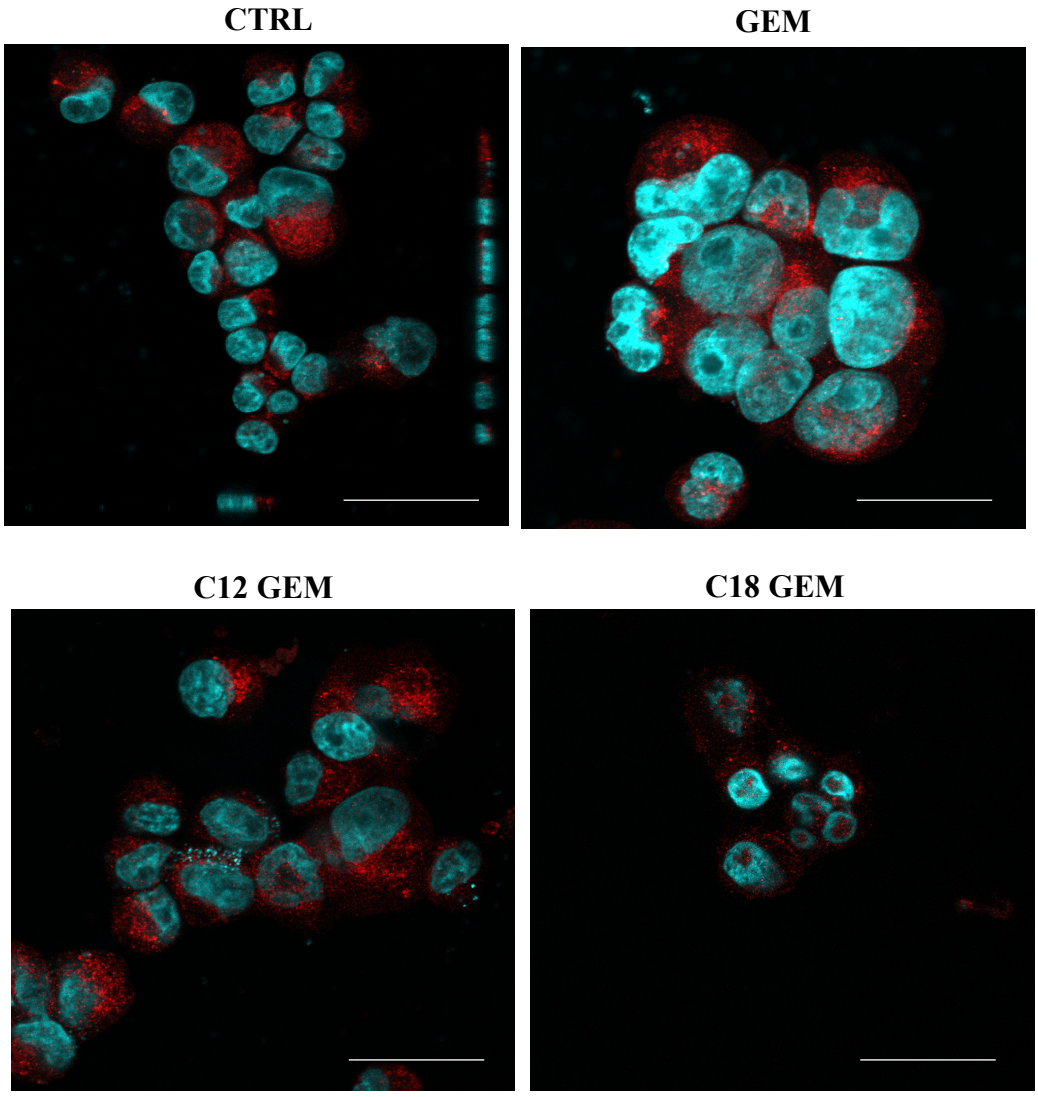


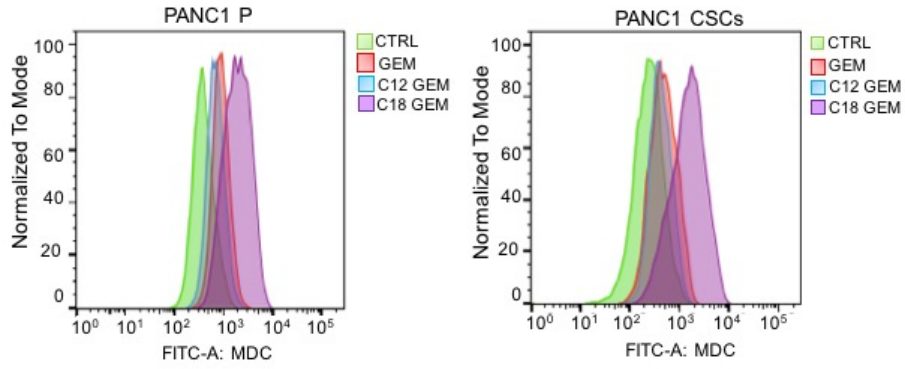
Fig. 22: Confocal microscopy images of Panc1 parental (A) and Panc1 CSCs (B) treated for 48 hours with GEM, C12 GEM or C18 GEM and incubated with AIF antibody and Dapi. Cyan channel shows nuclei labeled with Dapi, red channel shows AIF localization. Scale bars, 40 μm.

Only in Panc1 CSCs did we found an evident presence of AIF (red spots) in the nucleus after treatment, in particular after C18 GEM treatment. Taken together, these data demonstrate that the same treatments induced different apoptotic mechanisms by involving PARP in Panc1 and AIF in Panc1 CSCs.

6.3.3 Induction of autophagy after C18 GEM treatment

To evaluate the autophagic response of cells to treatments with GEM and its pro-drugs, we analyzed the amount of autophagosomes by flow cytometry, labeling the cells with monodansylcadaverine (MDC), a specific marker of autophagic vacuoles. MDC accumulates in these vacuoles due to a combination of ion trapping and specific interactions with membrane lipids. We found that C18 GEM induced an increase of monodansylcadaverine uptake and thus an increase of the amount of autophagosomes, in both cell lines (Fig. 23 A and 23 B).

A)



B)

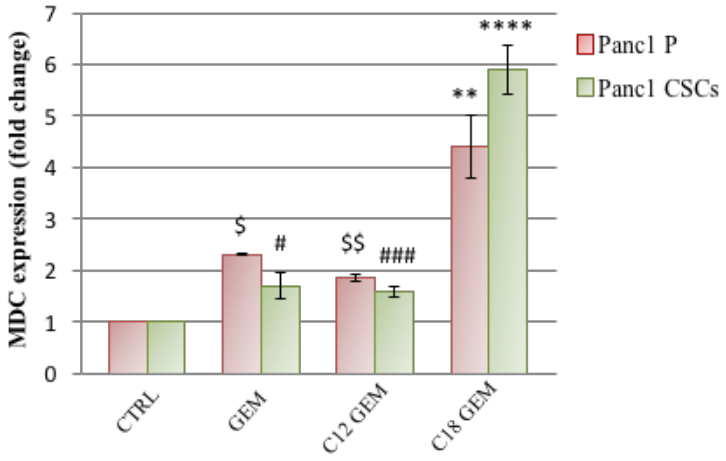


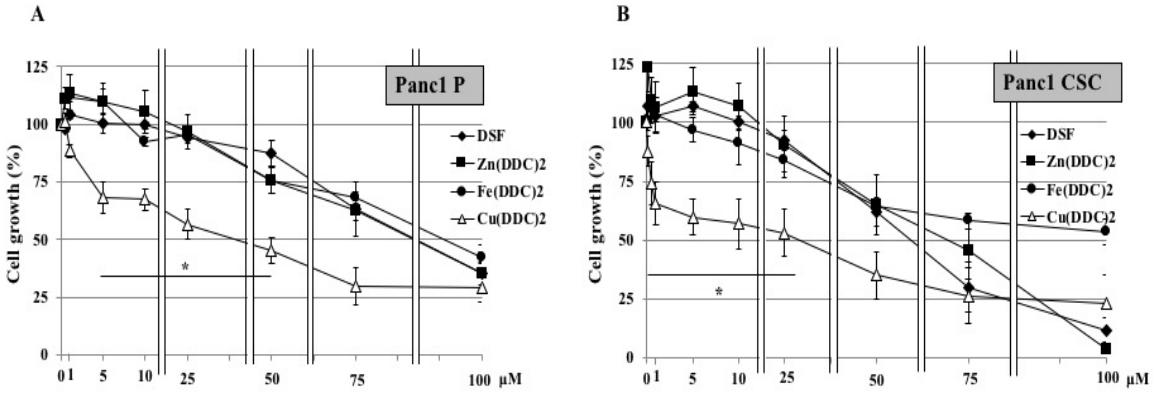
Fig 23: Analysis of autophagy performed through monodansylcadaverine (MDC) assay. A) Representative FACS histograms of Panc1 P and Panc1 CSCs untreated or treated for 48 hours with GEM, C12 GEM or C18 GEM and labeled with MDC; B) Histogram of the median fluorescence intensity of Panc1 parental cells and Panc1 CSCs treated for 48 hours with GEM, C12 GEM or C18 GEM and labeled with MDC. Values are the means (±SEM) of three independent experiments and are reported as a fold change relative to control. Statistical analysis: * CTRL vs treated; $ or # GEM, C12 GEM vs C18 GEM.

Taken together, the data described above indicate that C18 GEM may be a potential therapeutic strategy for the specific killing of CSCs. All the treatments tested induce a PARP-dependent apoptosis in Panc1 cells and a mechanism of caspase-independent apoptosis mediated by AIF in Panc1 CSC. Furthermore, C18 GEM increases the autophagosomes formation as a response of the cells to stress induced by drug treatment. Regarding the intracellular uptake mechanisms, the entrance of the lipophilic formulations and of GEM is dependent on nucleoside transporters in Panc1 cells, while in Panc1 CSCs the mechanism is yet not clear and further studies are needed.

7. Identification of CSC specific therapy with disulfiram formulations

7.1 Cytotoxicity of Disulfiram and conjugates on Panc1 cells and Panc1 CSCs

DSF acts as antitumoral drug and represents a possible candidate as anti-CSC agent in glioblastoma and breast cancer (179, 180). As widely reported in literature, DSF and dithiocarbamate are able to form stable complexes with metals such as copper, zinc, gold and iron (181). To evaluate the antitumor activity of DSF or $Zn(DDC)_2$, $Cu(DDC)_2$, $Fe(DDC)_2$ on Panc1 parental cell line or the derived Panc1 CSCs, both cell lines were treated at dose ranging from 0 to 100 μM of these compounds. Cell viability was evaluated after 24 and 72 hours (Fig. 24) of treatment. In both cell lines $Cu(DDC)_2$ was significantly more efficacious than DSF, $Zn(DDC)_2$ and $Fe(DDC)_2$, inducing a concentration-dependent reduction of cell growth.



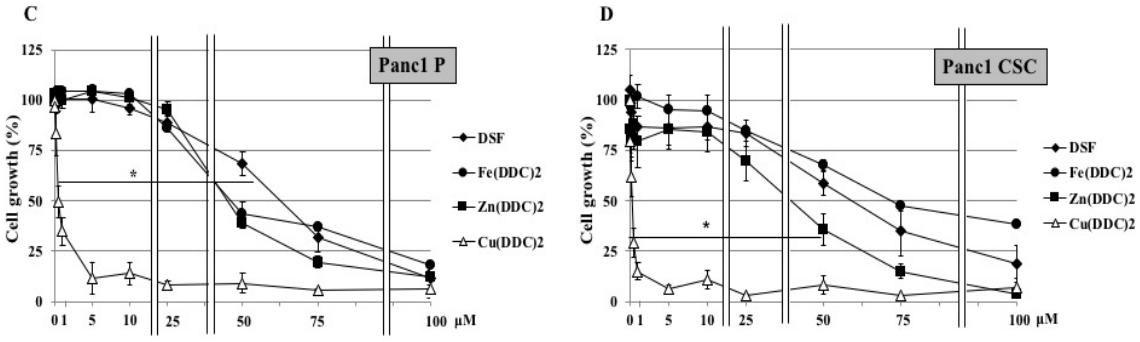


Fig. 24: Effect of DSF, $Zn(DDC)_2$, $Fe(DDC)_2$, $Cu(DDC)_2$ on Panc1 P (A and C) and Panc1 CSCs (B and D) cell growth. Cells were seeded in 96-wells plates and treated after 24 hours with increasing concentrations of DSF formulations for 24 h (A and B) or 72 h (C and D). Cell growth was determined using the Resazurin cell viability assay. Values are the means ±SEM of three independent experiments each performed in triplicate. Statistical analysis: DSF or $Zn(DDC)_2$ or $Fe(DDC)_2$ versus $Cu(DDC)_2$, * $p < 0.05$.

Interestingly, after 24 hours Panc1 CSCs were more sensitive than Panc1 cells to DSF treatment (Table 2). Treatments of 72 hours with $Cu(DDC)_2$ showed a greater inhibition of cell growth resulting in a total mortality even at low drug doses both in Panc1 cells and Panc1 CSCs (Fig. 24 C and D), with IC_{50} values of 0.68 ± 0.16 and 0.35± 0.03, respectively (Table 2).

These results demonstrate that $Cu(DDC)_2$ possesses the strongest antitumor activity in our cell line models. For this reason and for its well documented anticancer activity (182, 183, 184) it was chosen for the encapsulation in liposomes.

7.2 $Cu(DDC)_2$ containing liposomes possess a strong anti-proliferative activity on Panc1 cells and Panc1 CSCs

To increase the effectiveness and the blood circulation time of DSF and $Cu(DDC)_2$, we next evaluated the cytotoxic activity of liposomes containing DSF and $Cu(DDC)_2$. These liposomal formulations were prepared in collaboration with the University of Turin. It has been demonstrated that the development of liposomes as drug delivery system overcomes problems related to the poor

solubility of several anticancer agents and shows efficacy in different tumour models, such as breast cancer and acute myeloid leukemia (185, 186). In a control experiment, cell viability was shown not to be altered by the presence of liposomes alone without drugs (data not shown). The liposomal formulation containing DSF (LipoDSF-5%PEG) was significantly more active than free DSF, only after 72 hours of treatment, as shown in the IC_{50} values in Table 2 and in cell growth fig. 25, in both cell lines. Moreover, Panc1 CSCs were more sensitive than Panc1 to LipoDSF-5%PEG treatment at both incubation times (table 2).

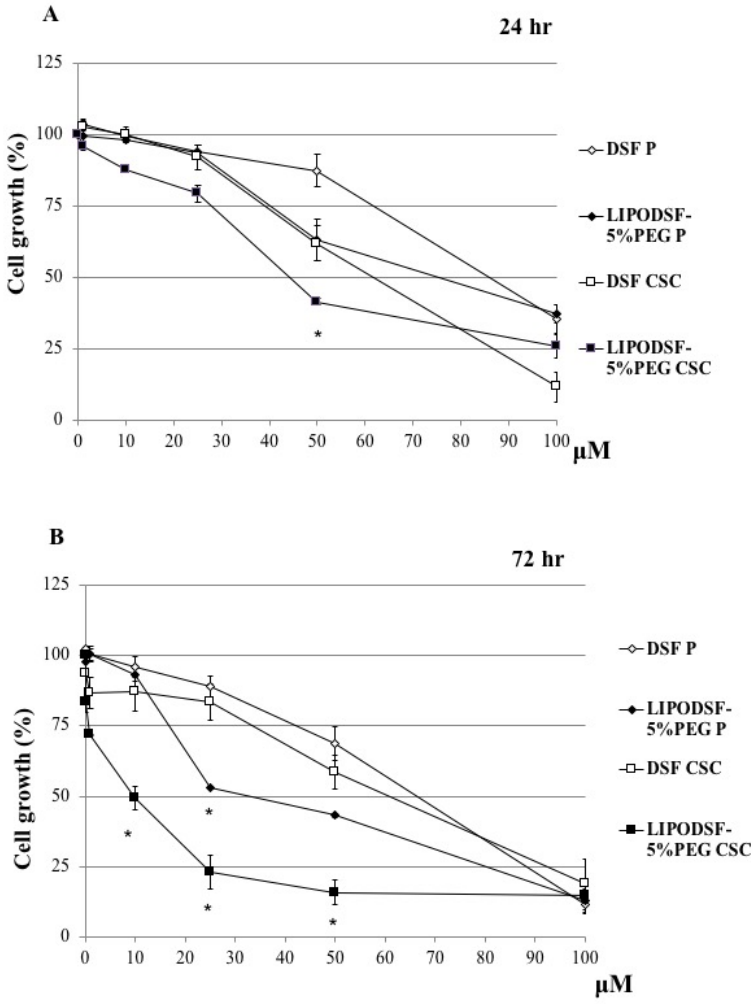
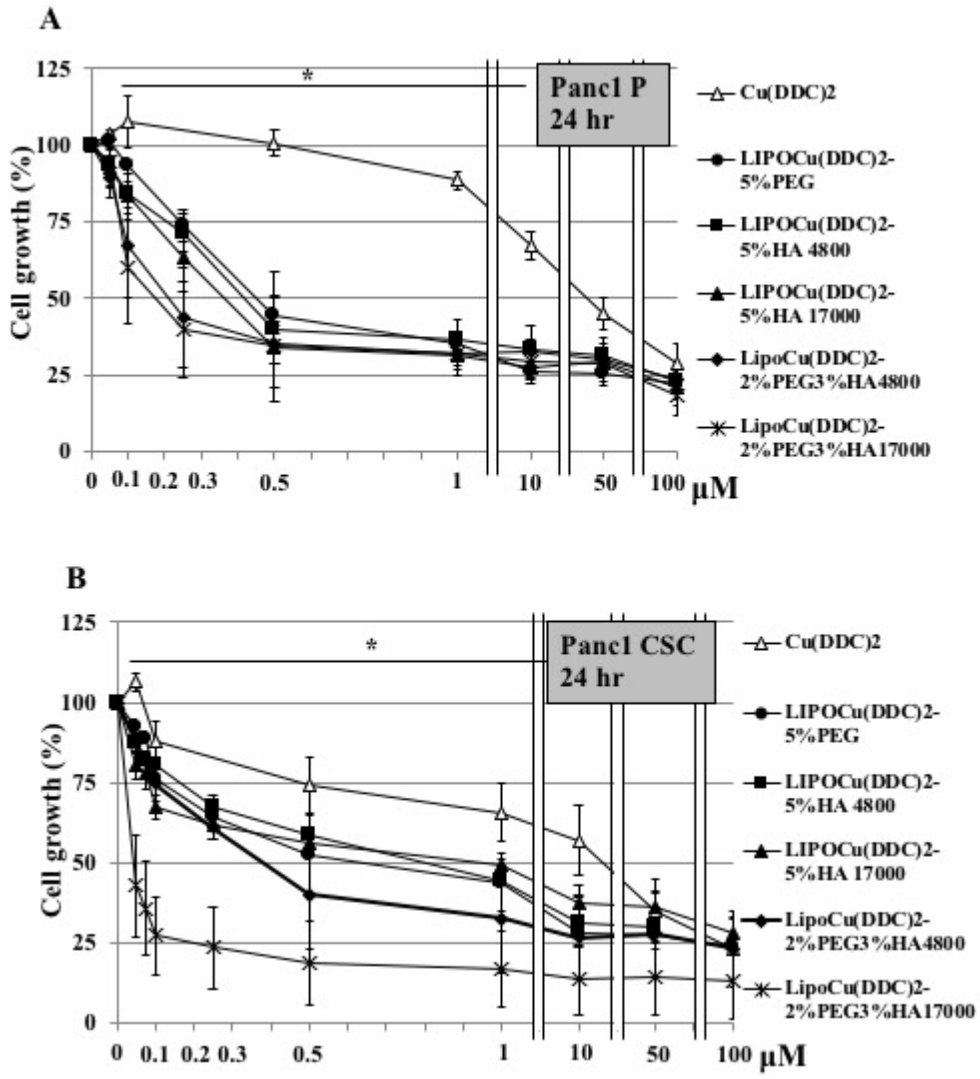


Fig. 25: Effect of DSF and LipoDSF-5%PEG on Panc1 P and Panc1 CSCs cell growth. Cells were seeded in 96-wells plates and treated after 24 hours with increasing concentrations of the drugs for 24h (A) or 72h (B). Statistical analysis: DSF versus LipoDSF-5%PEG on Panc1 P or on Panc1 CSC * $p < 0.05$.

Loading of $Cu(DDC)_2$ into the liposomes strongly increased the anti-proliferative effect of $Cu(DDC)_2$, making it a potent compound with an excellent antitumour activity.

As shown in Fig. 26, Lipo$Cu(DDC)_2$-5%PEG demonstrated a higher anti-proliferative activity than $Cu(DDC)_2$ in Panc1 and Panc1 CSCs, resulting in a concentration dose-dependent reduction of cell growth and lower IC_{50} values (Table 2).



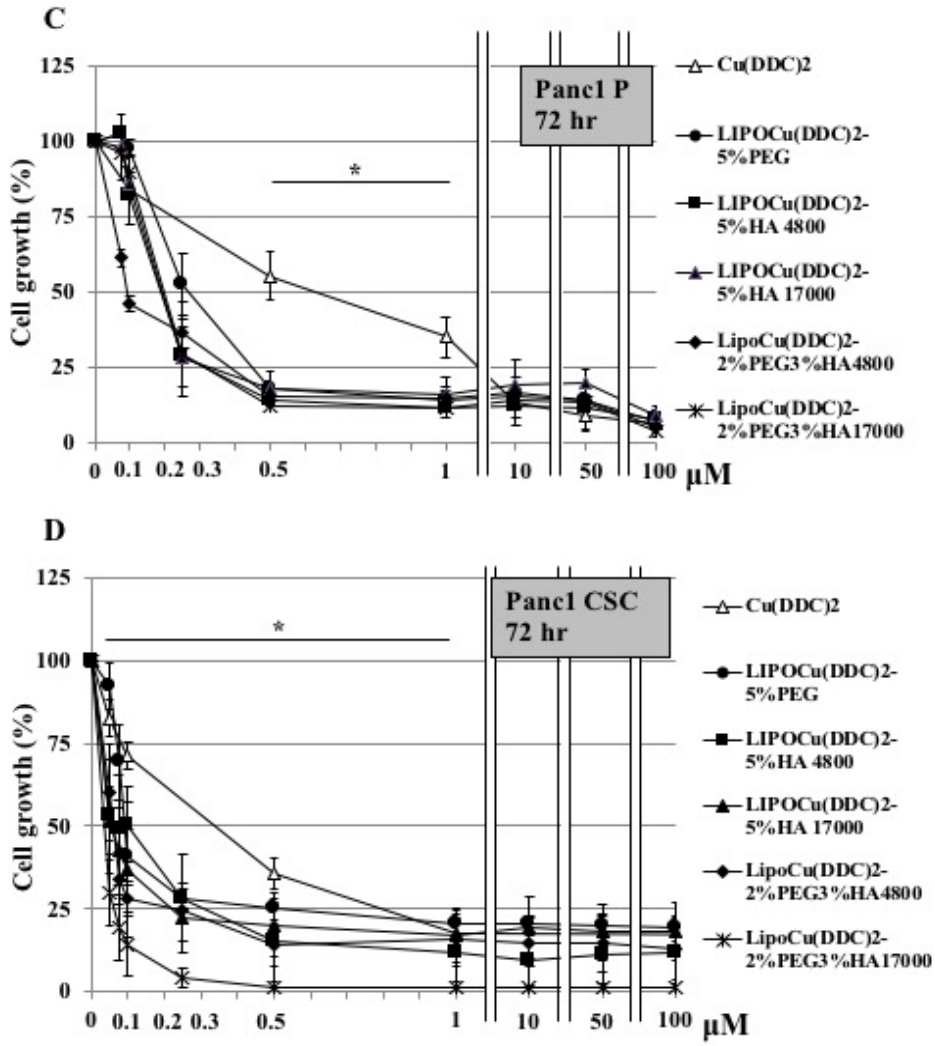


Fig. 26: Effect of $Cu(DDC)_2$ and of liposome formulations Lipo$Cu(DDC)_2$-5%PEG, Lipo $Cu(DDC)_2$-5%HA 4800, Lipo$Cu(DDC)_2$-5%HA 17000, Lipo$Cu(DDC)_2$-2%PEG-3%HA 4800, Lipo$Cu(DDC)_2$-2%PEG-3%HA 17000 on Panc1 P (A and C) and Panc1 CSCs (B and D) cell growth. Cells were seeded in 96-well plates and treated after 24 hours with increasing concentrations of $Cu(DDC)_2$ formulations for 24 h (A and B) or 72 h (C and D). Cell growth was determined using the Resazurin cell viability assay. Values are the means ±SEM of three independent experiments each performed in triplicate. Statistical analysis: liposome formulations versus $Cu(DDC)_2$, * $p < 0.05$.

To investigate the impact of hyaluronic acid (HA)-coating on the targeting capacity to CD44-expressing tumour cells, we next evaluated the anti-proliferative effect of $Cu(DDC)_2$ loaded in liposomes coated with HA at two different

molecular weights, 4800 and 17000 Da, and/or with different percentage of PEG. Liposomal formulations containing either PEG or HA determined a similar inhibition of cell proliferation, whereas the presence of both PEG and HA further increased the anti-proliferative activity of $Cu(DDC)_2$ at 24 h (Fig. 26 and Table 2), in both cell lines. Furthermore, after 72 hours of treatment, Panc1 CSCs were more sensitive to liposome formulations with HA17000 and/or PEG compared to Panc1 cells (Table 2).

	IC_{50} 24h (µM)		**IC_{50} 72h (µM)**	
	P	**CSCs**	**P**	**CSCs**
DSF	85.5±6.0	60.8±5.9*	65.3±4.2	61.0± 10.5
$Zn(DDC)_2$	86.6±2.4	70.3±10.6	45.2±1.2	41.5±7.6
$Cu(DDC)_2$	43.6±10.8$	27.4±7.0$	0.68±0.16$	0.35±0.03$
LipoDSF -5%PEG	73.8±4.2	44.4±0.1*	33.1±0.8#	10.3±1.9#*
Lipo$Cu(DDC)_2$-5%PEG	0.46±0.02#	0.68±0.16#	0.28±0.03#	0.08±0.006#*
Lipo$Cu(DDC)_2$-5%HA4800	0.48±0.001#	0.80±0.31#	0.18±0.003#	0.11±0.08#
Lipo$Cu(DDC)_2$-5%HA17000	0.34±0.09#	0.97±0.44#	0.18±0.006#	0.04±0.001#*
Lipo$Cu(DDC)_2$-2%PEG3%HA4800	0.20±0.09#	0.38±0.11#	0.08±0.004#	0.06±0.01#
Lipo$Cu(DDC)_2$-2%PEG3%HA17000	0.14±0.05#£	0.03±0.004#£	0.20±0.01#	0.02±0.004#*

Table 2: IC50 values (expressed as µM) on Panc1 P and Panc1 CSC at 24h and 72h after the indicated treatments as determined by Resazurin assay, mean ± SEM. P-values < 0.05 were indicated as $ DSF or $Zn(DDC)_2$ vs $Cu(DDC)$; # DSF and DSF conjugates vs liposomal complex; £ Lipo$Cu(DDC)_2$-5%PEG vs Lipo$Cu(DDC)_2$-2%PEG-3%HA17000 * P vs CSC.

Interestingly, examining the anti-proliferative effect at a concentration as low as 0.1 μM, Panc1 CSCs were highly sensitive to liposomal formulations, while Panc1 cells were completely resistant (Fig. 27).

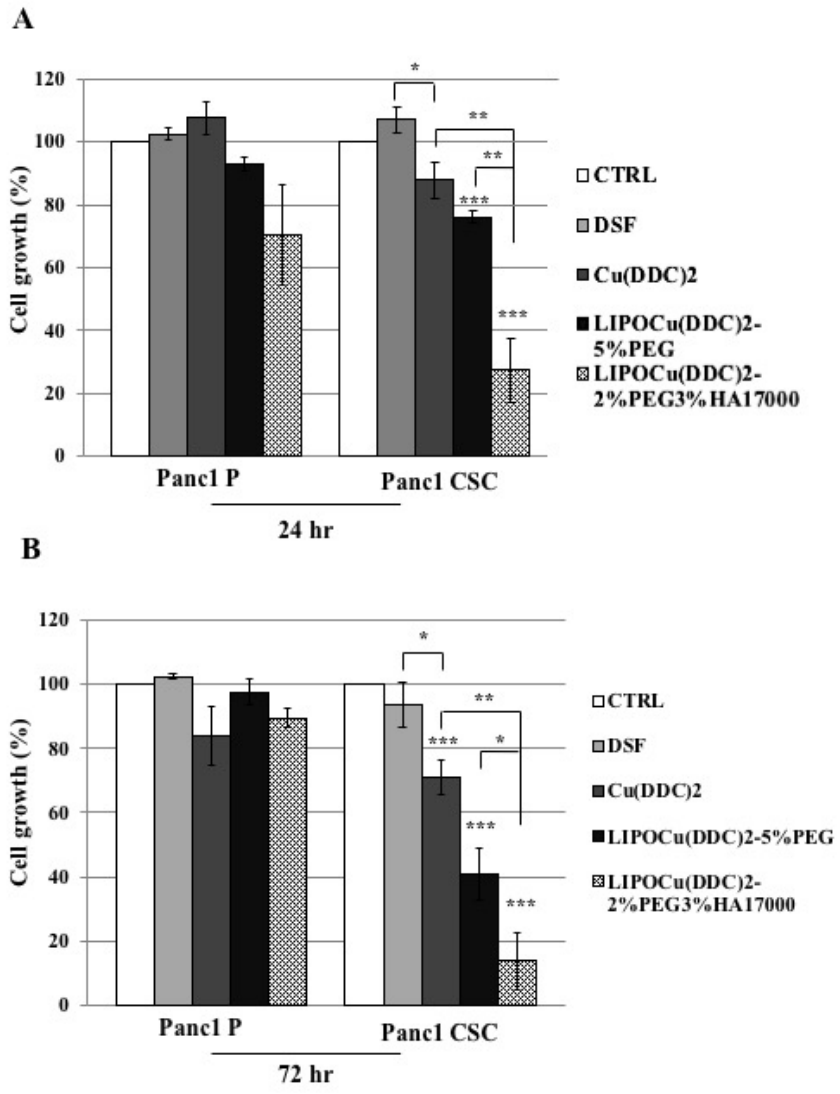


Fig. 27: Effect of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG and Lipo$Cu(DDC)_2$-2%PEG-3%HA 17000 on Panc1 P and Panc1 CSCs cell growth. Cells were treated with 0.1 μM of drugs for 24h (A) or 72h (B). Statistical analysis: CTRL versus treated, or as indicated in figure * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

These results demonstrate that liposomes coated with 2%PEG and 3% HA17000 are the most effective tested compound on Panc1 CSCs, suggesting an innovative formulation that could be used in a CSC- targeted therapy in PDAC. Furthermore, these results suggest that liposomes increase the effectiveness of the drugs and could prevent their degradation, allowing a cytotoxic prolonged activity over time.

7.3 Cytotoxicity of DSF and $Cu(DDC)_2$ containing liposomes on cells derived from PDAC patients

To evaluate the anti-proliferative activity of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG or lipo$Cu(DDC)_2$-2%PEG-3% HA17000 on pancreatic cancer cells obtained directly from patients, the primary cells 12556, A6L, C75, C76 and C102 were cultured as spheres, as described in materials and methods, and treated with 0.1 μM of the formulations mentioned above. Cell viability was evaluated after 24 h of treatment. In fig. 28, we show that the liposome formulations containing $Cu(DDC)_2$ were more effective than free drugs on first generation spheres and even more on second generation spheres. These data are particularly interesting since they confirm the strong anti-proliferative effect of $Cu(DDC)_2$ containing liposomes, obtained with Panc1 cell lines, on cells derived from PDAC patients having stem like features.

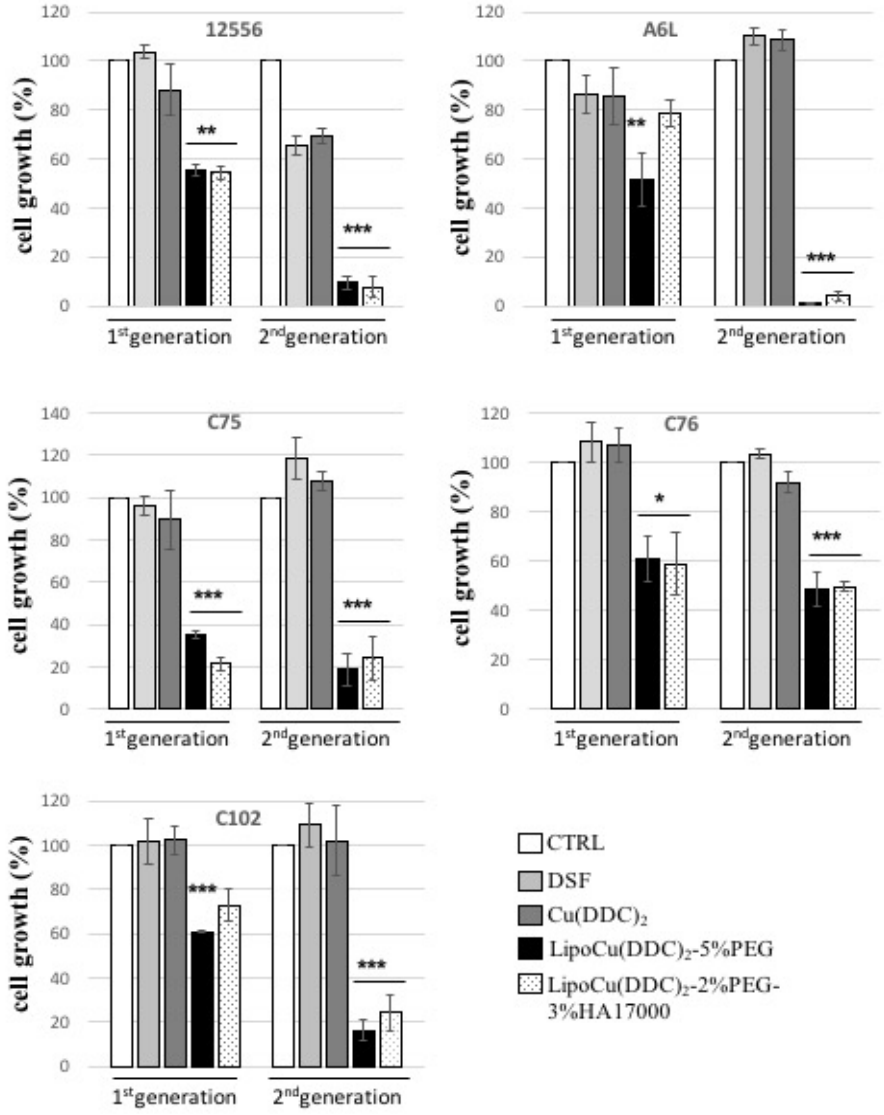


Fig. 28: Effect of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG and Lipo$Cu(DDC)_2$-2%PEG-3%HA 17000 on cell growth of PDAC PDX-derived culture cells (12556, A6L) and CTC-derived cells (C75, C76, C102) cultured as first and second generation spheres. Cells were treated with 0.1μM of drugs for 24h. Cell growth was determined using the Resazurin cell viability assay. Values are the means ±SEM of three independent experiments each performed in triplicate. Statistical analysis: CTRL versus treated, or as indicated in figure * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Since the copper-drug complexes have not been approved for the use in patients because of their extremely low solubility, it is difficult to establish their utility in preclinical models or patients. For this reason, the liposome formulations are a good strategy to make the $Cu(DDC)_2$ complexes suitable for intravenous administration. Moreover, we demonstrate the initial evidence that the liposome formulations seem to have a promising potential as CSC-targeting agents.

7.4 Effect of DSF and $Cu(DDC)_2$ containing liposomes on sphere formation capability

In the context of CSC features, the sphere formation capability is regularly studied *in vitro* and used to identify new ways for targeting CSCs. We used a specific method for culturing primary human pancreatic cancer cells isolated from tissues resected during surgery as tumour spheres of first or second generation. In fig. 29, we reported the effect of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5%PEG and Lipo$Cu(DDC)_2$-2%PEG-3%HA17000 on the *in vitro* sphere formation capability. We found that liposome formulations at 0.1µM decreased the number of first generation spheres formed, while DSF and $Cu(DDC)_2$ treatment affected slightly or didn't affect the sphere number.
The first generation spheres were subsequently passaged into second generation spheres and also the formation of these spheres was drastically reduced after the treatment with the liposome formulations.

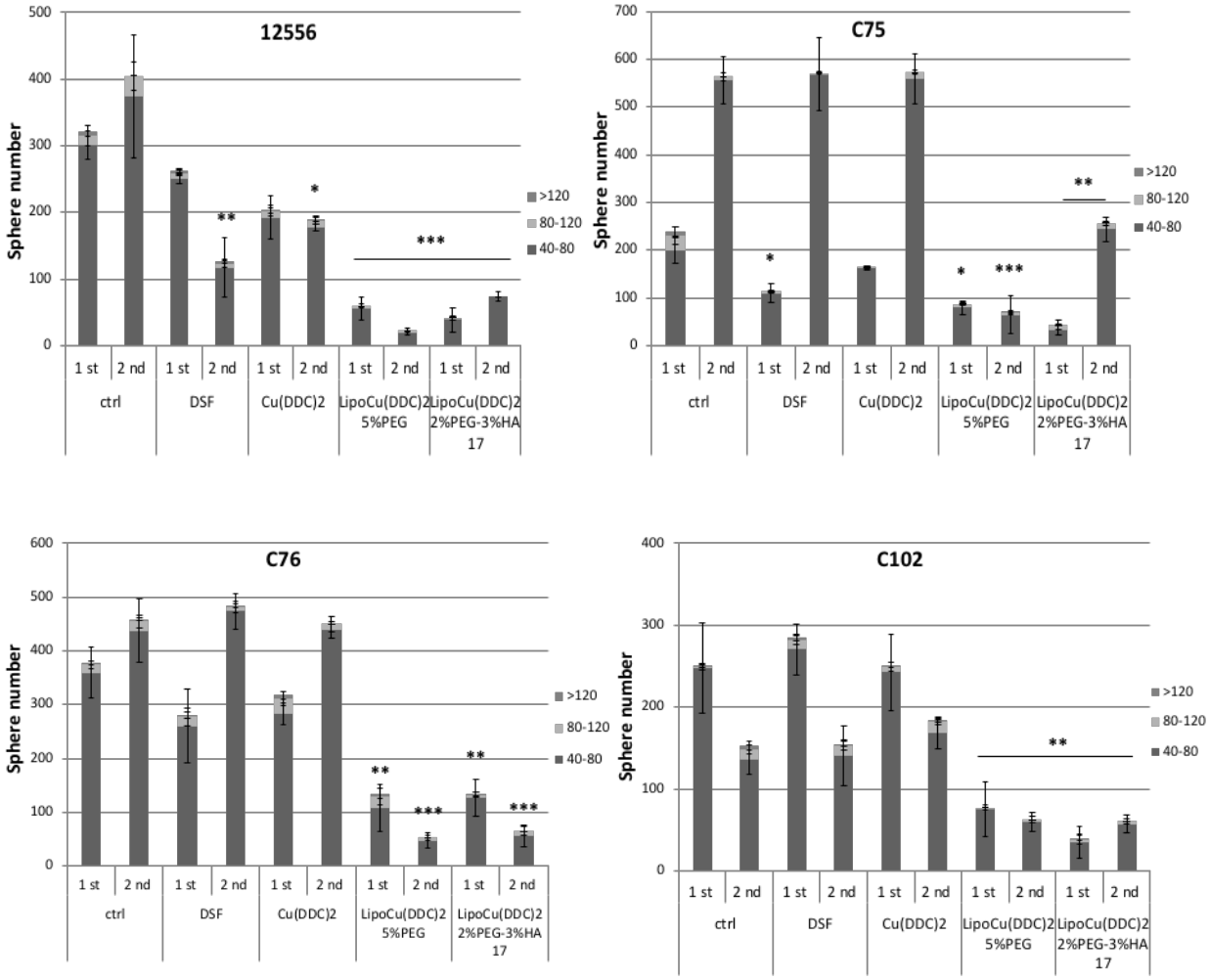


Fig. 29: Effect of DSF, $Cu(DDC)_2$, Lipo$Cu(DDC)_2$-5% PEG and Lipo$Cu(DDC)_2$-2% PEG-3%HA17000 on 12556, C75, C76, and C102 spheres formation capability. Cells were seeded in 24-well plates for first or second generation spheres and treated after 3 days with 0.1μM of drugs for 4 days. Spheres number was determined through sphere formation assay performed by CASY Counter and reported as spheres of 40-80 μm , 80-120μm and >120 of diameter. Statistical analysis: CTRL versus treated, or as indicated in figure * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Also the cellular morphology was affected by liposome formulation treatment (Fig. 30) and the spheres lost their typical shape becoming similar to small cell aggregates.

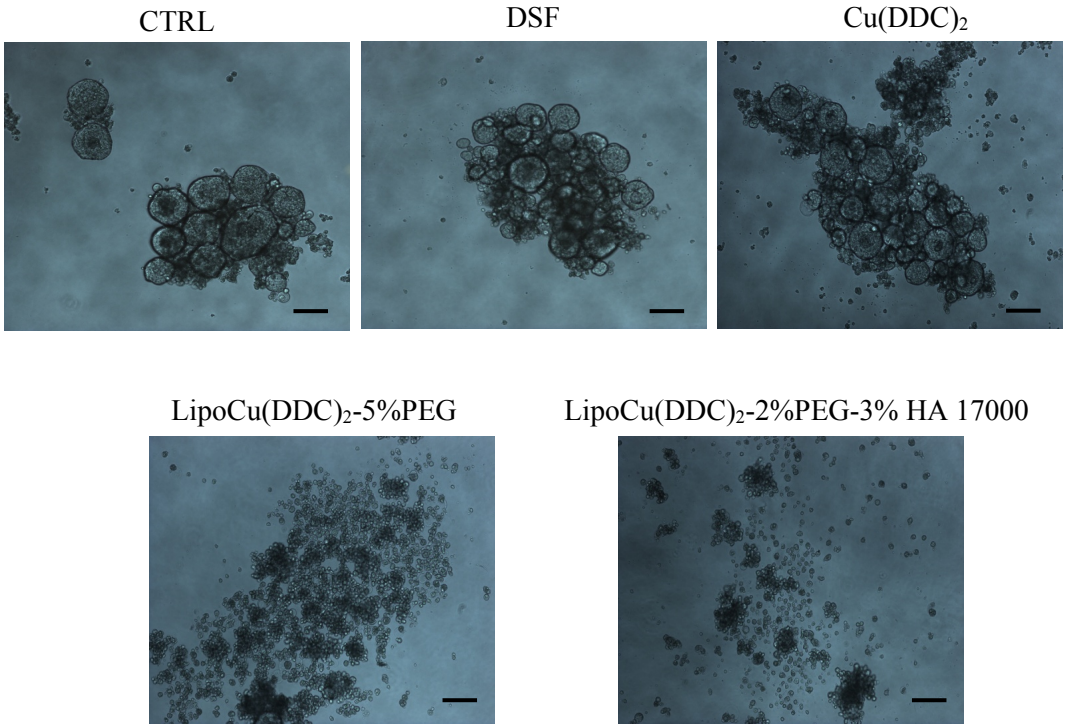


Fig. 30: Representative images of PDAC spheres. Cells were seeded in 24-well plates and treated after 3 days with 0.1µM of drugs for 4 days. The pictures have been acquired at day 7 with a 10X objectives. Scale bars, 50µm

Thus, our data demonstrate a significant effect of liposome formulations on the self-renewal capacity of primary pancreatic cancer stem like cells.

8. DISCUSSION AND CONCLUSION

In the last years, cancer stem cell biology represents one of the most controversial fields of modern molecular oncology. Since CSC hypothesis has been confirmed for leukemia in 1994 (187), other evidence has also emerged for solid tumours, including PDAC, indicating that also these tumours are sustained and promoted by cells with features of stem cells and self-renewal capacity (188). CSCs have several advantages over the differentiated cancer cell population, including high resistance to radio- and chemotherapy. For this reason, researches on the development of alternative CSCs-targeted therapeutic approaches are of great importance. Several studies are providing increasing evidence that direct targeting of pancreatic CSCs in combination with the elimination of the more differentiated cancer cells increases the efficacy of the treatment, as indicated by a longer survival in preclinical xenograft models (189). These studies were focused on the inhibition of the most important regulatory pathways that are relevant for the self-renew ability of CSCs, and on the capability of immune-based treatments to target pancreatic CSCs. Moreover, since acquired resistance to treatment is due to genetic and epigenetic alterations, specific drugs for the epigenetic regulatory machinery have been considered (190, 191). Thus, many therapeutic strategies have been designed to specifically target CSCs, but with limited success (192). Currently, nanoparticle formulations have been designed to effectively destroy these cells or chemical modification of drugs has been devised to improve their stability and solubility (193, 194). Nanoparticles can sequester chemotherapeutic agents at high concentrations and release them within the cancer cells, achieving high cell selectivity by targeting agents on the surface of nanoparticles. It has been demonstrated that this approach provides encouraging results in the inhibition of multiple types of CSCs by targeting specific markers (CD44, ALDH, CD133) or specific signaling pathways (Notch, Hedgehog and others) (195). Furthermore, nanoparticles have been developed to overcome the resistance of cancer cells to chemotherapy (196). Reddy et al., reported that a nanoparticle formulation of GEM, the 4-(*N*)-tris-nor-squalenoyl-GEM was more cytotoxic than GEM in human and murine leukemia cell lines (197).

In this work, we have developed two different therapeutic strategies for the treatment of PDAC and for CSC specific killing:

1. A pro-drug approach that involves the use of GEM conjugated with the fatty acid chains, C12 GEM and C18 GEM, for improving GEM stability;
2. A targeted drug delivery system that involves the development of PEGylated liposomes containing DSF or $Cu(DDC)_2$ and liposomes selective for pancreatic CSCs, using HA as targeting agent.

It has been demonstrated that GEM is rapidly deaminated in blood, liver, kidney and other tissues, showing a very short half-life (198). Different approaches have been tried to improve the GEM metabolic stability and its "in vivo" cytotoxic activity, such as the synthesis of an acyl moiety that protects the drug from rapid inactivation and improves its antitumour activity compared to the pure drug (199). Recently, the antitumor activity of GEM 4-(N)-acyl derivatives (C12 GEM and C18 GEM) and their loading into nanoparticles have been studied. Malfanti et al. demonstrated that C12 GEM was more toxic than GEM in human ovarian carcinoma cells and the prodrug cytotoxicity was reduced after encapsulation into nanoparticles (200). The C18 GEM activity was studied *in vivo* on human colorectal adenocarcinoma (HT-29) and nasopharyngeal carcinoma (KB 396p) cells (201). The animals treated with C18 GEM had a smaller tumour mass and higher percentage of regression than mice treated with GEM alone. Exploring the lipophilic pro-drug strategy, we demonstrated that Panc1 CSCs were more sensitive to C18 GEM compared to standard treatment with GEM or C12 GEM, and respect to Panc1 parental cell line. Furthermore, the two cell lines exhibited different intracellular uptake mechanisms of the drugs. In Panc1 parental cell line in contrast with CSCs derived cells, the lipophilic formulations and GEM alone were dependent on nucleoside transporters for entering into the cells. Furthermore, in both cell lines C18 GEM was also dependent on fatty acid translocase CD36 for entering into the cells, while in Panc1 CSCs GEM and C12 GEM crossed the membrane by a mechanism that should still be identified. The inhibition data of membrane transporters suggest a different membrane composition between Panc1 and CSCs that influences the uptake of drugs and

then their antitumor activity. Finally, we characterized the cell growth inhibition mechanisms, highlighting another peculiar feature of CSCs regarding the apoptotic response to treatment. In Panc1 parental cells, drug treatments induced a PARP-dependent apoptosis, while in Panc1 CSCs they activated a mechanism of caspase-independent apoptosis mediated by AIF. Furthermore, we demonstrated that C18 GEM increased the autophagosomes formation as a response of the cells to stress induced by drug treatment. These data highlight the possible use of pro-drugs of GEM as a therapeutic strategy in the treatment of PDAC, because of their high efficiency in killing CSCs with the final aim to permanently remove the tumor and prevent recurrence.

The second therapeutic approach described in this work has demonstrated an even greater efficacy towards CSC containing PDAC and is based on the use of PEGylated liposome formulations coated with HA and containing the potent antiproliferative $Cu(DDC)_2$ complex. The anti-alcoholism drug DSF is a Cu ionophore and has been shown to act as antitumoral drug by inducing oxidative stress, especially when complexed with Cu (202). It has been demonstrated that the cytotoxic activity of DSF, after its degradation to DDC, against U87 and U251 glioblastoma cells, A549 lung cancer line and MDA-231BR breast cancer cells is copper dependent (203). Despite its potent anticancer activity, the use of DSF-based cancer therapy in clinic is hampered by its instability in gastric juice and bloodstream, and poor solubility in biological fluid. To overcome these limitations, the encapsulation of DSF in liposomes is crucial to protect it from degradation and renders it suitable for intravenous administration (204). Furthermore, the development of liposomes capable of specifically recognizing the target cells, strongly increases the therapeutic efficacy of the drug (205). Wehbe et al. have resolved, for the first time, the issue related to the high insolubility of the diethyldithiocarbamate-copper complexes by synthesizing them inside liposomes (203). These liposomal formulations have been characterized and the rate of $Cu(DDC)_2$ formation inside the liposome has been quantified, resulting directly related to the amount of encapsulated copper. The liposomes containing DDC and copper have proved to be highly cytotoxic against breast

cancer stem cells *in vitro* and *in vivo*, giving rise to ROS activation and inhibition of NFκB pathway (206).

In this work, DSF-metal complexes were prepared in collaboration with the University of Turin and their anti-proliferative activity was tested on pancreatic cancer cells. Among these complexes, the most effective on our cell models, $Cu(DDC)_2$, was chosen for the encapsulation in PEGylated formulations or in liposomes with HA at two different MW (HA_{4800} and HA_{17000}). In a previous work, it has been demonstrated that liposomes decorated with HA show a strong affinity towards pancreatic tumour cells overexpressing CD44 on their plasma membrane (207). The anti-proliferative activity of liposomes was evaluated using pancreatic CSCs derived from cell lines or patients. Taken together, our results demonstrate that the loading of $Cu(DDC)_2$ into the liposomes strongly increases the anti-proliferative effect of $Cu(DDC)_2$. Coating with HA generally improves the anti-proliferative activity of $Cu(DDC)_2$ containing liposomes only in the presence of PEG. Impressive data were obtained using primary cells with a stem like phenotype directly derived from PDAC patients in which these formulations show a high capacity to inhibit cell proliferation and sphere formation capability.

9. REFERENCES

1) Ducreux M, Sa Cuhna A, Caramella C, Hollebecque A, Burtin P, Goéré D. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and followup. Annals of Oncology, 2015; doi:10.1093/annonc/mdv295.
2) Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014; doi: 10.1158/0008-5472.CAN-14-0155.
3) Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin. 2017; doi: 10.3322/caac.21387.
4) J.P. Neoptolemos, D.D. Stocken, H. Friess, C. Bassi, J.A. Dunn, H. Hickey, H. Beger, L. Fernandez-Cruz, C. Dervenis, F. Lacaine, M. Falconi, P. Pederzoli, A. Pap, D. Spooner, D.J. Kerr, M.W. Buchler. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N Engl J Med. 350, 2004; 1200-1210.
5) Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, Kamiyama M, Hruban RH, Eshleman JR, Nowak MA, Velculescu VE, Kinzler KW, Vogelstein B, Iacobuzio-Donahue CA. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010; 467(7319):1114-7.
6) Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res, 2000;6(8):2969-72.
7) Matthaei H, Schulick RD, Hruban RH, Maitra A. Cystic precursors to invasive pancreatic cancer. Nat Rev Gastroenterol Hepatol. 2011; doi: 10.1038/nrgastro.2011.2.
8) Basturk O, Hong SM, Wood LD, Adsay NV, Albores-Saavedra J, Biankin AV, Brosens LA, Fukushima N, Goggins M, Hruban RH, et al. A revised classification system and recommendations from the Baltimore consensus meeting for neoplastic precursor lesions in the pancreas. Am J Surg Pathol. 2015. doi: 10.1097/PAS.0000000000000533.
9) Kawada N, Uehara H, Takada R, Yamai T, Fukutake N, Katayama K, Takenaka A, Nagata S, Tomita Y. Microinvasion of high-grade pancreatic intraepithelial neoplasia. Case Rep Gastroenterol. 2013; doi: 10.1159/000346693.
10) Wang J, Paris PL, Chen J, Ngo V, Yao H, Frazier ML, Killary AM, Liu CG, Liang H, Mathy C, Bondada S, Kirkwood K, Sen S. Next generation sequencing

of pancreatic cyst fluid microRNAs from low grade-benign and high grade-invasive lesions. Cancer Lett. 2015; doi: 10.1016/j.canlet.2014.09.029.

11) Suzuki R, Ohira H, Irisawa A, Bhutani MS. Pancreatic cancer: early detection, diagnosis, and screening. Clin J Gastroenterol. 2012; doi: 10.1007/s12328-012-0327-0.

12) Di Marco M, Astolfi A, Grassi E, Vecchiarelli S, Macchini M, Indio V, Casadei R, Ricci C, D'Ambra M, Taffurelli G, Serra C, Ercolani G, Santini D, D'Errico A, Pinna AD, Minni F, Durante S, Martella LR, Biasco G. Characterization of pancreatic ductal adenocarcinoma using whole transcriptome sequencing and copy number analysis by single-nucleotide polymorphism array. Mol Med Rep. 2015; doi: 10.3892/mmr.2015.4344.

13) Witkiewicz AK, McMillan EA, Balaji U, Baek G, Lin WC, Mansour J, Mollaee M, Wagner KU, Koduru P, Yopp A, et al. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. Nat Commun. 2015; doi: 10.1038/ncomms7744.

14) Lu L, Zeng J. Evaluation of K-ras and p53 expression in pancreatic adenocarcinoma using the cancer genome atlas. PLoS One. 2017; doi: 10.1371/journal.pone.0181532.

15) Rhim AD, Stanger BZ. Molecular biology of pancreatic ductal adenocarcinoma progression: aberrant activation of developmental pathways. Prog Mol Biol Transl Sci. 2010; doi: 10.1016/B978-0-12-385233-5.00002-7.

16) Liu J, Ji S, Liang C, Qin Y, Jin K, Liang D, Xu W, Shi S, Zhang B, Liu L, Liu C, Xu J, Ni Q, Yu X. Critical role of oncogenic KRAS in pancreatic cancer. Mol Med Rep. 2016; doi:10.3892/mmr.2016.5196.

17) Mann KM, Ying H, Juan J, Jenkins NA, Copeland NG. KRAS-related proteins in pancreatic cancer. Pharmacol Ther. 2016; doi: 10.1016/j.pharmthera.2016.09.003.

18) Erkan M, Hausmann S, Michaiski CW, Fingerle AA, Dobritz M, Kleeff J, Friess H. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. Nat Rev Gastroenterol Hepatol. 2012; doi: 10.1038/nrgastro.2012.115.

19) Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res. 2006; 66(19):9339-44.

20) Nguyen, L. V., Vanner, R., Dirks, P. Eaves, CJ. Cancer stem cells: an evolving concept. Nature Rev Cancer. 2012; doi: 10.1038/nrc3184.
21) Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006; 444(7120):756-60.
22) Li, X. et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J. Natl Cancer Inst. 2008; doi: 10.1093/jnci/djn123.
23) Diehn, M. et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature. 2009; doi: 10.1038/nature07733.
24) McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneuty in cancer evolution. Cancer Cell. 2015; doi: 10.1016/j.ccell.2014.12.001.
25) Valent P. et al. Cancer stem cell definitions and terminology: the devil is in the details. Nat Rev Cancer. 2012; doi: 10.1038/nrc3368.
26) Polyak, K. & Weinberg, R. A. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer. 2009. doi: 10.1038/nrc2620.
27) Yilmaz, M. & Christofori, G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 2009; doi: 10.1007/s10555-008-9169-0.
28) Mani, S. A. et al. The epithelial–mesenchymal transition generates cells with properties of stem cells. Cell. 2008; doi: 10.1016/j.cell.2008.03.027.
29) Wellner, U. et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat. Cell Biol. 2009; doi: 10.1038/ncb1998.
30) Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. Nat. Rev. Cancer. 2013; doi: 10.1038/nrc3599.
31) Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol. 2017; doi: 10.1038/nrclinonc.2017.44.
32) Hermann PC, Bhaskar S, Cioffi M, Heeschen C. Cancer stem cells in solid tumors. Semin Cancer Biol. 2010; doi: 10.1016/j.semcancer.2010.03.004.
33) Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature. 2004; 432(7015):396-401.
34) Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of

human colon-cancer-initiating cells. Nature. 2007; 445(7123):111-5.

35) Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell. 2007; doi: 10.1016/j.stem.2007.06.002.

36) C. Li, D.G. Heidt, P. Dalerba, C.F. Burant, L. Zhang, V. Adsay, M. Wicha, M.F. Clarke, D.M. Simeone. Identification of pancreatic cancer stem cells. Cancer research. 2007; 67(3):1030-7.

37) Jimeno A, Feldmann G, Suarez-Gauthier A et al. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol Cancer Ther. 2009; doi: 10.1158/1535-7163.MCT-08-0924.

38) Lonardo E, Frias-Aldeguer J, Hermann PC et al. Pancreatic stellate cells form a niche for cancer stem cells and promote their self-renewal and invasiveness. Cell Cycle. 2012; doi: 10.4161/cc.19679.

39) Sainz B, Jr., Martin B, Tatari M et al. ISG15 is a critical microenvironmental factor for pancreatic cancer stem cells. Cancer Res 2014; doi: 10.1158/0008-5472.CAN-14-1354.

40) Ito D, Fujimoto K, Mori T, Kami K, Koizumi M, Toyoda E, Kawaguchi Y, Doi R. In vivo antitumor effect of the mTOR inhibitor CCI-779 and gemcitabine in xenograft models of human pancreatic cancer. Int J Cancer. 2006; 118(9):2337-43.

41) Smith KB, Tran LM, Tam BM, Shurell EM, Li Y, Braas D, Tap WD, Christofk HR, Dry SM, Eilber FC, Wu H. Novel dedifferentiated liposarcoma xenograft models reveal PTEN down-regulation as a malignant signature and response to PI3K pathway inhibition. Am J Pathol. 2013; doi: 10.1016/j.ajpath.2013.01.002.

42) Jung J, Lee CH, Seol HS, Choi YS, Kim E, Lee EJ, Rhee JK, Singh SR, Jun ES, Han B, Hong SM, Kim SC, Chang S. Generation and molecular characterization of pancreatic cancer patient-derived xenografts reveals their heterologous nature. Oncotarget. 2016; doi: 10.18632/oncotarget.11530.

43) Kure S, Matsuda Y, Hagio M, Ueda J, Naito Z and Ishiwata T. Expression of cancer stem cell markers in pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinomas. Int J Oncol. 2012; doi: 10.3892/ijo.2012.1565.

44) Lonardo E, Hermann PC, Heeschen C. Pancreatic cancer stem cells—Update and future perspectives. Mol Oncol. 2010; doi: 10.1016/j.molonc.2010.06.002.

45) Miranda-Lorenzo I, Dorado J, Lonardo E et al. Intracellular autofluorescence: A biomarker for epithelial cancer stem cells. Nat Methods. 2014; doi: 10.1038/nmeth.3112.

46) Paulson AS, Tran Cao HS, Tempero MA, Lowy AM. Therapeutic advances in pancreatic cancer. Gastroenterology. 2013; doi: 10.1053/j.gastro.2013.01.078.

47) Ellenrieder V, König A, Seufferlein T. Current standard and future perspectives in first- and second-line treatment of metastatic pancreatic adenocarcinoma. Digestion. 2016; doi: 10.1159/000447739.

48) Koay EJ, Truty MJ, Cristini V, Thomas RM, Chen R, Chatterjee D, et al. Transport properties of pancreatic cancer describe gemcitabine delivery and response. J Clin Invest. 2014; doi: 10.1172/JCI73455.

49) Ju HQ, Gocho T, Aguilar M, Wu M, Zhuang ZN, Fu J, Yanaga K, Huang P, Chiao PJ. Mechanism of overcoming intrinsic resistance to gemcitabine in pancreatic ductal adenocarcinoma through the redox modulation. Mol Cancer Ther. 2015; doi: 10.1158/1535-7163.MCT-14-0420.

50) Milella M, Gelibter AJ, Pino MS, Bossone G, Marolla P, Sperduti I, Cognetti F. Fixed-dose-rate gemcitabine: a viable first-line treatment option for advanced pancreatic and bilary tract cancer. Oncologist. 2010; doi: 10.1634/theoncologist.2008-0135.

51) Arora S, Bhardwaj A, Singh S, Srivastava SK, McClellan S, Nirodi CS, et al. An undesired effect of chemotherapy: gemcitabine promotes pancreatic cancer cell invasiveness through reactive oxygen species-dependent, nuclear factor kappaB- and hypoxia-inducible factor 1alpha-mediated up-regulation of CXCR4. J Biol Chem. 2013; doi: 10.1074/jbc.M113.484576.

52) Pauwels B, Korst AE, Pattyn GG, Lambrechts HA, Van Bockstaele DR, Vermeulen K, Lenjou M, De Pooter CM, Vermorken JB, Lardon F. Cell cycle effect of gemcitabine and its role in the radiosensitizing mechanism in vitro. Int J Radiat Oncol Biol Phys. 2003; 57(4):1075-83.

53) Yong-Xian G, Xiao-Huan L, Fan Z, Guo-Fang T. Gemcitabine inhibits proliferation and induces apoptosis in human pancreatic cancer Panc1 cells. J Cancer Res Ther. 2016; doi: 10.4103/0973-1482.191615.

54) L. de Sousa Cavalcante, G. Monteiro. Gemcitabine: metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. Eur. J. Pharmacol. 2014; doi: 10.1016/j.ejphar.2014.07.041.

55) Susanne Sebens and Heiner Schafer. The Tumor Stroma as Mediator of Drug Resistance - A Potential Target to Improve Cancer Therapy? Current Pharmaceutical Biotechnology. 2012; 13(11):2259-72.
56) Moore MJ, et al. 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. J Clin Oncol. 2007; 25(15):1960-6.
57) Cunningham D, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol. 2009; doi: 10.1200/JCO.2009.24.2446.
58) Von Hoff DD, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013; doi: 10.1056/NEJMoa1304369.
59) Gourgou-Bourgade S, et al. Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer: results from the PRODIGE 4/ACCORD 11 randomized trial. J Clin Oncol. 2013; doi: 10.1200/JCO.2012.44.4869.
60) Huttunen, K.; Raunio, H.; Rautio, J. Prodrugs: Design and clinical applications. Pharmacol. Rev. 2011, 50(4):387-94.
61) Abet V, Filace F, Recio J, Alvarez-Builla J, Burgos C. Prodrug approach: An overview of recent cases. Eur J Med Chem. 2017. doi 10.1016/j.ejmech.2016.10.061.
62) Han, H.K.; Amidon, G.L. Targeted prodrug design to optimize drug delivery. AAPS PharmSci. 2000; 2(1):E6.
63) Stella VJ, Nti-Addae KW. Prodrug strategies to overcome poor water solubility. Adv Drug Deliv Rev. 2007; 59(7):677-94.
64) Beaumont, K.; Webster, R.; Gardner, I.; Dack, K. Design of ester prodrugs to enhance oral absorption of poorly permeable compounds: Challenges to the discovery scientist. Curr. Drug Metab. 2003; 4, 461–485.
65) Castelli F, Sarpietro MG, Ceruti M, Rocco F, Cattel L. Characterization of lipophilic gemcitabine prodrug-liposomal membrane interaction by differential scanning calorimetry. Mol Pharm. 2006; 3(6):737-44.
66) Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, et al. Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol. 2007; doi: 10.1038/nnano.2007.387.
67) A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki. Liposome: classification,

preparation, and applications. Nanoscale research letters. 2013; doi: 10.1186/1556-276X-8-102.

68) Dash TK, Konkimalla VB. Polymeric modification and its implication in drug delivery: poly- ε- caprolactone (PLC) as a model polymer. Mol Pharm. 2012; doi: 10.1021/mp3001952.

69) Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today. 2005; 10(21):1451-8.

70) Vail DM, Amantea MA, Colbern GT, Martin FJ, Hilger RA, Working PK. Pegylated liposomal doxorubicin: a proof of principle using preclinical animal models and pharmacokinetics studies. Semin Oncol. 2004; 31(6 Suppl 13):16-35.

71) Torchilin VP. Passive and active drug targeting: drug delivery to tumors as an example. Handb Exp Pharmacol. 2010; doi: 10.1007/978-3-642-00477-3_1.

72) P.P. Deshpande, S. Biswas, V.P. Torchilin. Current trends in the use of liposomes for tumor targeting. Nanomedicine (London, England). 2013; doi: 10.2217/nnm.13.118.

73) W. Alshaer, H. Hillaireau, J. Vergnaud, S. Ismail, E. Fattal. Functionalizing liposomes with anti-CD44 Aptamer for Selective Targeting of Cancer Cells. Bioconjugate chemistry. 2015; doi: 10.1021/bc5004313.

74) Xiao-Ping Li, Xiao-Wei Zhang, Lei-Zhen Zheng, Wei-Jian Guo. Expression of CD44 in pancreatic cancer and its significance. Int J Clin Exp Pathol. 2015; 8(6):6724-31.

75) G. Mattheolabakis, L. Milane, A. Singh, M.M. Amiji. Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. Journal of drug targeting. 2015; doi: 10.3109/1061186X.2015.1052072.

76) Johansson B. A review of the pharmacokinetics and pharmacodynamics of disulfiram and its metabolites. Acta Psychiatr Scand Suppl. 1992; 369: 15–26.

77) D. Chen, Q.C. Cui, H. Yang, Q.P. Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts via inhibition of the proteasome activity. Cancer research. 2006; 66(21):10425-33.

78) V.T. Cheriyan, Y. Wang, M. Muthu, S. Jamal, D. Chen, H. Yang, L.A. Polin, A.L. Tarca, H.I. Pass, Q.P. Dou, S. Sharma, A. Wali, A.K. Rishi. Disulfiram suppresses growth of the malignant pleural mesothelioma cells in part by inducing apoptosis. PloS one. 2014; doi: 10.1371/journal.pone.0093711.

79) J.Y. Kim, Y. Cho, E. Oh, N. Lee, H. An, D. Sung, T.-M. Cho, J.H. Seo.

Disulfiram targets cancer stem-like properties and the HER2/Akt signaling pathway in HER2-positive breast cancer. Cancer letters. 2016; doi: 10.1016/j.canlet.2016.05.026.

80) P. Liu, S. Brown, T. Goktug, P. Channathodiyil, V. Kannappan, J.P. Hugnot, P.O. Guichet, X. Bian, A.L. Armesilla, J.L. Darling, W. Wang, Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells. British journal of cancer. 2012; doi: 10.1038/bjc.2012.442.

81) Lovborg H, Oberg F, Rickardson L, Gullbo J, Nygren P, Larsson R. Inhibition of proteasome activity, nuclear factor-KappaB translocation and cell survival by the antialcoholism drug disulfiram. Int J Cancer. 2006; 118(6):1577-80.

82) Viola-Rhenals M, Patel KR, Jaimes-Santamaria L, Wu G, Liu J, Dou QP. Recent advances in Antabuse (Disulfiram): the importance of its metal-binding ability to its anticancer activity. Curr Med Chem. 2017. doi: 10.2174/0929867324666171023161121.

83) Liu P, Brown S, Goktug T, Channathodiyil P, Kannappan V, Hugnot JP, Guichet PO, Bian X, Armesilla AL, Darling JL, Wang W. Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells. Br J Cancer. 2012; doi: 10.1038/bjc.2012.442.

84) Barbieri E, Di Fiore PP, Sigismund S. Endocytic control of signaling at the plasma membrane. Curr Opin Cell Biol. 2016; doi: 10.1016/j.ceb.2016.01.012.

85) Section 15.2. Overview of membrane Transport proteins. Molecular cell biology. 4th edition.

86) Falcone S, Cocucci E, Podini P, Kirchhausen T, Clementi E, Meldolesi J. Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events. Journal of Cell Science. 2009. 119 (Pt 22): 4758–69

87) Siamon Gordon. Phagocytosis: an immunobiologic process. Immunity, Vol 44, Issue 3, 2016, 463-475.

88) Sorkin, Alexander; Puthenveedu, Manojkumar A. Yarden, Yosef; Tarcic, Gabi, eds. Clathrin-mediated endocytosis. Springer New York. pp. 1–31

89) N.V. Popova, I.E. Deyev, and A.G. Petrenko. Clathrin-mediated endocytosis and adaptor proteins. Acta Naturae. 2013; 5(3):62-73

90) A.I. Ivanov. Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Methods Mol. Biol. 2008; doi: 10.1007/978-1-59745-178-9_2.

91) Al Soraj M, He L, Peynshaert K, Cousaert J, Vercauteren D, Braeckmans K, De Smedt SC, Jones AT. J Control Release. 2012; doi: 10.1016/j.jconrel.2012.03.015.

92) Vercauteren D1, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. Mol Ther. 2010; doi: 10.1038/mt.2009.281.

93) Ivanov AL. Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Methods Mol Biol. 2008; doi: 10.1007/978-1-59745-178-9_2.

94) Owen DM, Magenau A, Williamson D, Gaus K. The lipid raft hypothesis revisited--new insights on raft composition and function from super-resolution fluorescence microscopy. Bioessays. 2012; doi: 10.1002/bies.201200044.

95) Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. Science. 2010; doi: 10.1126/science.1174621.

96) Bian F, Xiong B, Yang X, Jin S. Lipid rafts, ceramide and molecular transcytosis. Front Biosci (Landmark Ed). 2016; 21:806-38.

97) Staubach S, Hanisch FG. Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. Expert Rev Proteomics. 2011; doi: 10.1586/epr.11.2.

98) Miguel A. Alonso, Jaime Millán. The role of lipid rafts in signalling and membrane trafficking in T lymphocytes. Journal of Cell Science. 2001; 114: 3957-3965.

99) Reeves VL, Thomas CM, Smart EJ. Lipid rafts, caveolae and GPI-linked proteins. Adv Exp Med Biol. 2012; doi: 10.1007/978-1-4614-1222-9_1.

100) P Lajoie and IR Nabi. Regulation of raft-dependent endocytosis. J Cell Mol Med. 2007; doi: 10.1111/j.1582-4934.2007.00083.x.

101) Partlow KC, Lanza GM, Wickline SA. Exploiting lipid raft transport with membrane targeted nanoparticles: a strategy for cytosolic drug delivery. Biomaterials. 2008; doi: 10.1016/j.biomaterials.2008.04.030.

102) Raphael Zidovetzki, Irena Levitan. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim Biophys Acta. 2007; doi: 10.1016/j.bbamem.2007.03.026.

103) Besenicar MP, Bavdek A, Kladnik A, Macek P, Anderluh G. Kinetics of cholesterol extraction from lipid membranes by methyl-beta-cyclodextrin—a surface plasmon resonance approach. Biochim Biophys Acta. 2008; doi:

10.1016/j.bbamem.2007.09.022.

104) Molina-Arcas M, Casado FJ, Pastor-Anglada M. Nucleoside transporter proteins. Curr Vasc Pharmacol. 2009;7(4):426-34.

105) Molina-Arcas M, Trigueros-Motos L, Casado FJ, Pastor-Anglada M. Physiological and pharmacological roles of nucleoside transporter proteins. Nucleosides Nucleotides Nucleic Acids. 2008; doi: 10.1080/15257770802145819.

106) Ritzel, M.W., Yao, S.Y., Ng, A.M., Mackey, J.R., Cass, C.E., Young, J.D. Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol. Membr. Biol. 1998; 15(4):203-11.

107) Ritzel, M.W., Ng, A.M., Yao, S.Y., Graham, K., Loewen, S.K., Smith, K.M., Ritzel, R.G., Mowles, D.A., Carpenter, P., Chen, X.Z., Karpinski, E., Hyde, R.J., Baldwin, SA Cass, C.E., Young, J.D. Molecular identification and characterization of novel human and mouse concentrative Na-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J. Biol. Chem. 2001; 276(4):2914-27.

108) Young JD, Yao SY, Sun L, Cass CE, Baldwin SA. Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins. Xenobiotica. 2008; doi: 10.1080/00498250801927427.

109) Pastor-Angglada M, Casado FJ, Valdès R, Mata J, Garcia- Manteiga J, Molina M. Complex regulation of nucleoside transporter expression in epithelial and immune system cells. Mol Membr Biol. 2001; 18(1):81-5.

110) Johnson ZL, Lee JH, Lee K, Lee M, Kwon DY, Hong J, Lee SY. Structural basis of nucleoside and nucleoside drug selectivity by concentrative nucleoside transporters. Elife. 2014; doi: 10.7554/eLife.03604.

111) Shimada T, Nakanishi T, Tajima H, Yamazaki M, Yokono R, Takabayashi M, Shimada T, Sawamoto K, Miyamoto K, Kitagawa H, Ohta T, Tamai I, Sai Y. Saturable hepatic extraction of gemcitabine involves biphasic uptake mediated by nucleoside transporters equilibrative nucleoside Transporter 1 and 2. J Pharm Sci. 2015; doi: 10.1002/jps.24498.

112) Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R, Mackey JR. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res. 2004; 10: 6956–6961.

113) Vincenzi B, Stacchiotti S, Collini P, Pantano F, Rabitti C, Perrone G, Iuliani M, Baldi A, Badalamenti G, Sanfilippo R, Santini D, Onetti Muda A, Gronchi A, Casali P, Dei Tos AP, and Tonini G. Human equilibrative nucleoside transporter 1 gene expression is associated with gemcitabine efficacy in advanced leiomyosarcoma and angiosarcoma. British journal of cancer. 2017; doi: 10.1038/bjc.2017.187.

114) Nakagawa N, Murakami Y, Uemura K, Sudo T, Hashimoto Y, Kondo N, Sueda T. Combined analysis of intratumoral human equilibrative nucleoside transporter 1 (hENT1) and ribonucleotide reductase regulatory subunit M1 (RRM1) expression is a powerful predictor of survival in patients with pancreatic carcinoma treated with adjuvant gemcitabine- based chemotherapy after operative resection. Surgery. 2013; doi: 10.1016/j.surg.2012.10.010.

115) Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, Iannopollo M, Bevilacqua G, Mosca F, Danesi R. Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. Cancer Res. 2006; 66: 3928–3935.

116) Jansen WJ, Pinedo HM, VanDer Wilt CL, Feller N, Bamberger U, Boven E. The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine in vitro and in human tumour xenografts. Eur J Cancer. 1995; 31A (13-14):2313-9.

117) Stremmel W., Pohl L., Ring A. & Herrmann T. A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. Lipids. 2001; 36, 981–9.

118) Dutta-Roy AK. Cellular uptake of long-chain fatty acids: role of membrane-associated fatty-acid-binding/transport proteins. Cell Mol Life Sci. 2000; 57(10):1360-72.

119) Joep F F Brinkmann, Nada A Abumrad, Azeddine Ibrahimi, Ger J van der Vusse, Jan F C Glatz. New insights into long-chain fatty acid uptake by heart muscle: a crucial role for fatty acid translocase/CD36. Biochem J. 2002; doi: 10.1042/BJ20020747.

120) Bonen A, Campbell SE, Benton CR, Chabowski A, Coort SL, Han XX, Koonen DP, Glatz JF, Luiken JJ. Regulation of fatty acid transport by fatty acid translocase/CD36. Proc Nutr Soc. 2004 ;63(2):245-9.

121) Abumrad NA, el-Maghrabi MR, Amri EZ, Lopez E, Grimaldi PA.

Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. J Biol Chem. 1993; 268:17665–8.

122) Yamashita S, Hirano K, Kuwasako T, Janabi M, Toyama Y, Ishigami M, Sakai N. Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis; insights from CD36-deficient patients. Mol Cell Biochem. 2007; 299:19–22.

123) Love-Gregory L, Abumrad NA. CD36 genetics and the metabolic complications of obesity. Curr Opin Clin Nutr Metab Care. 2011; doi: 10.1097/MCO.0b013e32834bbac9.

124) Hale JS, Li M, Sinyuk M, et al. Context dependent role of the CD36--thrombospondin--histidine- rich glycoprotein axis in tumor angiogenesis and growth. PloS one. 2012; doi: 10.1371/journal.pone.0040033.

125) Kaur B, Cork SM, Sandberg EM, et al. Vasculostatin inhibits intracranial glioma growth and negatively regulates in vivo angiogenesis through a CD36-dependent mechanism. Cancer research. 2009; doi: 10.1158/0008-5472.CAN-08-1166.

126) James S. Hale, Balint Otvos, Maksim Sinyuk et al. Cancer stem cell-specific scavenger receptor CD36 drives glioblastoma progression. Stem Cells. 2014; doi:10.1002/stem.1716.

127) Coort, S. L., Willems, J., Coumans, W. A., van der Vusse, G. J., Bonen, A., Glatz, J. F., and Luiken, J. J. Sulfo-N-succinimidyl esters of long chain fatty acids specifically inhibit fatty acid translocase (FAT/CD36) - mediated cellular fatty acid uptake. Mol. Cell. Biochem. 2002; 239, 213–219.

128) Harmon CM, Abumrad NA. Binding of sulfosuccinimidyl fatty acids to adipocyte membrane proteins: isolation and amino-terminal sequence of an 88-kD protein implicated in transport of long-chain fatty acids. J Membr Biol. 1993; 133:43–9.

129) T. G. Cotter. Apoptosis and cancer: the genesis of a research field. Nature Reviews Cancer. 2009; doi: 10.1038/nrc2663.

130) Salvesen GS. Caspases and apoptosis. Essays Biochem. 2002; 38:9-19.

131) Taylor, R. C., Cullen, S. P. & Martin, S. J. Apoptosis: controlled demolition at the cellular level. Nat. Rev. Mol. Cell Biol. 2008; 9, 231–241.

132) Gabriel Ichim & Stephen W. G. Tait. A fate worse than death: apoptosis as an oncogenic process. Nature Reviews Cancer. 2016; doi:10.1038/nrc.2016.58.

133) Tait, S. W. & Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. 2010; doi: 10.1038/nrm2952.

134) Li, H., Zhu, H., Xu, C. J. & Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998; 94, 491–501.

135) Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998; 94, 481–490.

136) Thomas Landes, Jean- Claude Martinou. Mitochondrial outer membrane permeabilization during apoptosis: The role of mitochondrial fission. Biochimica et Biophysica Acta. 2011; doi: 10.1016/j.bbamcr.2011.01.021.

137) Colin Adrain, Emma M. Creagh, and Seamus J. Martin. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J. 2001; doi: 10.1093/emboj/20.23.6627.

138) Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem R, Penninger JM, Mak TW. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell. 1998; 94(6):739-50.

139) Schile AJ, García-Fernández M, Steller H. Regulation of apoptosis by XIAP ubiquitin-ligase activity. Genes Dev. 2008; doi: 10.1101/gad.1663108.

140) Bröker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. Clin Cancer Res. 2005;11(9):3155-62.

141) Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prévost MC, Leber B, Andrews D, Penninger J, Kroemer G. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J. 2000; 14(5):729-39.

142) Judy Lieberman. Granzyme A activates another way to die. Immunol Rev. 2010; doi: 10.1111/j.0105-2896.2010.00902.x.

143) Goping IS, Barry M, Liston P, Sawchuk T, Constantinescu G, Michalak KM, Shostak I, Roberts DL, Hunter AM, Korneluk R, Bleackley RC. Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity. 2003;18(3):355-65.

144) D.Hanahan and R.A.Weinberg. The hallmarks of cancer. Cell. 2000; 100(1):57-70.

145) Strasser, A., Harris, A. W., Bath, M. L. & Cory. S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature. 1990; 348(6299):331-3.

146) Finch, A. et al. Bcl-xL gain of function and p19 ARF loss of function cooperate oncogenically with Myc in vivo by distinct mechanisms. Cancer Cell. 2006; 10, 113–120.

147) Yonish-Rouach, E. et al. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature. 1991; 352, 345–347.

148) Montero, J. et al. Drug-induced death signaling strategy rapidly predicts cancer response to chemotherapy. Cell. 2015; doi: 10.1016/j.cell.2015.01.042.

149) Alcaide, J. et al. The role and prognostic value of apoptosis in colorectal carcinoma. BMC Clin. Pathol. 2013; doi: 10.1186/1472-6890-13-24.

150) Dawson, S. J. et al. BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. Br. J. Cancer. 2010; doi: 10.1038/sj.bjc.6605736.

151) Mizushima, N. Autophagy: process and function. Genes Dev. 2007; 21, 2861–2873.

152) Gautam Das, Bhupendra V, Shravage and Eric H. Baehrecke. Regulation and Function of Autophagy during Cell Survival and Cell Death. Cold Spring Harb Perspect Biol. 2012; doi: 10.1101/cshperspect.a008813.

153) Mizushima, N., Yoshimori, T. & Ohsumi, Y. The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. 2011; doi: 10.1146/annurev-cellbio-092910-154005.

154) Klionsky, D. J. et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016; doi: 10.1080/15548627.2015.1100356.

155) Liu L, Liao JZ, He XX, Li PY. The role of autophagy in hepatocellular carcinoma: friend or foe. Oncotarget. 2017; doi: 10.18632/oncotarget.17202.

156) Li YY, Feun LG, Thongkum A, Tu CH, Chen SM, Wangpaichitr M, Wu C, Kuo MT, Savaraj N. Autophagic mechanism in anti-cancer immunity: its pros and cons for cancer therapy. Int J Mol Sci. 2017; doi: 10.3390/ijms18061297.

157) Galluzzi, L. et al. Autophagy in malignant transformation and cancer progression. EMBO. 2015; doi: 10.15252/embj.201490784.

158) Amaravadi, R., Kimmelman, A. C. & White, E. Recent insights into the function of autophagy in cancer. Genes Dev. 2016; doi: 10.1101/gad.287524.116.

159) Zhu J, Zheng Y, Zhang H, Zhu J, Sun H. Low concentration of chloroquine enhanced efficacy of cisplatin in the treatment of human ovarian cancer dependent on autophagy. Am J Transl Res. 2017; 9(9):4046-4058.

160) Tang ZH, Cao WX, Guo X, Dai XY, Lu JH, Chen X, Zhu H, Lu JJ. Identification of a novel autophagic inhibitor cepharanthine to enhance the anti-cancer property of dacomitinib in non-small cell lung cancer. Cancer Lett. 2017; doi: 10.1016/j.canlet.2017.10.001.

161) Amaravadi, R. K. et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. 2007; 117, 326–336.

162) Yang, Y. P. et al. Application and interpretation of current autophagy inhibitors and activators. Acta Pharmacol. Sin. 2013; doi: 10.1038/aps.2013.5.

163) Mulcahy Levy, J. M. et al. Autophagy inhibition overcomes multiple mechanisms of resistance to BRAF inhibition in brain tumors. eLife. 2017; doi: 10.7554/eLife.19671.

164) Hongxing Ye, Mantao Chen, Fei Cao, Hongguang Huang, Renya Zhan and Xiujue Zheng. Chloroquine, an autophagy inhibitor, potentiates the radiosensitivity of glioma initiating cells by inhibiting autophagy and activating apoptosis. BMC Neurol. 2016; doi: 10.1186/s12883-016-0700-6.

165) X Sui, R Chen, Z Wang, Z Huang, N Kong, M Zhang, W Han, F Lou, J Yang, Q Zhang, X Wang, C He and H Pan. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis. 2013; doi: 10.1038/cddis.2013.350

166) Wang JD, Levin PA. Metabolism, cell growth and the bacterial cell cycle. Nature Reviews Microbiology. 2009; doi: 10.1038/nrmicro2202.

167) Senderowicz AM. Small-molecule cyclin-dependent kinase modulators. Oncogene. 2003; 22:6609–6620.

168) Jeong YJ, Hoe HS, Cho HJ, Park KK, Kim DD, Kim CH, Magae J, Kang DW, Lee SR, Chang YC. Suppression of c-Myc enhances p21WAF1/CIP1 - mediated G1 cell cycle arrest through the modulation of ERK phosphorylation by ascochlorin. J Cell Biochem. 2017; doi: 10.1002/jcb.26366

169) Dash BC, El-Deiry WS. Cell cycle checkpoint control mechanisms that can be disrupted in cancer. Methods Mol Biol. 2004; 280:99-161.

170) Ho A, Dowdy SF. Regulation of G (1) cell-cycle progression by oncogenes and tumor suppressor genes. Curr Opin Genet Dev. 2002;12(1):47-52.

171) Efeyan A, Serrano M. p53: guardian of the genome and policeman of the oncogenes. Cell Cycle. 2007; 6(9):1006-10.

172) Shi Z, Azuma A, Sampath D, Li YX, Huang P, Plunkett W. S-Phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxystaurosporine. Cancer Res. 2001; 61(3):1065-72.

173) Dalla Pozza E, Dando I, Biondani G, Brandi J, Costanzo C, Zoratti E, Fassan M, Boschi F, Melisi D, Cecconi D, Scupoli MT, Scarpa A, Palmieri M. Pancreatic ductal adenocarcinoma cell lines display a plastic ability to bi-directionally convert into cancer stem cells. Int J Oncol. 2015; doi: 10.3892/ijo.2014.2796.

174) Enza Lonardo, Michele Cioffi, Patricia Sancho, Shanthini Crusz, Christopher Heeschen. Studying Pancreatic Cancer Stem Cell Characteristics for Developing New Treatment Strategies. J. Vis. Exp. 2015; doi:10.3791/52801.

175) Moog, R, Burger, A. M, Brandl M, Schqler J, Shubert R, Unger C, Fiebig, H. H, Massing U. Change in pharmacokinetic and pharmacodynamic behavior of gemcitabine in human tumor xenografts upon entrapment in vesicular phospholipid gels. Cancer Chemother. Pharmacol. 2002, 49, 356-366.

176) Jennica L. Zaro. Lipid-based drug carriers for prodrugs to enhance drug delivery. AAPS J. 2015 Jan; 17(1): 83–92. Published online 2014 Oct 1. doi: 10.1208/s12248-014-9670-z.

177) Johnson ZL, Lee JH, Lee K, Lee M, Kwon DY, Hong J, Lee SY. Structural basis of nucleoside and nucleoside drug selectivity by concentrative nucleoside transporters. Elife. 2014 Jul 31;3:e03604. doi: 10.7554/eLife.03604.)

178) Bold RJ, Chandra J, McConkey DJ. Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. Ann Surg Oncol. 1999 Apr-May;6(3):279-85.

179) P. Liu, S. Brown, T. Goktug, P. Channathodiyil, V. Kannappan, J.P. Hugnot, P.O. Guichet, X. Bian, A.L. Armesilla, J.L. Darling, W. Wang, Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells. British journal of cancer, 107 (2012) 1488-1497.

180) J.Y. Kim, Y. Cho, E. Oh, N. Lee, H. An, D. Sung, T.-M. Cho, J.H. Seo, Disulfiram targets cancer stem-like properties and the HER2/Akt signaling pathway in HER2-positive breast cancer, Cancer letters, 379 (2016) 39-48.

181) D. Buac, S. Schmitt, G. Ventro, F.R. Kona, Q.P. Dou. Dithiocarbamate-based coordination compounds as potent proteasome inhibitors in human cancer cells. Mini reviews in medicinal chemistry. 2012; 12, 1193-1201.

182) X. Guo, B. Xu, S. Pandey, E. Goessl, J. Brown, A.L. Armesilla, J.L. Darling, W. Wang. Disulfiram/copper complex inhibiting NFkappaB activity and potentiating cytotoxic effect of gemcitabine on colon and breast cancer cell lines. Cancer letters. 2010; doi: 10.1016/j.canlet.2009.09.002.

183) S.S. Brar, C. Grigg, K.S. Wilson, W.D. Holder, Jr., D. Dreau, C. Austin, M. Foster, A.J. Ghio, A.R. Whorton, G.W. Stowell, L.B. Whittall, R.R. Whittle, D.P. White, T.P. Kennedy. Disulfiram inhibits activating transcription factor/cyclic AMP-responsive element binding protein and human melanoma growth in a metal-dependent manner in vitro, in mice and in a patient with metastatic disease. Molecular cancer therapeutics. 2004; 1049-1060.

184) E. Dalla Pozza, M. Donadelli, C. Costanzo, T. Zaniboni, I. Dando, M. Franchini, S. Arpicco, A. Scarpa, M. Palmieri. Gemcitabine response in pancreatic adenocarcinoma cells is synergistically enhanced by dithiocarbamate derivatives. Free radical biology & medicine. 2011; doi: 10.1016/j.freeradbiomed.2011.01.001.

185) Han SM, Baek JS, Kim MS, Hwang SJ, Cho CW. Surface modification of paclitaxel-loaded liposomes using d-α-tocopheryl polyethylene glycol 1000 succinate: Enhanced cellular uptake and cytotoxicity in multidrug resistant breast cancer cells. Chem Phys Lipids. 2018 Mar 14;213:39-47. doi: 10.1016/j.chemphyslip.2018.03.005.

186) Kim M, Williams S. Daunorubicin and Cytarabine liposome in newly diagnosed therapy-related Acute Myeloid Leukemia(AML) or AML with Myelodysplasia-related changes. Ann Pharmacother. 2018 Mar 1:1060028018764923. doi: 10.1177/1060028018764923.

187) Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994; doi: 10.1038/367645a0.

188) Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer. 2008; doi: 10.1038/nrc2499.

189) Mueller MT, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, et al. Combined targeted treatment to eliminate tumorigenic cancer stem

cells in human pancreatic cancer. Gastroenterology. 2009; doi: 10.1053/j.gastro.2009.05.053.

190) Zahreddine H., Borden K. L. Mechanisms and insights into drug resistance in cancer. Front. Pharmacol. 2013; doi: 10.3389/fphar.2013.00028.

191) Biancotto C., Frige G., Minucci S. Histone modification therapy of cancer. Adv. Genet. 2010; doi: 10.1016/B978-0-12-380866-0.60013-7.

192) Krishnamurthy S., Ke X., Yang Y. Y. Delivery of therapeutics using nanocarriers for targeting cancer cells and cancer stem cells. Nanomedicine (London, England). 2015; doi: 10.2217/nnm.14.154.

193) Lu B, Huang X, Mo J, Zhao W. Drug delivery using nanoparticles for cancer stem-like cell targeting. Front Pharmacol. 2016; doi: 10.3389/fphar.2016.00084.

194) Andrew R. Burke, Ravi N. Singh, David L. Carroll, Frank M. Torti, Suzy V. Torti. Targeting cancer stem cells with nanoparticle-enabled therapies. J Mol Biomark Diagn. 2012; doi: 10.4172/2155-9929.S8-003.

195) Hong I. S., Jang G. B., Lee H. Y., Nam J. S. Targeting cancer stem cells by using the nanoparticles. Int. J. Nanomed. 2015; doi: 10.2147/IJN.S88310.

196) Hu CM, Zhang L. Therapeutic nanoparticles to combat cancer drug resistance. Curr Drug Metab. 2009;10:836–841.

197) Reddy LH, Dubernet C, Mouelhi SL, Marque PE, Desmaele D, Couvreur P. A new nanomedicine of gemcitabine displays enhanced anticancer activity in sensitive and resistant leukemia types. J Control Release. 2007;124:20–27.

198) Bouffard DY, Laliberte J, Momparler RL. Kinetic studies on 2',2'-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Bio- chem Pharmacol. 1993; 45(9):1857-61.

199) Zhang, J.A. Ahmad, I. Liposomal gemcitabine compositions for better drug delivery. WO 2004017944, March 4, 2004.

200) Malfanti A , Miletto I , Bottinelli E , Zonari D, Blandino G , Berlier G and Arpicco S. Delivery of Gemcitabine Prodrugs Employing Mesoporous Silica Nanoparticles. Molecules 2016, 21(4), 522; doi:10.3390/molecules21040522.

201) Brusa P, Immordino ML, Rocco F and Cattel L. Antitumor Activity and Pharmacokinetics of Liposomes Containing Lipophilic Gemcitabine Prodrugs. Anticancer Research, 2016. 27: 195-200.

202) Allensworth JL, Evans MK, Bertucci F, Aldrich AJ, Festa RA, Finetti P,

Ueno NT, Safi R, Mc Donnell DP, Thiele DJ, Van Laere S, Devi GR. Disulfiram (DSF) acts as a copper ionophore to induce copper-dependent oxidative stress and mediate anti-tumor efficacy in inflammatory breast cancer. Mol Oncol. 2015 Jun;9(6):1155-68. doi: 10.1016/j.molonc.2015.02.007.

203) Wehbe M, Anantha M, Backstrom I, Leung A, Chen K, Malhotra A, Edwards K, Bally MB. Nanoscale reaction vessels designed for synthesis of copper-drug complexes suitable for preclinical development. PLoS One. 2016 Apr 7;11(4):e0153416. doi: 10.1371/journal.pone.0153416.

204) Wehbe M, Anantha M, Shi M, Leung AW, Dragowska WH, Sanche L, Bally MB. Development and optimization of an injectable formulation of copper diethyldithiocarbamate, an active anticancer agent. Int J Nanomedicine. 2017 May 31;12:4129-4146. doi: 10.2147/IJN.S137347.

205) Medina OP, Zhu Y, Kairemo K. Targeted liposomal drug delivery in cancer. Curr Pharm Des. 2004;10(24):2981-9.

206) Peng Liu, Zhipeng Wang, Sarah Brown, Vinodh Kannappan, Patricia Erebi Tawari, Wenguo Jiang, Juan M. Irache, James Z. Tang, Stephen Britland, Angel L. Armesilla, John L. Darling, Xing Tang, Weiguang Wang. Liposome encapsulated disulfiram inhibits NFKB pathway and targets breast cancer stem cells in vitro and in vivo. Oncotarget. 2014 Sep; 5(17): 7471–7485. doi: 10.18632/oncotarget.2166.

207) E. Dalla Pozza, C. Lerda, C. Costanzo, M. Donadelli, I. Dando, E. Zoratti, M.T. Scupoli, S. Beghelli, A. Scarpa, E. Fattal, S. Arpicco, M. Palmieri, Targeting gemcitabine containing liposomes to CD44 expressing pancreatic adenocarcinoma cells causes an increase in the antitumoral activity, Biochimica et biophysica acta, 2013, 1396-1404.

10. ANNEXES

During the period of my PhD, I have collaborated in a research project focused on the study of new therapeutic strategies for the treatment of PDAC. In particular, I studied the biological features of cancer stem cells that constitute a distinct population of quiescent cells that are resistant to standard therapy and are responsible for metastasis and relapses. A part of the project was carried out in the Prof. Heeschen's lab at the Barts Cancer Institute in London, where I spent a period of six months. In this period, I continued to study the effect of new therapeuthic strategies on cells directely derived from PDAC patients and cultured as tumour spheres having stem like features.

During the period of my PhD, I have been collaborating in the following publications:

1) The scaffolding protein NHERF1 sensitizes EGFR-dependent tumor growth, motility and invadopodia function to gefitinib treatment in breast cancer cells. Bellizzi A, Greco MR, Rubino R, Paradiso A, **Forciniti S,** Zeeberg K, Cardone RA, Reshkin SJ. - Int J Oncol. 2015 Mar;46(3):1214-24. doi: 10.3892/ijo.2014.2805.
2) Secreted molecules inducing epithelial-to-mesenchymal transition in cancer development.
   Dalla Pozza E., **Forciniti S.**, Palmieri M., Dando I. Semin Cell Dev Biol. 2017 Jun 30. pii: S1084-9521(16)30486-4. doi: 10.1016/ j.semcdb.2017.06.027. Review.
3) Extracellular Matrix composition modulates PDAC parenchymal and stem cell plasticity and behavior through the secretome.
   Biondani G., Zeeberg K., Greco M.R., Cannone S., Dando I., Dalla Pozza E., Mastrodonato M., **Forciniti S.,** Casavola V., Palmieri M., Reshkin S.J. and Cardone R.A. FEBS J. 2018 Apr 16. doi: 10.1111/febs.14471.