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In vitro and in vivo inhibition of Chk1 sensitize lung cancer stem cells to chemotherapy

Susanne Svensson

Coordinator and tutor

Prof. Daniele Condorelli

Tutor

Prof. Ruggero de Maria

Abstract

Development of resistance to radiation and chemotherapy turns the treatment of solid cancers into a therapeutic challenge. One of the most exciting breakthroughs being explored in cancer research today is the concept of cancer stem cells (CSCs). CSCs are a minority of cells within a tumor that are the source of tumor cell renewal and thereby determine the behavior of tumors, including proliferation, spreading and response to therapy. CSCs are highly resistant to conventional treatment and are therefore emerging as the preferred target of drug therapies in order to obtain eradication of tumors.

In this study, we examined the activation of the DNA damage response pathway in CSCs derived from non-small cell lung cancer (NSCLC-CSCs) and their differentiated counterparts after treatment with chemotherapeutic agents commonly used in clinic for lung cancer treatment. Our data show that NSCLC-CSCs possess a highly active DNA damage pathway compared to differentiated progeny and preferentially activate the checkpoint kinase Chk1 in response to DNA damage caused by chemotherapy. This indicates that Chk1 is most likely the main player of drug resistance in NSCLC-CSCs and its targeting might yield significant therapeutic gains. We demonstrate that chemical Chk1 inhibitors dramatically induce NSCLC-CSC death *in vitro* in combination with DNA damaging drugs. Cell death is induced through a premature activation of the cell cycle regulatory proteins Cdc2 and Cyclin B1, which in turn forces cells with damaged DNA to enter aberrant mitosis, a mechanism known as mitotic catastrophe. Moreover, our results indicate that final cell death occurs through apoptosis. Combination therapy studies have been successfully carried out also *in vivo*. Chk1 inhibition enhanced the antitumoral effect of conventional chemotherapy in mice xenograft tumor models by increasing tumor latency, potently abrogating tumor growth and reducing tumor mass. We also found a significant reduction of NSCLC-CSCs in xenograft-derived cells, confirming that combination treatment actually targets and reduces the NSCLC-CSCs compartment *in vivo*.

The importance of DNA repair as a resistance mechanism in cancer is a clinically relevant topic and we believe that the combination of selective Chk1 inhibitors with anti-cancer drugs could represent a new therapeutic approach for targeting NSCLC-CSCs and thereby for effective treatment of lung tumors.

Abbreviations

ABC	ATP binding cassette
AC	adenocarcinoma
AML	acute myeloid leukaemia
Apaf-1	apoptotic protease-activating factor-1
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3 related
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
caspases	cysteine-dependent aspartate-specific protease
CDK	cyclin-dependent kinase
CEA	carcinoembryonic antigen
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CKs	cytokeratins
CSC	cancer stem cell
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DSB	DNA double-strand break
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
HLA	human leukocyte antigen
HSP-90	heat shock protein-90
H&E	hematoxylin and eosin
IC_{50}	half maximal inhibitory concentration
LCC	large cell carcinoma
LCSC	lung cancer stem cell
NSCLC	non-small cell lung cancer
NSCLC-CSC	non-small cell lung cancer stem cell
PI	propidium iodide
PIKK	phosphoinisitide 3-kinase related protein kinase

PI3K	phosphatidylinositol-3-kinase
PS	phosphatidylserine
Rb	Retinoblastoma
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
SFM	serum-free medium
SHH	sonic hedgehog
SP	side population
TNF	tumor necrosis factor

Table of Contents

1. Introduction

1.1. Lung cancer

1.1.1. The human airway epithelium

The epithelium lining the human airways is important for gas transport and exchange, as well as regulation of host defence and reparation following tissue damage [1]. To accomplish these functions, the lung epithelium has evolved with different types of cells in different zones, such as basal mucous secretory cells of the trachea and bronchi, Clara cells of bronchioles, type 1 and type 2 pneumocytes of alveoli [1,2].

There are evidence supporting the presence of undifferentiated multipotent stem cells in each of the epithelial compartments of the lung and that these cells give rise to the mature differentiated cells of the lung [2-4].

1.1.2. General aspects of lung cancer

Lung cancer is one of the most common malignancies worldwide and a leading cause of cancer-related deaths [5-11]. Despite advances in lung cancer therapy, the average five-year survival rate is only 15% and early metastasis has become increasingly common [6,7]. The poor prognosis depends on late diagnosis and high rate of recurrence, which result in an advanced stage of cancer at the time of diagnosis, for which chemotherapy and radiotherapy have limited efficacy [11].

Lung cancer can be divided into two main subgroups that show major differences in histopathologic and genetic characteristics; small cell lung cancer (SCLC) and non-SCLC (NSCLC), where NSCLC accounts for more than 80% of all lung cancer cases [6,9-11]. NSCLC is further subdivided into squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC) [6,7,9]. ACs are histologically heterogeneous peripheral masses that metastasize early, in many cases while the primary tumor is still a symptomless peripheral lesion [6,12]. SCCs are characterized by centrally located endobronchial masses, that unlike adenocarcinomas generally metastasize late in the disease course [6,12]. LCCs are poorly differentiated, usually large peripheral masses associated with early metastases [6,12]. SCLCs have a rapid

growth rate and are clinically aggressive. They are usually centrally located and are associated with early metastases. Despite their responsiveness to chemotherapy, small cell carcinomas often are advanced at the time of diagnosis, and patients have a poor prognosis [6,12].

1.1.3. Risk factors

Smoking is the predominant risk factor for all types of lung cancer. The effect of pipe and cigar use on the risk of lung cancer is similar to that of light cigarette smoking. Passive smoke exposure is also a risk factor and noteworthy is that passive smoking during childhood increases lung cancer risk in adulthood [6,9,10].

Environmental and occupational risk factors include exposure to asbestos, radon, arsenic, chromium, nickel, vinyl chloride, and ionizing radiation [6,10]. Lung cancer could also be a long-term effect of exposure to air pollution [10].

1.1.4. Symptoms of lung cancer

More than 90% of patients with lung cancer show symptoms at the time of diagnosis [13]. Most patients present with nonspecific symptoms, including fatigue, weakness and weight loss. A minority of patients present with direct signs and symptoms related to the primary tumor, to intrathoracic spread or to distant metastasis [6,13]. The most commonly presenting symptom caused by a primary lung tumor is cough. Other symptoms caused by the primary tumor include shortness of breath, hemoptysis (coughing up blood) and chest pain, which occurs in up to 50% of patients at diagnosis [6,13]. Intrathoracic spread of lung cancer, either by direct extension or lymphatic spread, produces a variety of symptoms and signs. These may be caused by involvement of the structures such as nerves, chest wall, vascular involvement and heart [13]. Approximately one third of patients present with symptoms as a result of distant metastases [13].

1.1.5. Diagnosis and staging of lung cancer

Diagnosis of lung cancer is based on methods such as chest radiograph, bronchoscopy, computer tomography scans and thoracotomy. Biopsy is often used to confirm the diagnosis of lung cancer and to identify the histological tumor type. The final

component of the diagnostic assessment is a functional evaluation of performance and pulmonary status of the patient [6].

Lung cancer staging, which can be determined based on the type of tumor identified and the presence or absence of metastatic disease, is an important factor affecting the possible treatment of lung cancer [6,9]. NSCLC is categorized using the TNM (tumor size – node involvement – metastasis status) staging system, whereas SCLC is categorized after a two-stage system defining tumors as being of limited stage or extensive stage [6].

1.1.6. Metastasis from lung cancer

The most common sites of distant metastasis from lung cancer are the bones, liver, brain, spinal cord, lymph nodes and skin.

The skeleton is one of the most common sites of metastasis in patients with lung cancer and the incidence of bone metastases is approximately 30-40% [14]. The primary symptoms resulting from metastatic bone disease include pain, pathologic fracture, vertebral malformation and spinal cord compression [13,14]. The prevention and treatment of bone metastases is mainly dependent on an effective treatment against the primary lung cancer tumor [14].

Liver metastasis occurs frequently with lung cancer and is associated with a very poor prognosis. Liver function is rarely abnormal until the metastases are numerous and large, which the give symptoms of weakness and weight loss [13].

Brain metastases occur in 10% of patients with lung cancer and are a significant cause of morbidity and mortality [13,15]. Spinal cord metastases are less common and tend to occur in patients with cerebral metastases [13].

NSCLC has a strong ability to metastasize to regional lymph nodes already at an early stages of tumor growth [16]. Detecting enlarged lymph nodes or subcutaneous nodules due to metastatic lung cancer is very helpful in facilitating both diagnosis and staging [13].

1.1.7. Genetic alterations associated with lung cancer

Lung cancers exhibit both common and type-specific genetic alterations. The profile of molecular and genetic alterations considerably differs between SCLC and NSCLC, as well as among the subtypes of NSCLC [9].

The most common genetic alteration occurs in p53, which is inactivated in nearly 50% of NSCLC and more than 70% of SCLC [9]. Activation of this tumor suppressor can be induced by carcinogenesis stresses or DNA damage, leading to the expression of downstream genes involved in cell cycle arrest, allowing DNA repair or initiation of apoptosis [17]. An essential effect of p53 inactivation that is significant in carcinogenesis is the avoidance of apoptosis and cell cycle arrest by neoplastic cells.

Rb gene alterations and protein loss are found in practically all SCLC, along with a normal p16/Ink4 gene and cyclin D1 protein expression. Rb mutations are infrequently detectable in NSCLC (15%). Instead, the Rb function is interrupted due to dysfunction of upstream components of the Rb pathway, either through p16/Ink4 gene silencing or by overexpression of cyclin D1 protein [18-20]. Rb protein is one of the critical regulators of the G_1 to S phase cell cycle transition and exhibits a growth suppressive function [20,21].

Activating mutations in epidermal growth factor receptor (EGFR) gene have been demonstrated in 40% to 80% of patients with NSCLC, and its overexpression correlates with a poor prognosis [10]. EGFR overexpression plays a significantly important role in SCC and AC carcinogenesis [22].

There are other less investigated genetic alterations that might contribute to lung cancer development or progression. K-ras gene mutations are rare in all lung cancers except AC, that show an alteration frequency of 30% and activation of PIK3 has been found in nearly half of SCC cases [9].

1.1.8. Treatment of lung cancer

Treatment of lung cancer differs according to the histologic type, the stage of the tumor and the patient's functional evaluation [6]. Surgical resection remains the most reliable and successful option for cure of lung cancer patients [10]. Patients with resected lung

cancer have a high risk of relapse and adjuvant chemotherapy is standard after complete resection of lung cancer [6,10]. Treatment for unresectable lung cancer usually involve radiotherapy and chemotherapy [6]. Drugs commonly used for lung cancer treatment include cisplatin and carboplatin (alkylating agents that bind to and causes crosslinking of DNA), etoposide and irinotecan (topoisomerase inhibitors causing DNA strand breaks), docetaxel and paclitaxel (agents stabilizing microtubules and thereby inhibits mitosis), and gemcitabine (interferes with the nucleic acids during DNA replication and inhibits DNA synthesis) [12,23,24]. Although chemotherapy is appropriate for many patients with lung cancer, there are signs that the use of traditional chemotherapeutic agents has reached a therapeutic plateau and new therapeutic targets, such as signal transduction and angiogenesis pathways, are necessary [10].

1.2. Cancer stem cells

1.2.1. General aspects

Solid tumors are composed of a heterogeneous population of cells with different proliferative, differentiative and tumor initiating potential [25]. To explain the heterogeneity of cells observed in cancer, two models of tumor cell proliferation and tumor expansion have been proposed; the stochastic model and the stem cell model (**Figure 1**) [26,27]. In the stochastic model, every cell has the same probability to proliferate extensively and to cause development and progression of malignancy. According to the stem cell model, only a small fraction of cells, the cancer stem cells (CSCs), have the potential to proliferate unlimitedly and to form tumors [26,27].

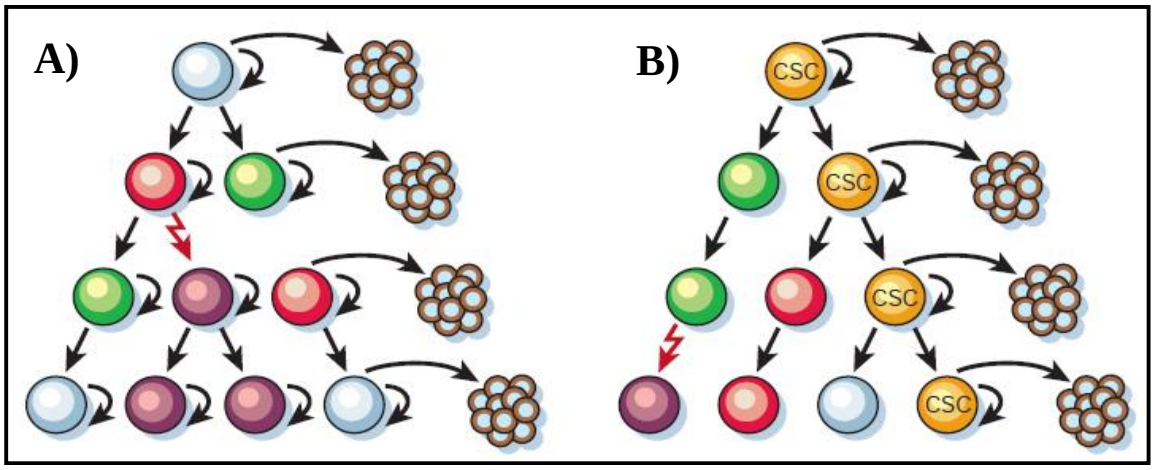


Figure 1. Two models of heterogeneity in solid tumors. Cancer cells are heterogenous in both models. In the stochastich model (**A**), all phenotypes have the same potential to proliferate, while in the stem cell model (**B**), only the CSCs have the ability to proliferate extensively and form new tumors [26].

CSCs (or cancer initiating cells) are a rare population of undifferentiated tumorigenic cells responsible for tumor initiation, maintenance, spreading and therapy resistance [7,9,26,28]. These cells display unlimited proliferative potential, ability to self-renew and capacity to generate a progeny of terminally differentiated cells that constitute the major tumor population (**Figure 2**) [7,11,25,29-32]. Within an established tumor, only the CSCs have the capacity to reproduce the original tumor when transplanted into immunodeficient mice [7,9,11,25,26,30,31,33,34].

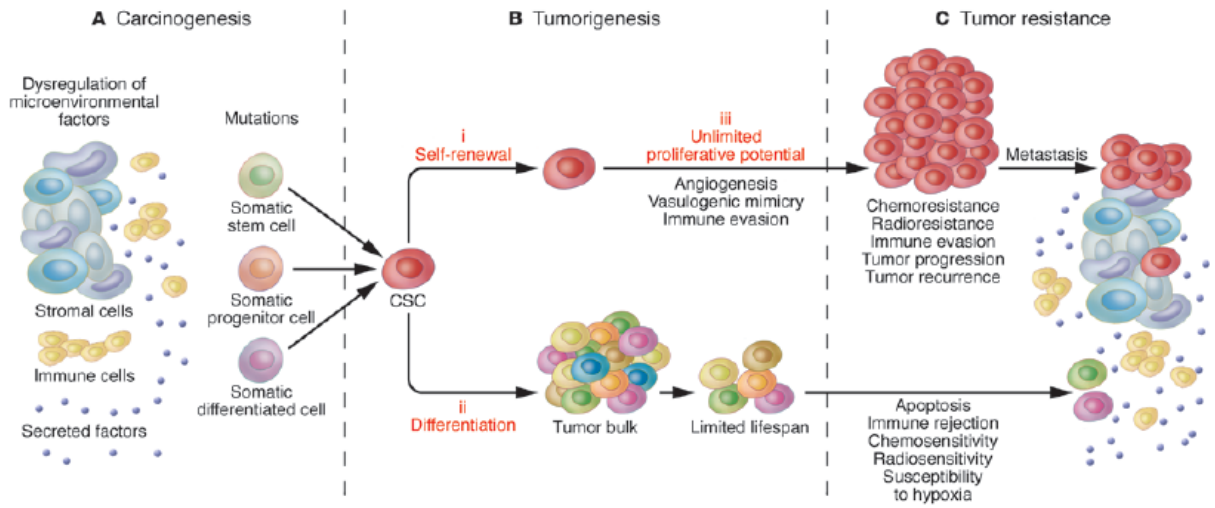


Figure 2. CSCs and their implication for carcinogenesis, tumorigenesis, and tumor resistance. A. Tumors can arise from somatic cells through genetic mutations of cancer-critical genes and microenvironmental factors can contribute to the carcinogenic process. **B.** CSCs are capable of driving tumorigenesis through: (**i**) their ability for long-term self-renewal, (**ii**) their capacity to differentiate into tumor bulk populations, and (**iii**) their unlimited potential for proliferation and tumorigenic growth. **C.** CSCs exhibit increased resistance to chemotherapeutic agents and most likely drive neoplastic progression, tumor recurrence, and metastasis [35].

The origin of CSCs is still unknown, they could arise from somatic stem cells that acquire tumorigenic properties, or they could evolve from more differentiated progenitor cells that transform and acquire stem cell-like properties [27,36,37]. Stem cells have the self-renewing machinery already activated and the fact that CSCs and normal stem cells share many signalling pathways strengthens the hypothesis of a stem cell origin of cancer [26,27,29].

1.2.2. Discovery of CSCs

The existence of CSCs was first proven in the context of acute myeloid leukaemia (AML). It was shown that only a small subset of human AML cells, phenotypically similar to normal haematopoietic stem cells, were clonogenic in culture and capable of forming AML when transplanted into immunodeficient mice [27,38,39].

Since the initial discovery of CSCs in AML, subpopulations of tumor cells with stem cell-like characteristics have also been identified and expanded from several solid tumors, including breast [33], brain [40-43], melanoma [44,45], prostate [46,47], head and neck [48], pancreas [49,50], colon [51,52] and lung [7].

1.2.3. Identification of CSCs

The CSCs comprise a very small fraction of the entire tumor cell population and it is necessary to use specific markers to distinguish CSCs from the bulk of more differentiated tumor cells [11,25,30,37].

The use of cell surface markers for identification of CSCs has been widely investigated in different cancer types (**Table 1**). Several studies have demonstrated that CD133 (prominin-1) is an important marker of CSCs and it has been successfully used to identify CSCs in brain tumors [42], colon carcinomas [51,52], pancreatic cancer [49] and lung cancer [7]. Al-Haaj et al.[33] demonstrated that the CD44+CD24-/low cell population in breast cancer is significantly enriched in cancer-initiating cells. In AML, CD34+ and CD38- cells have highly enhanced tumor initiating potential [38,39].

Cancer type	Cell surface markers
AML	CD34+, CD38−
B-ALL	CD34+, CD38−, CD19+
Ph1-ALL	CD34+, CD38−
Blast-crisis CML	CD34+, CD38+, CD123+
Myeloproliferative disorder	CD117+
Glioblastoma	CD133+
Medulloblastoma	CD133+
Pilocytic astrocytoma	CD133+
Anaplastic ependymoma	CD133+
Breast cancer	CD44+, $CD24^{-/low}$
Prostatic cancer	CD44+, integrin $alpha2beta1^{high}$, CD133+
Ovarian cancer	CD44+, MyD88+
Colon cancer	CD133+, CD44+, CD166+, $E\text{-}CAM^{high}$
Pancreatic cancer	CD133+, CD44+, CD24+
Hepatocellular cancer	CD133+
Head and neck squamous cell carcinoma	CD44+
Bone sarcomas	Stro-1+, CD105+, CD44+
Metastatic melanoma	CD20+
Lung cancer	CD133+

Table 1. Cell surface markers for identification of CSCs [37].

Another functional approach used to distinguish CSCs within a population is by using Hoechst 33342. Uptake of the dye occurs universally in all cells, but efflux occurs only in specific cells. Cells with capacity to efflux the dye were first identified in the mouse bone marrow and they were termed side population (SP) cells as they fell to the side of the majority of positively stained cells in a FACS analysis [53]. SP cells have been identified in for example, skin, lung, liver, heart, brain and mammary gland and in

normal tissues the SP cells express high levels of stem cell genes and possess differentiation potential [25]. The Hoechst efflux requires the expression of the ABCG2 gene, a member ATP binding cassette (ABC) transporter superfamily [25,54].

Recently, Jiang et al. [55] reported that ALDH1-positive lung cancer cells exhibited properties consistent with those of tumor stem cells, including high ability of proliferation, self-renewal and differentiation, resistance to chemotherapy as well as expression of the CSC surface marker CD133. They also demonstrate that ALDH1-positive cells were able to induce tumor growth *in vivo*.

The selection of candidate markers for identifying CSCs can be very difficult, especially in the case of solid tumors where the normal tissue developmental hierarchy has not been characterized. A widely accepted assay to validate a candidate CSC population is by tumor initiation and serial transplantation in immunocompromised mice [37,56]. However, it is essential to confirm that these xenografts are phenotypically identical to patient tumors [56].

1.2.4. Signaling pathways in CSCs

Important signaling pathways that regulate self-renewal, proliferation and differentiation in stem cells include Bmi-1, Sonic Hedgehog (SHH), Notch and Wnt/β-catenin [28,57-59]. Involved in the process of self-renewal and proliferation is also the tumor suppressors, such as p53, PTEN, INK4A and ARF, that can inhibit proliferation, block self-renewal and regulate cell responses to DNA damage [28,58,60-62].

Signaling pathways regulating self-renewal of CSCs are poorly characterized, but based on the similarities between normal stem cells and CSCs and on the fact that signaling pathways often are deregulated in cells undergoing neoplastic transformation, the Bmi-1, SHH, Notch and Wnt/β-catenin pathways are thought to be implicated also in CSC regulation [28,59].

A few studies concerning signaling pathways activated in CSCs from solid tumors have been performed. Malanchi et al. [63] reported that Wnt/β-catenin signaling is essential to sustaining the CSC phenotype and to maintain skin tumorigenesis. It has been demonstrated that Wnt signaling activity is implicated in colon CSCs [64] and that

overexpression of Bmi-1 and inappropriate activation of SHH pathway might lead to medulloblastoma development [65].

1.2.5. Therapeutic implications of CSCs

CSCs are thought to be more resistant to chemo- and radiotherapy than the committed differentiated cells of the tumor population [26,28,31,66]. The difficulty in eradicating solid tumors may be due to the fact that existing therapies target the bulk of the tumor cells, while they fail to kill CSCs effectively [25,26,67]. The remaining CSCs could quickly allow regrowth of the tumor [26,31,37,66,67]. It has actually been shown that ionizing radiation treatment of human glioma cultures and glioma xenografts enriches the CD133+ CSC population [5,40].

Several properities of CSCs contribute to their resistance to chemotherapy. Firstly, CSCs are often found in a quiescent state or have a very low proliferation rate and are hence resistant to cell cycle specific chemotherapeutic agents, as well as chemotherapy that affect proliferating cells [5,68-70]. Secondly, stem cells are able to efficiently repair DNA damage, hence being more resistant to radiation and chemotherapy, including DNA damaging agents such as alkylating agents [40,68,71]. Thirdly, CSCs have impaired apoptosis pathway and express higher levels of antiapoptotic proteins, such as Bcl-2, compared to differentiated cells [28,32,68,72,73]. Finally, CSCs have high expression levels of drux efflux transporter proteins, such as p-glycoprotein, ABCG2 and other members of the ABC superfamily [5,66,74-77].

1.2.6. Lung cancer stem cells

Eramo et al. [7] identified lung cancer stem cells (LCSCs) as a rare population of CD133+ cells in both SCLC (SCLC-CSCs) and NSCLC (NSCLC-CSCs). LCSCs could be unlimitedly expanded and maintained *in vitro* as tumor spheres, while differentiated tumor cells lost the CD133 antigen and were only able to proliferate for 4 weeks before declining in number.

CD133+ lung cells showed higher tumorigenic potential than their CD133-counterparts and were able to generate tumor xenografts in immunocompromised mice, with morphological and immunohistological features closely resembling the original tumor [5,7]. LCSCs were shown to be resistant to chemotherapeutic drugs like cisplatin,

etoposide, paclitaxel and gemcitabine [5,7], which can explain the poor therapeutic effect of conventional chemotherapy on lung cancer patients. Even if tumor burden can be reduced by chemotherapy, the CD133+ tumor cells might be spared and enriched [5].

An increased expression level of genes involved in maintenance of stemness, like Oct3/4 and Nanog, was observed in CD133+ LCSCs compared with the CD133- counterpart [5,7,74].

1.3. DNA damage and the DNA damage response pathway

1.3.1. General aspects

All cells are constantly under assault of different forms of DNA lesions. The damage can be classified as either endogenous (normal metabolic processes) or exogenous damage (environmental factors) like radiation, chemicals, UV, chemotherapy [78]. Unrepaired DNA damage can impede a cells ability to carry out its functions and may result in mutations, increased likelihood of tumor formation or even cell death [79]. Maintenance of genome stability depends on the appropriate response to DNA damage and cells have therefore evolved complex signaling networks, that coordinates cell cycle transitions, DNA replication, DNA repair and apoptosis [79-84]. In response to DNA damage, the DNA damage response (DDR) pathway is activated. The signaling pathways activated during DDR have three aims: (1) to block the cell cycle progression and cell division; (2) to increase accessibility to the damaged sites to the DNA repair machinery and (3) to induce apoptosis to cells with damaged DNA that cannot be repaired [85]. The DDR pathway consists of three main components; sensor proteins that recognize damaged DNA, signal transducers that relay and amplify the damage signal (ATR and ATM) and finally, the effectors that regulate the commitments of the cell (Chk1, Chk2 and p53) (**Figure 3**) [79,84,86].

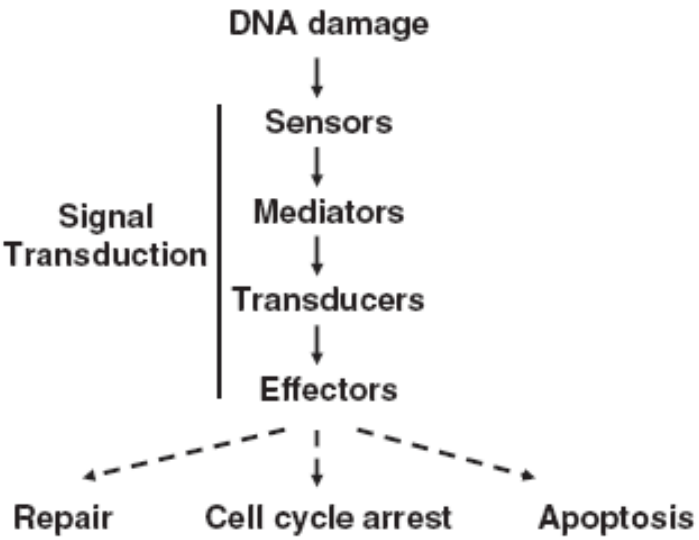


Figure 3. Organization of the DNA damage response pathway. DNA damage is regocnized by sensor proteins, thereafter the signal is transmitted to transducers (ATM and ATR) that finally regulate the effectors (Chk1 and Chk2) [87].

1.3.2. ATM and ATR

The major regulators of the DDR pathway are the protein kinases ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) that belong to the phosphoinisitide 3-kinase related protein kinases (PIKKs) [79,81,83,84,86]. Signals initiated by sensor proteins rapidly transduce to ATM and ATR kinases, which both phosphorylate a great number of substrates, initiating a cascade that results in cell cycle arrest, DNA repair or apoptosis [79,81].

DNA damage induces phosphorylation of ATM at Serine 1981, which in turn leads to activation of several key targets in the DDR pathway, including Chk2 and p53 [87-90]. ATM is mainly recruited and activated in response to DNA double-strand breaks (DSBs) [79,81-83,87,91] and the first response to DSBs is phosphorylation of histone H2A.X (γ-H2A.X) [88]. Cells lacking ATM could fail to perform many of the cellular responses necessary in response to DNA damage [84]. On the other hand, cells lacking ATM and ATM null mice are viable, suggesting that ATM is not essential for a normal cell cycle or differentiation [84,87].

ATR is activated in response to a broad range of damage, including UV, stalled DNA replication forks, DSBs and to a lesser extent, ionizing radiation (IR) [79,83,84,86,92,93]. Once ATR is activated, it phosphorylates Chk1, initiating a signal transduction cascade that leads to cell cycle arrest [87,92]. ATR deficiency in mice results in early embryonic death [87], it is essential for the viability of human and mouse cells [81,91] and ATR-/- blastocyst cells die in culture with a phenotype resembling mitotic catastrophe [84].

ATM and ATR have been shown to act either together or separately to organize the responses to specific types of DNA damage or stalled replication [83]. They share a number of phosphorylation substrates and their major functions in cell cycle control are overlapping and redundant [81,84]. ATM is activated rapidly irrespective of the cell cycle, whereas ATR is activated more slowly and predominantly in S and G_2 phase cells [81].

1.3.3. Chk1 and Chk2

Checkpoint kinases 1 and 2 (Chk1 and Chk2) are located downstream of ATM and ATR [79,84,86]. Chk1 and Chk2 are structurally unrelated but share a number of overlapping substrates, although it is clear that they have distinct roles in directing the response of the cell to DNA damage [79,84,93]. Once activated, the effects of Chk1 and Chk2 are mediated to proteins like Cdc25A, Cdc25B, Cdc25C, and p53 [79].

Chk1 is a Serine/Threonine kinase that is primarily responsible for initiating cell cycle arrest in response to DNA damage, allowing time for DNA repair and cell survival [79,84,86]. The Chk1 protein plays a fundamental role in cell cycle checkpoint control and is important in regulation of the S phase and G_2-M phase checkpoints [79,84,87]. In response to DNA damage, Chk1 is quickly phosphorylated at Serine 317/345 [81,87,91-95]. Serine 345 can be phosphorylated by ATR both *in vivo* and *in vitro* in response to UV [84]. Chk1-deficiency has been demonstrated to result in a premature onset of mitosis and Chk1-deficient mice die at an early embryonic stage of development [87,96,97].

Chk2 is also a Serine/Threonine kinase that is required for cell cycle arrest in response to DNA damage [84,86]. Chk2 is activated primarily by ATM in response to IR-induced DNA damage or to DSBs by phosphorylation of Threonine 68 [80,87,93]. Chk2 is not necessary for embryonic development [87] and Chk2-deficient mice are viable and seem normal [79]. However, tissues derived from Chk2-deficient mice show significant defects in G_1-S checkpoint and IR-induced apoptosis [79].

In response to DSBs, ATM has been proposed to influence the activation and phosphorylation of Chk1 indirectly, through the regulation of DSB processing and subsequent activation of ATR, indicating that crosstalk takes place between the ATR and ATM signaling cascades [81,83,90,93,95,98,99].

1.4. Cell cycle checkpoints

1.4.1. General aspects

The cell cycle checkpoints mediate and regulate progression through the cell cycle and prevent the initiation of downstream events when defects are detected [100,101]. The checkpoints inspect the integrity and replication status of the genetic material before cells decide to replicate their DNA [91]. Defects in cell cycle checkpoints can lead to accumulation of mutations and chromosomal aberrations, which in turn can contribute to tumorigenesis [80,102].

In response to DNA damage, interactions of the ATM/ATR pathways converge to regulate the cell cycle network and induce cell cycle arrest in order to provide time for DNA repair. Cell cycle arrest is induced by inactivating members of the Cdc25 family and thereby modulating the activity of Cyclins and Cyclin dependent kinases (CDKs) [103-105]. The CDKs are the key regulator proteins of the cell cycle and they control the transitions between different stages of the cell cycle [97,104,105]. The CDKs are positively regulated by Cyclins and at different phases of the cell cycle, diverse Cyclin-CDK complexes are responsible for blocking cell cycle progression (**Figure 4**), or alternatively inducing cell death if damage cannot be properly repaired [80,82,91].

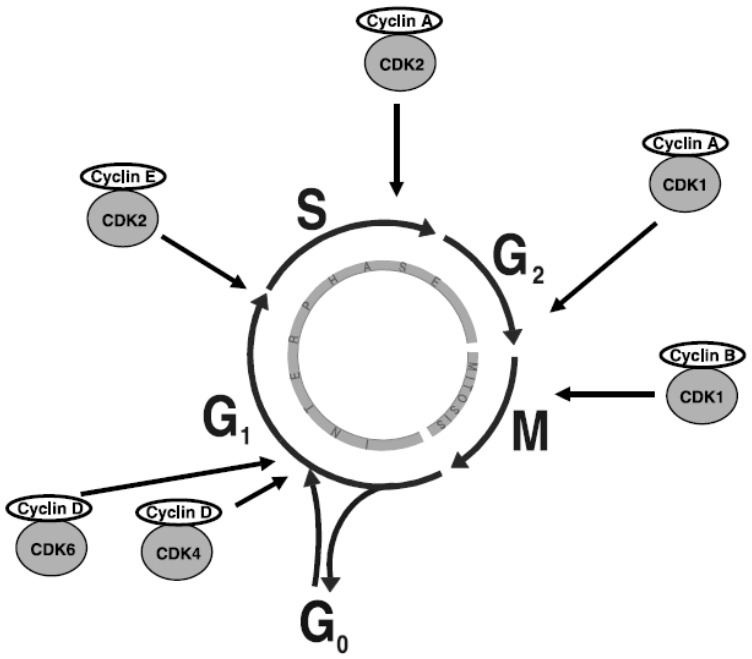


Figure 4. The different phases of the cell cycle and the sites of activity for the diverse CDK/Cyclin complexes [105].

1.4.2. The G_1 checkpoint

At the G_1 checkpoint, cells make decisions whether to replicate DNA and complete the cell division cycle or not [80]. DNA damage induces G_1 arrest in a mechanism that involves two different responses mediated by Chk1/Chk2; one initial rapid p53-independent response followed by a delayed, more sustained G_1 arrest maintained by p53–p21 signaling [79,80].

The immediate p53-independent response only lasts for a few hours and functions by decreasing abundance and activity of Cdc25A [80,86]. This results in inhibitory phosphorylation of CDK2, and thus inhibition of Cyclin E–CDK2 activity leading to the blockage of G_1/S transition [80].

The delayed p53-dependent response occurs at transcriptional level [86]. p53 becomes post-translationally modified and stabilized, which results in an induced expression of genes required for cell cycle arrest [80,83]. Among the genes induced by p53 is the CDK inhibitor p21, capable of inhibiting the CDK2-cyclin E complex and thus preventing the progression from G_1 phase to S phase of the cell cycle [80,83,86,102,105].

1.4.3. The S phase checkpoint

During DNA replication, cells must ensure that there are no abnormalities that could cause a incorrect copy of the genome. When abnormalities or DNA damage are detected, the S phase checkpoint slows down the rate of DNA synthesis, prevents new replication and stabilizes stalled replication forks [86,106].

The S phase checkpoint mechanisms respond in different manners to DNA damage, depending on the type of induced damage. In response to UVC, the S phase checkpoint response seems to be ATR-dependent [106]. However, in response to DNA DSBs, the S phase checkpoint requires ATM, but is p53-independent [79,80,106,107]. The ATM-Chk2 signaling cascade degrades Cdc25A, which leads to inhibition of the CDK2 kinase activity and thereby cell cycle arrest [80,106-108]. Chk1 has also been shown to retard progression through S phase via phosphorylation and degradation of Cdc25A [109].

1.4.4. The G_2 checkpoint

The G_2 checkpoint represents the last barrier that can block the entry into mitosis of a cell with damaged DNA. The G_2/M transition of the cell cycle is controlled by the Cdc2 (CDK1)/Cyclin B complex, whose activity is essential for both the G_2/M transition of the cell cycle and completion of mitosis [79,84,100,102,110].

In response to DNA damage, a series of events will cause an arrest in G_2 phase. DNA damage-induced Chk1/Chk2 activation leads to Cdc25C phosphorylation on Serine 216, promoting its binding to 14-3-3 proteins, thereby inhibiting its activity by nuclear export and sequestration in the cytoplasm [83,87,97,103,105,110]. Cdc2 will be kept in its inactivated form through inhibitory phosphorylation on Tyrosine 15 and prevent activation of Cdc2-Cyclin B complex [97,100,105]. Cyclin B is kept inactivate until the beginning of the prophase, by active translocation from the nucleus to the cytoplasm [105]. When conditions are appropriate for mitotic entry, Cdc2 is dephosphorylated by Cdc25, leading to Cdc2 activation and initiation of mitosis (**Figure 5**) [87,97,100].

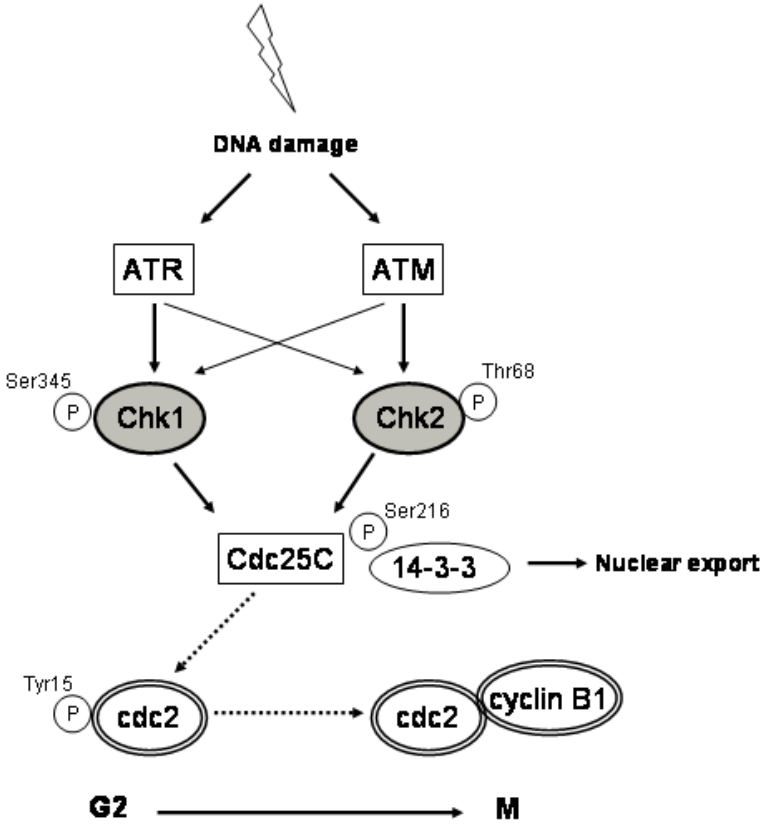


Figure 5. The G_2 checkpoint. After detection of DNA damage, the G_2 checkpoint primarily functions to block Cdc2-Cyclin B1 activity and thereby prevent progression through the cell cylce.

1.5. Cell Death

1.5.1. General aspects of cell death

Programmed cell death is central to the development and homeostasis of multicellular organisms [111]. Three types of cell death have been distinguished in mammalian cells by morphological criteria. Type I cell death, better known as apoptosis, type II cell death, also known as autophagic cell death and finally, type III cell death, more known as necrosis [111,112]. Another form of cell death, termed mitotic catastrophe, has been discovered to be fundamentally different from type I – type III cell deaths [113].

1.5.2. Apoptosis

Apoptosis is a highly regulated form of cell death, which is morphologically characterized by cellular shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, exposure of phosphatidylserine (PS) on the cell surface, and finally the formation of apoptotic bodies (**Figure 6**) [112-115]. In multicellular organisms, apoptosis is essential for development, tumor suppression, immune function and maintenance of homeostasis [36].

Two major pathways can induce apoptosis, the intrinsic pathway, which is controlled by mitochondrial membrane permeabilization, and the extrinsic pathway, in which death receptors trigger the apoptotic cascade [36,111]. The central players in both pathways are the caspases (cysteine-dependent aspartate-specific proteases) [36,116,117]. Caspase-2, -8, -9 and -10 are regarded as initiator caspases, whereas caspase- 3, -6 and -7, serve as effector caspases [114,117,118].

The intrinsic pathway is activated in the presence of cellular stresses such as growth factor withdrawal, cytotoxic drugs and DNA damage [36,111]. Initiation of the intrinsic pathway induces permeabilization of the outer mitochondrial membrane and the release of cytochrome C into the cytoplasm. Upon release, cytochrome C binds to Apaf-1 (apoptotic protease-activating factor-1), which in turn triggers the apoptotic cascade by activating procaspase-9 and forming the apoptosome complex. This complex activates the downstream effector caspases leading to DNA fragmentation and cell death [36,114,118].

The extrinsic pathway, or death receptor pathway, is activated through the TNF (tumor necrosis factor) family of cytokine receptors. This pathway is activated when a death ligand binds to the extracellular domains of the death receptor and leads directly to caspase activation [36].

1.5.3. Necrosis

In contrast to apoptosis, necrosis is a more uncontrolled form of cell death usually as a consequence of pathological traumas or harsh conditions. Morphologically, necrosis is characterized by vacuolization of the cytoplasm, loss of membrane integrity and cellular swelling (**Figure 6**) [111,114,115]. Necrosis is usually associated with inflammation, since the cellular contents leak into the extracellular environment [111]. There are growing evidence supporting the idea that necrosis can be a regulated form of cell death and that necrotic death can be induced by DNA damage via PARP-1, a protein involved in DNA damage repair [112,114].

1.5.4. Autophagy

The primary function of autophagy is to recycle proteins and organelles through degradation by lysosomal proteases and it is probably initiated as a survival response to cellular stress-associated damage or nutrient deprivation [111,114]. Various forms of environmental stress induce autophagy, which eventually results in either caspase-dependent or caspase-independent cell death [111].

Autophagy is recognized by the formation of autophagosomes, double membrane autophagic vacuoles that eventually fuse with lysosomes to form autolysosomes, where sequestered cellular components are digested (**Figure 6**) [111,114,115,119].

1.5.5. Mitotic catastrophe

Mitotic catastrophe is defined as a type of cell death that is caused by aberrant mitosis and is characterized by enlarged multinucleated cells, incomplete nuclear condensation, chromosome alignment defects, unequal DNA separation or mitosis in the presence of DNA damage (**Figure 6**) [114,115,120,121].

In mammalian cells and particularly in tumor cells, mitotic catastrophe is mainly associated with deficiencies in cell cycle checkpoints [113,114,121]. G_2/M regulatory proteins have been shown to be associated with mitotic catastrophe. High expression levels of proteins that promote entry of mitosis (such as Cdc2 and Cyclin B) as well as inhibition or knockout of proteins that prevent premature mitosis (such as ATR, ATM, Chk1, Chk2 and 14-3-3σ) can induce mitotic catastrophe [114]. Defective mitotic spindle checkpoints are also linked to mitotic catastrophe, since such defects usually lead to missegregation of chromosomes [113,114].

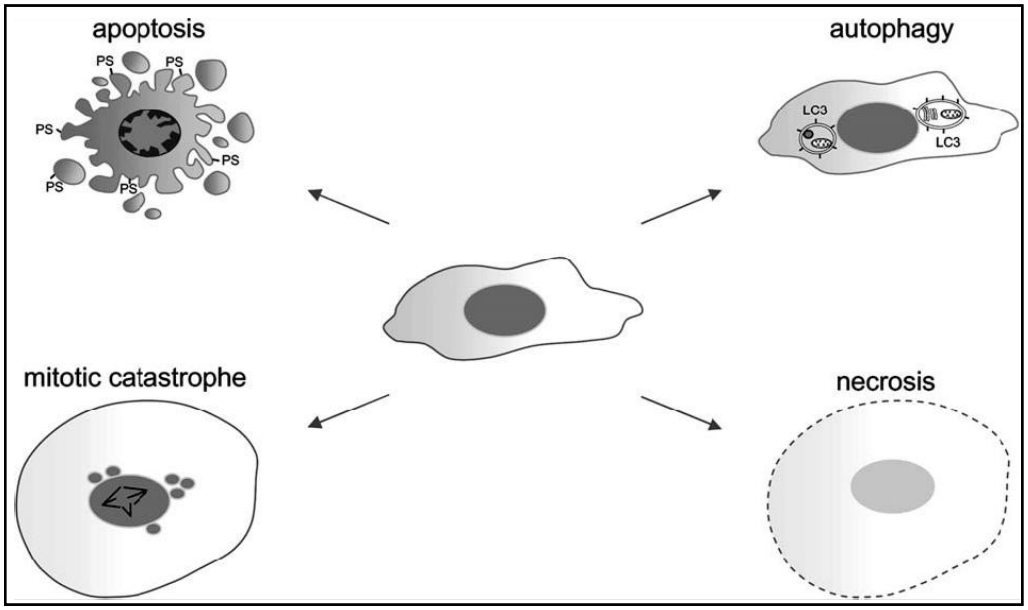


Figure 6. Morphological characteristics of apoptosis, autophagy, mitotic catastrophe and necrosis. Apoptosis is characterized by membrane blebbing, cytoplasmic shrinkage, chromatin condensation, exposure of phosphatidylserine (PS) on the cell surface, and finally the formation of apoptotic bodies. Death by autophagy is characterized by the double-membrane vesicles containing cytosolic organelles. Cells dying from mitotic catastrophe are usually large with multiple micronuclei and contain uncondensed chromosomes. During necrosis, cells swell and loose their membrane integrity [114].

1.6. Checkpoint inhibitors in cancer therapy

1.6.1. General aspects

DNA damaging therapies are among the most common cancer treatments. Due to the efficacy of these anticancer treatments, DNA damaging agents are likely to remain a standard treatment of many cancers in the future [79]. Since the majority of these agents are used at the maximum tolerated dose, DNA damaging agents often cause significant side effects. Toxicities to different organs are commonly observed and many patients develop resistance and therefore become refractory to treatment [79,122].

Through increased knowledge about the DDR pathway and cell cycle regulation, the cell cycle checkpoints have emerged as attractive therapeutic targets for anticancer therapy. Tumors may be sensitized to DNA damaging agents by targeting the cell cycle checkpoints, since abrogation of cell cycle arrest prevents cancer cells from repairing DNA damage, thereby forcing them into mitotic catastrophe and apoptosis [123].

1.6.2. Cell cycle abrogation as an anticancer strategy

The rationale behind using cell cycle abrogation to target cancer cells depends on the difference in functional cell cycle checkpoints between normal cells and tumor cells. Many malignant cells suffer from defects in various tumor suppressor genes, including p53 and Rb pathway, and have therefore a defective G_1 checkpoint mechanism [123,124]. Cells deficient in the G_1 checkpoint are highly dependent on the S and G_2 checkpoints to maintain cell cycle arrest and for repair of DNA damage [123,124]. In normal cells, DNA damage would arrest cells mostly in G_1 phase, whereas p53-deficient tumors, accounting for over half of all tumor types, would have to rely on the S or G_2/M checkpoints [125]. Therefore, G_2 cell cycle arrest abrogation could be used to specifically sensitize tumor cells to DNA damaging agents [79,124].

1.6.3. Candidate targets for cell cycle abrogation

The ideal S or G_2 checkpoint abrogator needs to be selective and targeting a molecule not involved in the G_1 checkpoint [123]. Different candidate targets for cell cycle arrest abrogation have been investigated, for example inhibition of ATM/ATR, activation

Cdc25C phosphatase, inhibition of the molecular chaperone heat shock protein-90 (HSP-90) and inhibition of Chk1 [123].

ATM /ATR inhibitiors are not specific G_2 checkpoint abrogators since ATM and ATR activate pathways involved in cell cycle checkpoints, apoptosis and DNA repair. However, ATM/ATR inhibition has been shown to disrupt the G_2 checkpoint, inducing damaged cells to undergo aberrant mitosis [123,126].

An alternative method of G_2 abrogation is to activate Cdc25C, resulting in de-phosphorylation and activation of Cyclin B/Cdc2 and subsequent cell cycle progression to mitosis. This can be achieved either through direct activation of Cdc25 or by inhibition of WEE1, a protein that opposes Cdc25 activity. The WEE1 inhibitor PD0166285 has demonstrated G_2 checkpoint abrogation in preclinical models [123,127].

An indirect and nonspecific method of checkpoint abrogation is provided by inhibition of HSP-90. In preclinical studies, the HSP-90 inhibitor 17-AAG has been shown to abrogate G_2/M arrest when combined with SN38 in p53-deficient cells and when combined with irradiation in human lung cancer cells [123].

The most relevant approach to checkpoint abrogation is by inhibition of Chk1 kinase. Chk1 plays a crucial role in the S phase checkpoint, the G_2/M checkpoint as well as for mitotic spindle checkpoint function. In this manner, Chk1 inhibitors are capable of not only enhancing the efficacy of DNA damaging agents that cause S or G_2 arrest, but also potentiating antimitotic agents [79,123]. Recent research have implicated checkpoint pathway activation as a major mechanism driving both chemoresistance and radioresistance, indicating that the use of Chk1 inhibitors will not only potentiate the efficacy of chemotherapy, but may also reduce drug resistance [40,79,128,129]. Based on the hypothesis that cells with defective G_1 checkpoint can be sensitized to chemotherapy by abrogating G_2 arrest, it is natural to believe that Chk1 inhibitors would preferentially enhance the cytotoxicity of DNA-damaging agents in cells with defective p53. Nonetheless, there are studies demonstrating that Chk1 inhibition also sensitizes p53-proficient cells, but the effects might be more pronounced in p53 mutant cell lines

[124,130-134]. The difference might in part relate to the different treatments and treatment schedules [135].

Based on the crosstalk between the Chk1 and Chk2 pathways, it has been claimed that it may be beneficial to target both Chk1 and Chk2 simultaneously. However, it has been shown that Chk1 is the only relevant checkpoint kinase as a cancer drug target and inhibition of other checkpoint kinases in addition to Chk1 has no benefit over inhibition of Chk1 alone [79,80,125]. Over the past decade many approaches to inhibit Chk1 have been made, for example by using small-molecule inhibitors and interference RNA, and several pharmaceutical compounds that target Chk1 are currently in clinical trials [79].

1.6.4. The Chk1 inhibitor UCN-01

The effects of inhibition of checkpoint pathways were first discovered in early work with caffeine, a nonspecific inhibitor of ATR and ATM, and with UCN-01 (7-Hydroxystaurosporine), an inhibitor of Chk1. It was shown that these agents could abrogate DNA damage-induced G_2 arrest and potentiate the cytotoxicity of DNA damaging agents [123,135-137]. UCN-01 has been shown to sensitize tumor cells to many agents, including, IR, cisplatin, camptothecin, gemcitabine and 5-fluoruoracil [24,138]. During phase I trials, the clinical utility of UCN-01 was found to be limited by its avid binding to human plasma proteins resulting in an unusually long half-life, however, the studies of UCN-01 collectively promoted Chk1 as a useful therapeutic target to induce enhanced cytotoxicity in tumor cells in response to DNA damaging agents [79,122,123,136,137].

1.6.5. The Chk1 inhibitor SB218078

The indolocarbazole SB218078 (**Figure 7A**), a molecule related UCN-01, was demonstrated as an inhibitor of Chk1, which potently abrogated Chk1-induced phosphorylation of Cdc25C (IC_{50} = 15 nM). SB218078 also inhibited Cdc2 and PKC, but at approximately 16-and 60-fold higher drug concentrations, respectively (IC_{50} = 250 nM and 1000 nM). This compound abrogates both camptothecin- and radiation-mediated cell cycle arrest and potentiates the cytotoxicity of camptothecin and topotecan in HeLa and HT-29 cells [122,137,139].

1.6.6. The Chk1 inhibitor AZD7762

AZD7762 (**Figure 7B**) is an ATP competitive Chk1/Chk2 inhibitor, currently in phase I clinical trials, that abrogates phosphorylation of a Cdc25C with an IC_{50} of 5 nmol/L [122,123,134]. A 1000-fold selectivity for Chk1 over Cdc2 has been shown. *In vitro*, treatment with AZD7762 resulted in abrogation of G_2 arrest induced by camptothecin or gemcitabine and a reduction in the concentration of DNA damaging agents required to inhibit cell growth of several different cancer cell lines. In mouse and rat xenograft models, AZD7762 potentiated both the efficacy of gemcitabine and irinotecan, causing tumor growth delays [122,123,134]. It has also been shown that AZD7762 sensitizes human tumor cells to radiation both *in vitro* and *in vivo* [130,131].

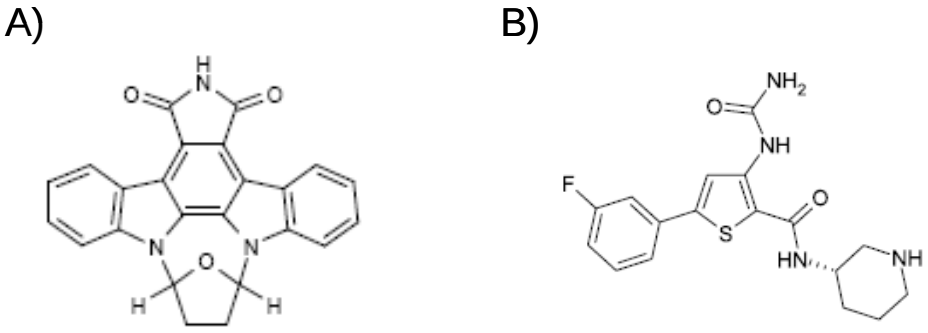


Figure 7. Chk1 inhibitors. Structures of the Chk1 inhibitors SB218078 (**A**) and AZD7762 (**B**) [134,139].

1.6.7. The Chk1 inhibitors XL-844 and PF-477736

Two other promising Chk1 inhibitors that have entered clinical trials are XL-844 and PF-477736. XL844 is a specific inhibitor of both Chk1 (IC_{50} = 2.2 nM) and Chk2 (IC_{50} = 0.2 nM) [122,123]. *In vitro* studies have shown potentiation of gemcitabine by abrogation of S phase arrest in multiple solid tumor cell lines and it has also been demonstrated to potentiate the effects of gemcitabine *in vivo* [109,122,123]. PF-477736 inhibits Chk1 with a 100-fold selectivity over Chk2 [122,123,140]. *In vitro*, PF-477736 induced checkpoint abrogation and potentiated the activity of a number of DNA-damaging agents, including gemcitabine, irinotecan and carboplatin, across several cell lines, with selectivity for p53-deficient cancer cell lines compared to p53-competent cells [122,123]. *In vivo*, PF-477736 enhanced the activity of gemcitabine and irinotecan in colon cancer xenograft models [123].

1.6.7. Validation of Chk1 inhibiton

It is necessary to be able to validate the inhibition of Chk1 in clinical studies and there are several possible markers for this purpose. Firstly, Cdc25C can be considered as marker for Chk1 inhibition, since Chk1 is known to phosphorylate Cdc25C following DNA damage and inhibition would cause a negative regulation. Secondly, an indirect but simpler way to evaluate Chk1 inhibition is to measure the extent of G_2-M checkpoint abrogation by using an antibody against phosphorylated histone H3, a marker of mitotic entry. Finally, activation of the DNA damage response as assayed by increased γ-H2AX levels compared to levels from chemotherapy alone can also be used as a downstream pharmacodynamic marker for Chk1 inhibition [123,135].

2. Aims

Despite the recent progress in molecularly targeted cancer therapies, advanced solid malignancies remain a therapeutic challenge, in part due to the development of resistance to radiation and chemotherapy. The purpose of this project was to study the cellular and molecular mechanisms of drug resistance in lung cancer tumors and convert it into the development of innovative therapies and clinical application for fighting lung cancer.

Identification of CSCs as an undifferentiated subpopulation of tumorigenic cells responsible for tumor maintenance, growth and spreading has become relevant for several human malignancies. To investigate the possibility that CSCs may represent the source of the drug-resistant subpopulation in lung tumors, our studies were specifically focused on the comparison of NSCLC-CSCs with their differentiated progenies to evaluate differences in response to chemotherapy and activation/regulation of the DNA damage response pathway.

The DNA damage response prevents cell cycle transition through surveillance mechanisms known as cell cycle checkpoints. Agents used for cancer treatment, such as cytotoxic chemotherapy and ionizing radiation (IR), also activate cell cycle checkpoints. Understanding how checkpoints are regulated is therefore important from the points of view of both tumorigenesis and cancer treatment.

We aimed to study the molecular hierarchy of the checkpoint signaling network in NSCLC-CSCs, especially the role of Chk1 in chemotherapy resistance and cancer therapy. Our approach was to treat NSCLC-CSCs with chemotherapy commonly used for lung cancer treatment in combination with chemical Chk1 inhibitors and thereafter evaluate the effects on DNA damage and reparation of damaged DNA, study changes in cell cycle profile and cell viability, characterize responsible molecular mechanisms and possible induction of cell death, and finally, to study the potential therapeutic effects of Chk1 inhibition on cancer progress *in vivo*.

3. Materials and Methods

3.1. Materials

3.1.1. Reagents

The following reagents were used:

- cisplatin (Teva, Petach Tikva, Israel)
- gemcitabine (Lilly, Fiesole, Italy)
- paclitaxel (Sigma-Aldrich, St. Louis, MO, USA)
- SB218078 (Calbiochem, Nottingham, UK)
- AZD7762 (Axon Medchem, Groningen, The Netherlands)

3.1.2. Antibodies

The following antibodies were used:

- Chk1 (Cell Signaling Technology, Danvers, MA, USA)
- phosphorylated Chk1 (Ser345) (Cell Signaling Technology)
- Chk2 (Cell Signaling Technology)
- phosphorylated Chk2 (Thr68) (Cell Signaling Technology)
- phosphorylated Cdc25C (Ser216) (Cell Signaling Technology)
- phosphorylated Cdc2 (Tyr15) (Cell Signaling Technology)
- ATM (5C2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
- phosphorylated ATM (0H11.E12) (Santa Cruz Biotechnology)
- Cyclin B1 (clone D-11) (Santa Cruz Biotechnology)
- phosphorylated H2A.X (Ser139) (Upstate-Millipore, Billerica, MA, USA)
- Caspase-2 (clone 35) (Upstate-Millipore)
- Caspase-3 (Upstate-Millipore)
- β-actin (AC-15) (Sigma-Aldrich)
- β-tubulin (TUB 2.1) (Sigma-Aldrich)
- anti-HLA class I (eBioscience, San Diego, CA, USA)
- PE-Cy5 anti-mouse CD45 (BD Pharmingen, San Diego, CA, USA)
- Cytokeratins 8/18 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK)

3.1.3. NSCLC-CSCs

NSCLC-CSCs from human adenocarcinoma (NSCLC-CSC #1 and NSCLC-CSC #4), human squamous cell carcinoma (NSCLC-CSC #2 and NSCLC-CSC #3) and human large cell neuroendocrine carcinoma (NSCLC-CSC #5), were obtained from patients who underwent surgical resection of lung tumors [7].

3.2. Methods

3.2.1. Isolation and culture of NSCLC-CSCs

Surgically resected lung tumors were washed several times and left overnight in DMEM:F12 medium supplemented with 500 Units/mL penicillin, 500 μg/mL streptomycin and 5 μg/mL amphotericin B. Tissue dissociation was carried out by enzymatic digestion with 1.5 mg/mL collagenase II (Gibco-Invitrogen, Carlsbad, CA, USA) and 20 μg/ml DNase I (Roche, Basilea, SW, USA), agitating for 2h at 37˚C. In the presence of high quantity of blood cells, hypotonic lysis with ammonium chloride was performed. Recovered cells were cultured at clonal density in a growth medium specifically adapted for NSCLC-CSCs. The complete serum-free medium (SFM) for NSCLC-CSCs consists of:

- 50 μg/mL insulin (Sigma-Aldrich)
- 100 μg/mL apo-transferrin (Sigma-Aldrich)
- 10 μg/mL putrescine (Sigma-Aldrich)
- 0.03 mM sodium selenite (Sigma-Aldrich)
- 2 μM progesterone (Sigma-Aldrich)
- 0.6% glucose (Sigma-Aldrich)
- 5 mM HEPES (Sigma-Aldrich)
- 0.1% sodium bicarbonate (Sigma-Aldrich)
- 0.4% BSA (ICN Biochemicals, Costa Mesa, CA, USA)
- 2 mM L-glutamine (PAA Laboratories GmbH, Pasching, Austria)
- 100 Units/mL penicillin (PAA Laboratories GmbH)
- 100 μg/mL streptomycin (PAA Laboratories GmbH)

All these reagents were dissolved in DMEM:F12 medium (Gibco-Invitrogen) and supplemented with 20 μg/mL epidermal growth factor (EGF) and 10 μg/mL basic

fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ, USA). Non-treated, sterile polystyrene flasks for suspension cell cultures (Nunc, Thermo Fischer Scientific, Rochester, NY, USA) were used to reduce cell adherence and support growth as undifferentiated tumor spheres. The medium was replaced or supplemented with fresh growth factors (20 μg/mL EGF and 10 μg/mL bFGF) twice a week, until cells started to grow as floating aggregates. Cultures were expanded by mechanical dissociation of spheres, followed by re-plating of single cells and residual small aggregates in complete fresh SFM.

3.2.2. Differentiation of NSCLC-CSCs

To obtain differentiation of NSCLC-CSCs, spheres were dissociated to obtain single cells and plated in DMEM (Gibco-Invitrogen) supplemented with 10% FBS for 24h, to allow cell attachment. DMEM was then replaced with Bronchial Epithelial Cell Growth medium (Lonza, Basel, Switzerland) for 72h to facilitate the progress of differentiation. Differentiated cells could thereafter be maintained in DMEM supplemented with 10% FBS for several weeks.

3.2.3. Treatment

For *in vitro* experiments, the following concentrations of chemotherapeutic drugs and Chk1 inhibitors were used:

- 5 ug/mL cisplatin
- 250 μM gemcitabine
- 30 ng/mL paclitaxel
- 20 nM SB218078
- 5 nM AZD7762

For *in vivo* studies we used:

- 60 mg/kg gemcitabine
- 3 mg/kg cisplatin
- 10 mg/kg AZD7762

Chk1 inhibitors for both *in vitro* and *in vivo* studies were added 8h after chemotherapy.

3.2.4. Cell viability assays

For chemoresistance comparison and cell viability studies, dissociated spheres and adherent differentiated cells were plated in 96 well plates at 5,000 cells/well in growth medium supplemented with cisplatin, gemcitabine or paclitaxel, for 96h. For cell viability studies, dissociated NSCLC-CSCs were seeded and treated as described above and in combination with Chk1 inhibitors. Cell viability was evaluated after 4 days of treatment by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide) assay (Promega, Madison, WI, USA) or CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to standard protocols and analyzed by a Victor 2 plate reader (Wallac, Turku, Finland).

3.2.5. Cell proliferation assays

A total of 75,000 dissociated NSCLC-CSCs were treated with cisplatin, gemcitabine or paclitaxel for 6 days. On day 3, cells were collected, counted and replated in the presence of chemotherapy. On day 6, cells were collected, counted and thereafter replated in fresh medium without chemotherapy. Cells were then counted and replated every 3 days until day 15, to study the effects of chemotherapy withdrawal. Viable cells were counted using trypan blue exclusion.

3.2.6. Cell cycle analysis

Cells were dissociated and treated with cisplatin, gemcitabine or paclitaxel for 48h. Thereafter, cells were dissociated with trypsin and stained with a PI staining solution (0.1% trisodium citrate, 9.65 mM NaCl, 0.3% NP40, 50 μg/mL PI, 200 μg/mL RNase A) for 30 minutes at room temperature. Cell cycle profile was acquired with a Facs Canto flow cytometer (Becton Dickinson, NJ, USA) and analyzed with FlowJo software (Tree Star Inc. www.FlowJo.com).

3.2.7. Immunofluorescence

NSCLC-CSCs were treated with cisplatin, paclitaxel, SB218078, AZD7762 or in combinations of drug and inhibitor for 48h or 96h. For CKs 8/18 staining, NSCLC-CSC spheres were fixed with 2% paraformaldehyde, attached to poly-L-lysine coated coverslips by sedimentation and permeabilized with 0.5% Triton X-100/PBS for 4h at 4°C. Differentiated progenies were cultured on coverslips to allow attachment, fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100/PBS for 2h at

4°C. Thereafter, slides were incubated with a FITC-conjugated CKs 8/18 antibody (Novocastra Laboratories Ltd) over night at 4°C. For anti-Cyclin B1 and anti-γ-H2A.X staining, treated NSCLC-CSCs were cyto-spun onto glass slides, fixed with 2% paraformaldehyde and then permeabilized with 0.1% Triton X-100/PBS for 1h at 37°C before incubation with Cyclin B1 (Santa Cruz Biotechnology) or γ-H2A.X (Ser139) (Upstate-Millipore) overnight at 4°C. Alexa Fluor 555 goat anti-mouse (1 μg/mL, Invitrogen) for 1h at RT was used as secondary antibody. TO-PRO-3 (4 μM, Invitrogen, Carlsbad, CA, USA) or DAPI (3 μM, Molecular Probes/Invitrogen) were used to visualize nuclei and Phalloidin-Alexa Fluor 488 (5 Units/mL, Molecular Probes/Invitrogen) was used to visualize cell membranes. Slides were analyzed using OLYMPUS FV-1000 confocal microscope with the Olympus objective Ultraplan Apochromatic 60X N.A.1.35 and the software Olympus Fluoview.

3.2.8. Western blot

NSCLC-CSCs were treated for 6h, 12h, 24h or 96h with cisplatin, gemcitabine, paclitaxel, SB218078, AZD7762 or in combinations of drug and Chk1 inhibitor. Whole cell lysates from treated cells were prepared in lysis buffer (NP40 1%, 20 mM TRIS (pH 7.2), 200 mM NaCl, Phosphatase Inhibitor Cocktail 1 (used 1:100, P2850, Sigma-Aldrich), Phosphatase Inhibitor Cocktail 2 (used 1:100, P5726, Sigma-Aldrich) and Protease Inhibitor Cocktail (used 1:100, P8340, Sigma-Aldrich)). 20 μg of whole cell extracts were subjected to 8-15% sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred onto Hybond-C membrane (Amersham Biosciences, Milan, Italy) and incubated with primary antibody over night at 4°C. Membranes were probed with secondary antibody for 1h at room temperature and detected using enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

3.2.9. Propidium iodide / Annexin V staining

NSCLC-CSCs were treated with cisplatin and SB218078 for 96h followed by 72h of cell culture in fresh medium. A total of 50,000 treated cells were washed with Annexin V-binding buffer (2.5 mM $CaCl_2$, 140 mM NaCl, 10 mM Hepes) and thereafter incubated with Annexin V Alexa Fluor 647 (1.25 nM, Invitrogen) for 15 minutes at room temperature. Cells were again washed with Annexin V-binding buffer, re-suspended in buffer containing 5 μg/mL PI and analysed by Facs Canto flow cytometer (Becton Dickinson).

3.2.10. May-Grünwald-Giemsa staining

NSCLC-CSCs were treated with cisplatin and SB218078 for 96h followed by 72h of cell culture in fresh medium. Treated cells were washed and cyto-spun onto glass slides. Slides were stained with concentrated giemsa staining (Sigma-Aldrich) for 30s and then with diluted giemsa staining (1:20) for 30 minutes.

3.2.11. Soft agar colony forming assays

Soft agar colony forming assays were carried out for dissociated NSCLC-CSCs treated with cisplatin or paclitaxel alone or in combinations with SB218078 or AZD7762 for 96h. Thereafter, cells were washed and 500 single cells were plated in the top agar layer in each well of a 24-well culture plate with 0.3% top agar layer and 0.4% bottom agar layer (SeaPlaque Agarose, Cambrex, New Jersey, USA). Cultures were incubated at 37°C for 20 days. Colonies from triplicate wells were stained with crystal violet (0.01% in 10% MetOH), visualized and counted under microscope and photographed with a Nikon D80 camera. For xenograft-derived cells, tumors were aseptically removed and dissociated. Recovered cells were extensively washed and plated in SFM for 3 days. Consequently, 500 cells for each treatment were plated in soft agar as described above.

3.2.12. *In vivo* studies

Female NOD-SCID mice were purchased from Charles River Laboratories Italia (Calco, LC, Italy). All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and within the protocols approved by the Istituto Superiore di Sanità. NSCLC-CSCs were dissociated, counted and resuspended in a mix of PBS and Matrigel (1:1). 50,000 NSCLC-CSCs were implanted subcutaneously into the right flank of each mouse in a volume of 0.1 to 0.2mL using a 25-gauge needle. Tumors were allowed to grow to the size of 100 to 200 mm^3 before the administration of compounds. Animals, 5 in each group, were dosed intraperitoneally with gemcitabine (60 mg/kg) or cisplatin (3 mg/kg) and intravenously with AZD7762 (10 mg/kg) every 3 days starting from day 0. Tumor growth was evaluated with an electronic caliper before every administration and volume was estimated by using the following formula: $a * b^2 / 2$, where a and b represents the tumor length and width (in mm), respectively. After 30 days, levels of human carcinoembryonic antigen (CEA) were measured in serum obtained by retro-orbital withdrawal by immunoluminometric technique using Vitros ECI analyzer (Ortho-

Clinical Diagnostics Inc. Rochester, NY, USA). Tumors were subsequently removed and weighed using a PL202-L Precision Balance (Mettler-Toledo, Novate Milanese MI, Italy). Immunohistochemistry was performed on formalin fixed paraffin-embedded tissue or frozen tissue. Paraffin sections (5 μm) were dewaxed in xylene and re-hydrated with distilled water. The slides were subsequently incubated with anti-γ-H2A.X (Upstate-Millipore). The reaction was performed using Elite Vector Stain ABC systems (Vector Laboratories) and DAB substrate chromogen (DakoCytomation), followed by counterstaining with hematoxylin. Human origin of the tumor xenografts was confirmed by FACS analysis with a PE conjugated anti-HLA class I antibody (eBioscience) and a PE-Cy5 anti-mouse CD45 antibody (BD Pharmingen) was used to exclude unspecific staining of mouse cells .

3.2.13. Statistical analysis

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., www.graphpad.com). The statistical significance of the results shown in Fig. 9A, 9B and 9C was evaluated by two-way ANOVA and Bonferroni's post-tests. The statistical significance of the results shown in Fig. 12C and 17H was evaluated by one-way ANOVA with Bonferroni's multiple comparison test while results in Fig. 14B, 16B, 17B, 17D and 17E were evaluated by repeated measured one-way ANOVA with Bonferroni's multiple comparison test. A P value <0.05 is represented by a single asterisk, a P value <0.01 is represented by a double asterisk, while three asterisks indicate $P<0.001$, all P values are two-sided.

4. Results

4.1. Isolation, culture and differentiation of NSCLC-CSCs

NSCLC-CSC clones were obtained from patients who underwent surgical resection of adenocarcinoma (NSCLC-CSC #1 and NSCLC-CSC #4), squamous cell carcinoma (NSCLC-CSC #2 and NSCLC-CSC #3) or large cell neuroendocrine carcinoma (NSCLC-CSC #5) and has been previously described by Eramo and colleagues [7]. After dissociation of tumor tissue, recovered cells could be unlimitedly propagated and maintained *in vitro* as three-dimensional, non-adherent tumor spheres/spheroids (**Figure 8A, left panel**) in serum-free medium containing EGF and bFGF.

Similarly to normal stem cells, CSCs possess the capacity to differentiate into progeny cancer cells. NSCLC-CSCs were induced to differentiate in medium supplemented with FBS as described in Materials and Methods, and upon differentiation, cells immediately lost the capability of forming spheres and started to grow as attached in a monolayer (**Figure 8A, right panel**).

To evaluate the *in vitro* differentiation status of progeny cells grown in serum-supplemented medium compared to undifferentiated NSCLC-CSCs, we performed immunofluorescence staining for cytokeratins 8/18 (CKs 8/18), which are acquired during epithelial cell differentiation [7]. The cells were counterstained with fluorescein-phalloidin, which binds to actin filaments in the cell membrane and DAPI that specifically stains nuclei. As shown in **Figure 8B**, there are considerably higher levels of CKs 8/18 in the differentiated counterparts than in NSCLC-CSCs.

Figure 8

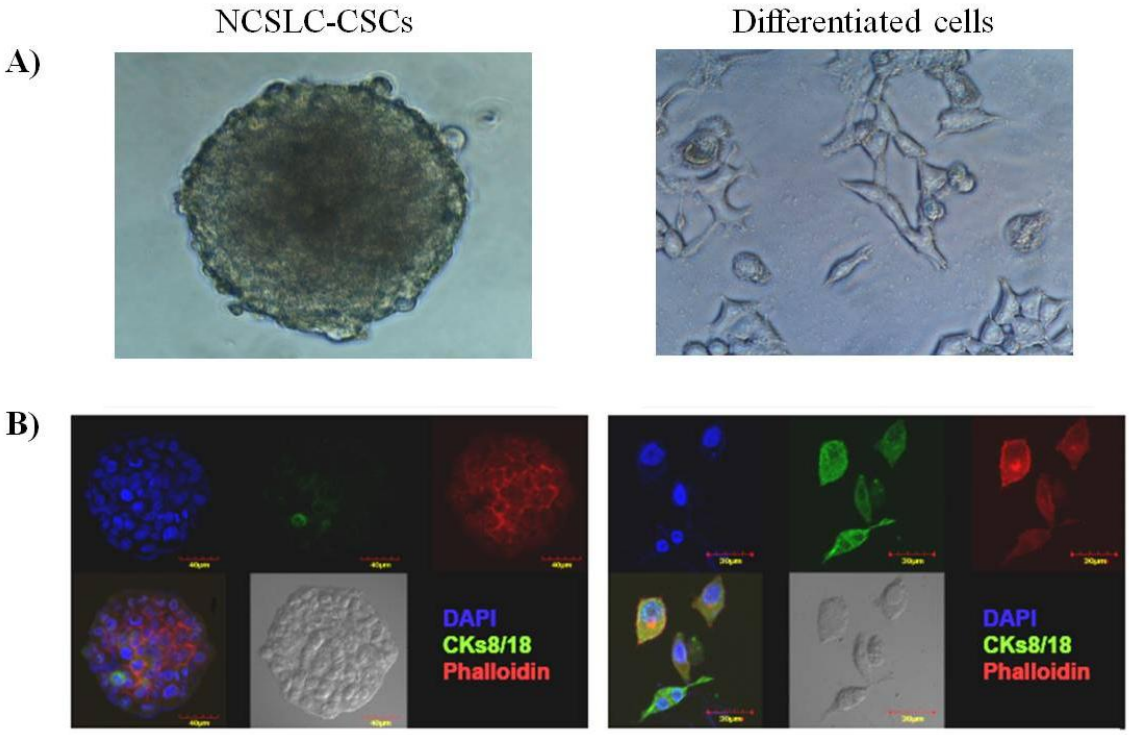


Figure 8. Characterization of NSCLC-CSCs and their differentiated progenies. A. Morphology of a LCSC sphere and differentiated progeny of NSCLC-CSC #1. **B**. Immunofluorescence was performed to evaluate the *in vitro* differentiation potential of NSCLC-CSCs. NSCLC-CSC spheres (left) and their differentiated progenies (right) were stained for CKs 8/18. Phalloidin and DAPI were used to visualize cell membranes and nuclei. Slides were analyzed with confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 40x.

4.2. NSCLC-CSCs are resistant to chemotherapy and efficiently repair chemotherapy-induced DNA damage

In spite of the variety of therapeutic approaches, lung cancer is the most common cause of cancer related mortality worldwide [8], which could be due to the fact that CSCs are largely resistant, *in vitro* and *in vivo*, to conventional chemotherapy [7,141]. To understand the basis of resistance to chemotherapy in NSCLC, we investigated the effects of commonly used chemotherapeutic agents on our five primary cultures of NSCLC-CSCs and compared them with their differentiated counterparts. Cisplatin, gemcitabine and paclitaxel were used at doses comparable with the higher plasma levels reached in treated lung cancer patients.

We observed that, unlike in the differentiated progenies, neither of the drugs induced noteworthy cell death in NSCLC-CSCs even after a long exposure (**Figure 9A**). The different sensitivity to anticancer agents of these two cell populations might account for the inability of current therapies to eradicate lung tumors. Many current cancer treatments target the most rapidly dividing differentiated cells, which represent the majority of the tumor bulk, resulting in a remarkable, but temporary tumor reduction.

Chemotherapy almost entirely interrupted proliferation and induced a transient growth arrest of NSCLC-CSCs that lasted until drug removal. Removal of chemotherapy induced cell proliferation with a profile similar to that of untreated cells, suggesting the ability of NSCLC-CSCs to recover after withdrawal of chemotherapy treatment (**Figure 9B**). In line with these observations, analysis of cell cycle profile in treated cells, revealed accumulation of NSCLC-CSCs at either S or G_2 phase depending on the anti-neoplastic agent used (**Figure 9C**). Specifically, the number of cells in G_2 significantly increased after cisplatin and paclitaxel treatment, whereas gemcitabine caused a significant accumulation in S phase.

Figure 9

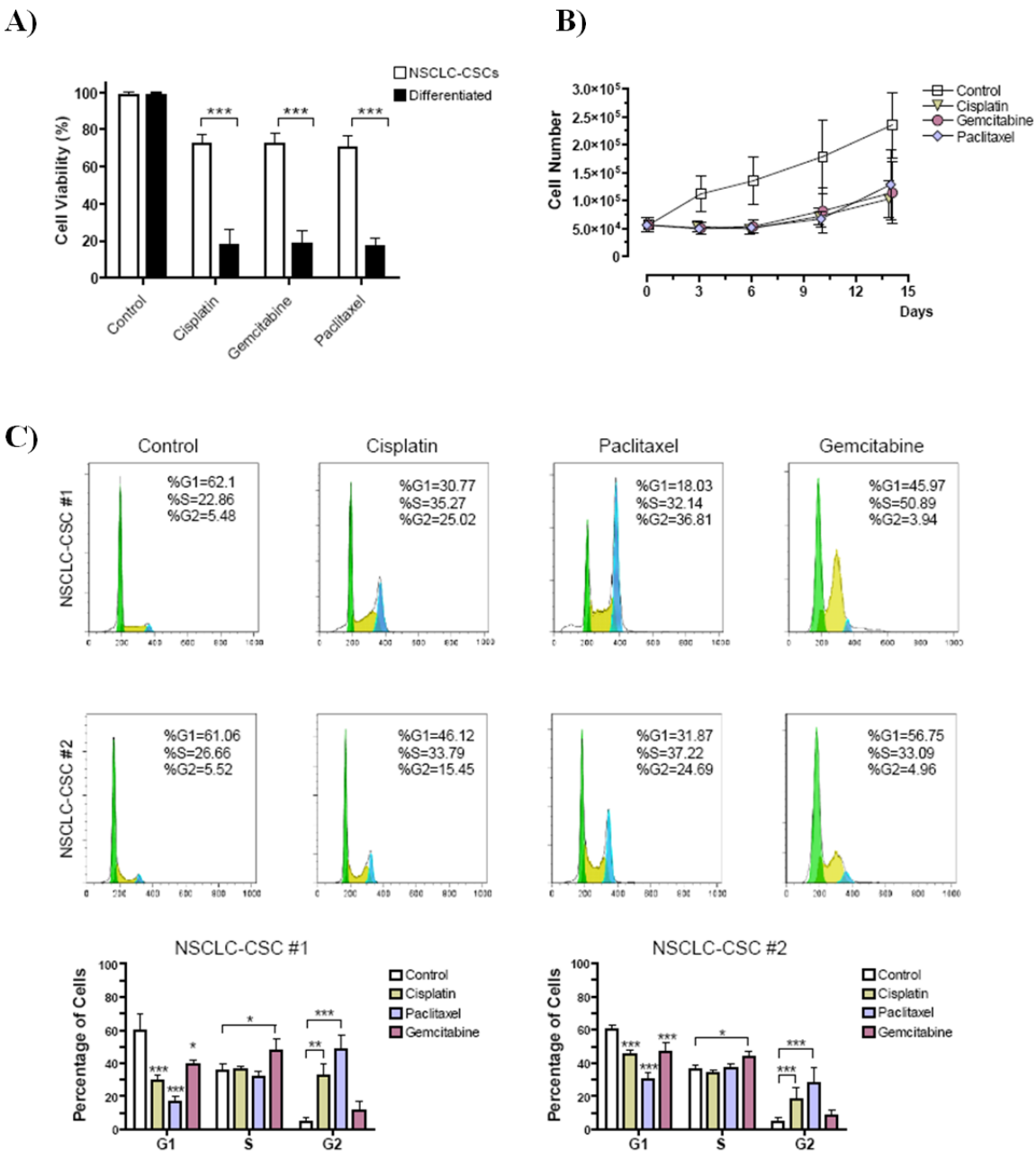


Figure 9. NSCLC-CSCs are resistant to conventional chemotherapy. A. Cell viability of five different NSCLC-CSC clones and differentiated progenies after 96h of chemotherapeutical treatment, measured by MTT assay. Data shown are mean value ± SD of three independent experiments. **B.** Proliferation of NSCLC-CSCs in the presence and after withdrawal of chemotherapy was evaluated as described in Materials and Methods. Briefly, cells were seeded at 50,000/ml and treated with chemotherapy for a total period of 6 days. On day 6, cells were washed and plated in fresh medium to study the effects after withdrawal of chemotherapy. Data are the results of three independent experiments performed on three different NSCLC-CSC lines **C.** Cell cycle profiles of control and treated NSCLC-CSCs after 48h of the indicated treatment (upper panel). The lower panel shows the distribution of cells between the different phases of the cell cycle. Three independent experiments ± SD is shown.

Cell cycle arrest may follow DNA damage and checkpoint activation and it is well known that many conventional anticancer treatments at least partly damage the DNA of cells. In response to DNA damage, DDR is activated and cells are arrested at cell cycle checkpoints to allow DNA to be repaired. We investigated whether exposure to cisplatin, gemcitabine or paclitaxel would induce damage in NSCLC-CSCs and thereby activate the DNA damage machinery. One of the earliest modifications of the chromatin structure in the damage response is phosphorylation of H2A.X at Serine 139 (γ-H2A.X), indicating the presence of DNA DSBs [88]. Both western blot and immunofluoresence staining demonstrate that a short exposure (6h) to either drug resulted in a considerable increase in γ-H2A.X (**Figure 10A and B**). However, the persistence of γ-H2A.X is not detectable or only slightly evident after 96h (**Figure 10A and B)**, suggesting that NSCLC-CSCs are able to efficiently repair the DNA damage induced by chemotherapy.

As expected, only a very small subset of the differentiated cells survived 96h of chemotherapeutical treatment. These cells displayed severely damaged DNA, demonstrated by an extensive γ-H2A.X expression (**Figure 10C**).

We concluded that, following DNA damage induced by chemotherapeutical treatment, cell cycle arrest allows repair of DNA and thereby support cell survival of NSCLC-CSCs.

Figure 10

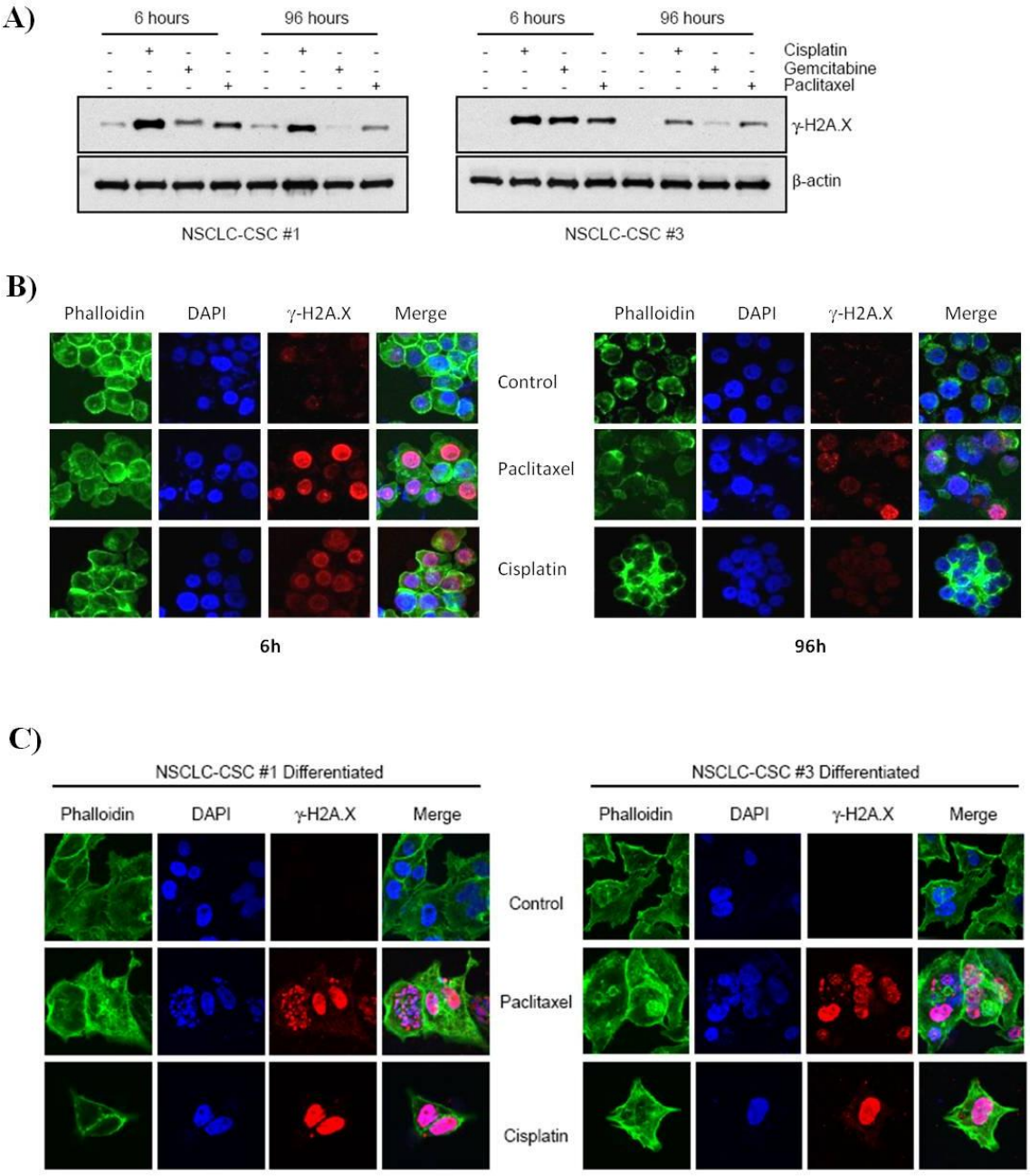


Figure 10. NSCLC-CSCs efficiently repair DNA damage. A. Western blot was used to evaluate chemotherapy-induced DNA damage and subsequent activation of H2A.X (γ-H2A.X) after 6h and 96h of treatment. β-actin served as control. **B and C.** Immunofluorescence for γ-H2A.X for NSCLC-CSC #1 treated as indicated for 6h or 96h (**B**) and for differentiated NSCLC-CSC #1 and #3 treated as indicated for 96h (**C**). DAPI and phalloidin were used to visualize nuclei and membranes. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 60x.

4.3. NSCLC-CSCs preferentially activate Chk1

Cell cycle checkpoints are controlled by the ATM and ATR sensor kinases that once activated, phosphorylate downstream effector proteins, such as Chk1 and Chk2, to initiate cell cycle checkpoints [79,81,83,84,86,98]. The activation of these checkpoints stimulates repair of DNA damage before it becomes replicated and passed on to daughter cells and therefore preserves the genomic integrity. As expected, short treatment (6h) of NSCLC-CSCs with either cisplatin, gemcitabine or paclitaxel, promptly induced phosphorylation of ATM, followed by a strong activation of Chk1 (Serine 345), but not Chk2 (**Figure 11A**). After 96h of treatment, Chk1 phosphorylation is still persistant and we could also observe phosphorylation of Chk2 (Threonine 68), but only after cisplatin and gemcitabine treatment (**Figure 11B**). These data indicate that cell cycle arrest induced by chemotherapy-damaged DNA is dominantly regulated by Chk1, but might at a later stage also involve Chk2.

It has been reported previously that CSCs have a highly active DNA damage pathway, with a greater checkpoint activation in response to DNA damage compared to bulk tumor cells [40]. We therefore compared the activation of Chk1 between NSCLC-CSCs and differentiated progeny. Interestingly, both basal and activation levels of Chk1 in NSCLC-CSCs were elevated compared to their differentiated counterparts, indicating not only an active DDR pathway, but also suggesting that NSCLC-CSCs can efficiently counteract DNA damage by Chk1 and Chk2 activation (**Figure 11C**).

Figure 11

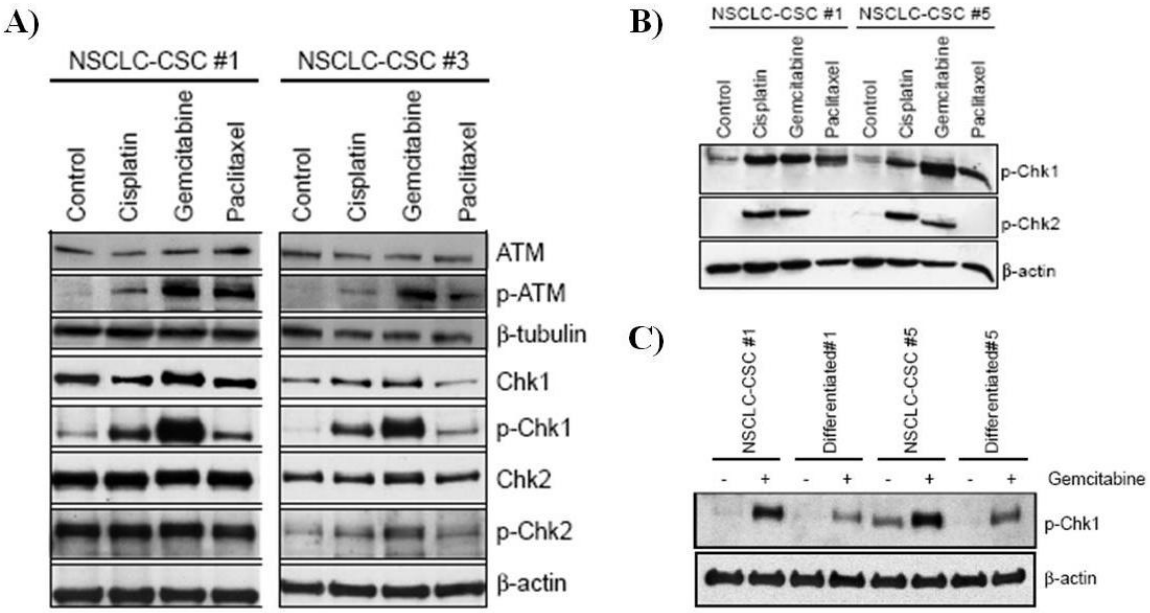


Figure 11. Chemotherapy activates the DNA damage response in NSCLC-CSCs. A and B. Activation of the DDR pathway was studied by western blot after 6h or 96h of treatment. Membranes were incubated with the indicated antibodies. **C.** Chk1 activation in NSCLC-CSC clones and differentiated progenies was evaluated by western blot after 24h exposure to gemcitabine. β-actin and β-tubulin antibodies were used to assess equal loading.

4.4. Chk1 inhibitors prevent DNA repair and increase the cytotoxicity of chemotherapy in NSCLC-CSCs

It is known that Chk1 phosphorylates the family of Cdc25 phosphatases, which in turn inhibit Cdc2 activity, thus maintaining the highly regulated order of cell-cycle progression. Inhibition of Chk1 would lead to the improper activation of Cdc2, resulting in checkpoint abrogation and aberrant entry into mitosis. We hypothesized that checkpoint abrogation with the specific Chk1 inhibitors SB218078 and AZD7762 [134,139] could increase the cytotoxicity of DNA damaging agents for treatment of NSCLC-CSCs. Both inhibitors are functioning downstream of Chk1 and as demonstrated by western blot after 24h of treatment, they are both able to abrogate Chk1-mediated phosphorylation of Cdc25C (**Figure 12A**). Chk1 inhibitors were always added 8h after chemotherapy treatment, to first achieve induction of DNA damage and checkpoint activation and thereafter efficiently abrogate checkpoints by the use of SB218078 or AZD7762.

We previously showed that NSCLC-CSCs efficiently repair DNA damage caused by cisplatin, gemcitabine or paclitaxel. However, when treating cells with chemotherapy in combination with either of the Chk1 inhibitors, DNA damage could not be repaired, demonstrated by the persistence of γ-H2A.X after 96h of treatment (**Figure 12B and C**). This suggests that NSCLC-CSCs treated with chemotherapy alone are able to repair DNA damage and consequently survive, while in the presence of Chk1 inhibitor, NSCLC-CSCs lose the ability to repair the DNA and may be targeted more effectively by anti-cancer drugs.

Accordingly, while having little activity as single agents, Chk1 inhibitors strongly reduced cell viability *in vitro* by increasing the cytotoxic effect of gemcitabine, cisplatin and paclitaxel (**Figure 12D**). Differentiated progeny died after 96h exposure to chemotherapy independently of the presence of Chk1 inhibitors (data not shown). These results confirm that NSCLC-CSCs are more resistant to chemotherapy and have a more efficient DNA repair activity compared to their differentiated progenies.

Figure 12

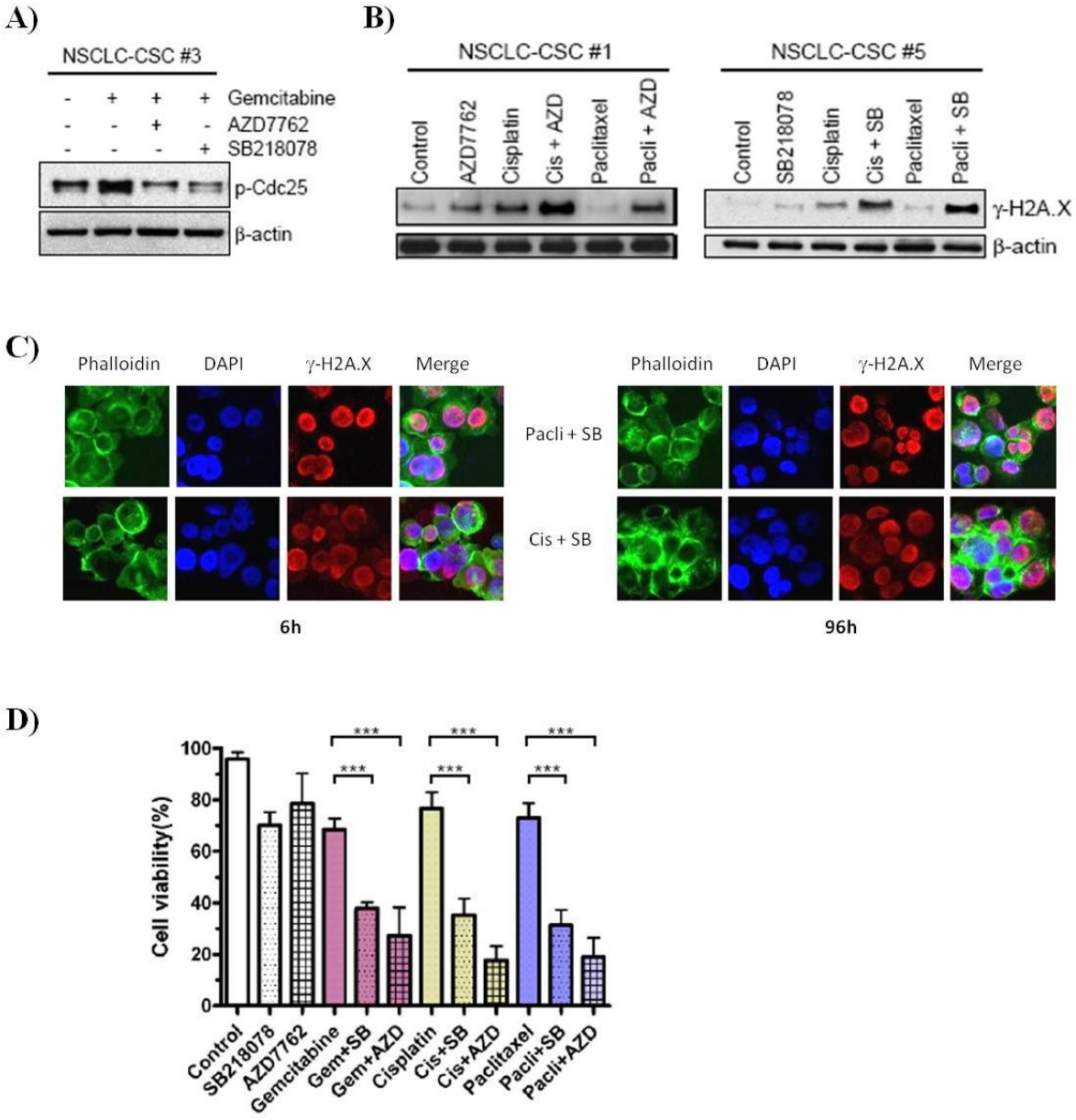


Figure 12. Chk1 inhibitors increase the cytotoxicity of chemotherapy in NSCLC-CSCs. A. Western blot for phosphorylated Cdc25C (p-Cdc25) after 24h of treatment with gemcitabine in combination with AZD7762 or SB218078. β-actin served as loading control. **B.** LCSCs treated for 96h as indicated were analyzed for γ-H2A.X expression by western blot with β-actin as loading control. **C.** Immunofluorescence for γ-H2A.X for NSCLC-CSC #1 treated for 6h or 96h as indicated. DAPI and phalloidin were used to visualize nuclei and membranes. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 60x. **D.** Cell viability of five different NSCLC-CSC clones after 96h treatment with chemotherapy alone or in combinations with Chk1 inhibitors, evaluated by MTT assay Mean value ± SD of three experiments is shown.

4.5. Chk1 inhibition induces premature activation of Cdc2/Cyclin B1

To obtain insight into the molecular mechanisms responsible for the increased DNA damage and cell death with the combination of chemotherapeutic drugs and Chk1 inhibitors, we analyzed the expression of Cdc2 and Cyclin B1, two cell-cycle regulatory proteins known to be controlled by Chk1. Cdc2 is a major target of Chk1 and is essential for both S and G_2/M phase transitions and it is kept in an inactivated state until the cell is ready for division. When Cdc2 forms a complex with Cyclin B1, a dividing cell is allowed to enter mitosis from G_2 phase. We found that inhibition of Chk1 after treatment with chemotherapy induced up-regulation of Cdc2 activity by de-phosphorylation and decreased expression of Cyclin B1 (**Figure 13A**), probably through nuclear translocation and subsequent degradation. These events lead to abrogation of the cell cycle arrest and aberrant mitotic entry before the completion of DNA repair.

We further investigated the role of Cyclin B1 in drug-induced cell cycle arrest in NSCLC-CSCs. Cyclin B1 accumulates in the cytoplasm through S and G_2 phases and translocates to the nucleus during prophase [142]. We observed that after 48h of cisplatin treatment, Cyclin B1 is prevalently located in the cytoplasm of NSCLC-CSCs, as a sign of cell cycle arrest. By contrast, in cells treated with both cisplatin and SB218078, Cyclin B1 translocated from the cytoplasm to the nucleus and forced cells to proceed through the cell cycle (**Figure 13B**).

Figure 13

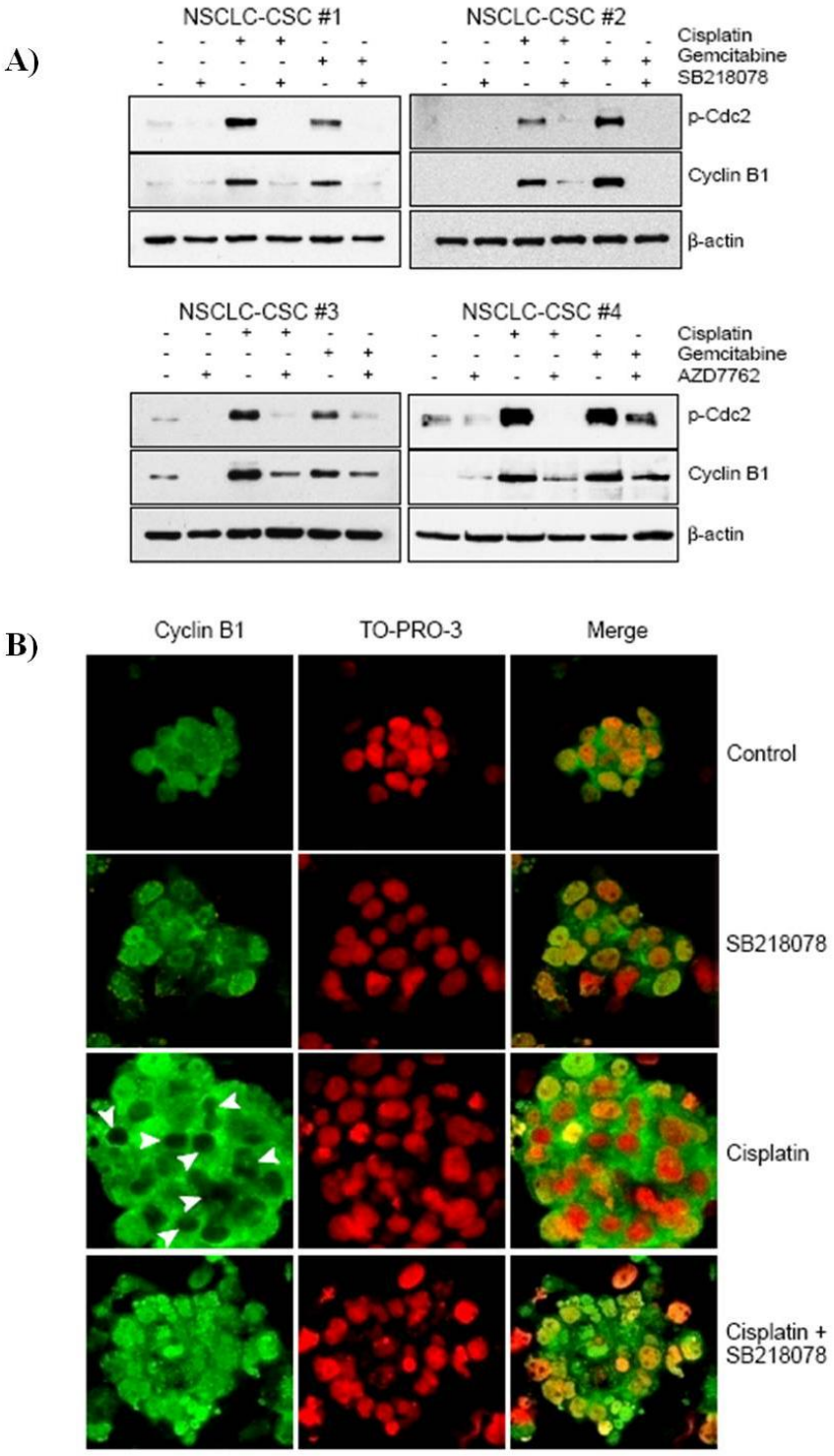


Figure 13. Chk1 inhibition induces premature Cdc2/Cyclin B1 activation. A. The cell-cycle regulatory proteins Cdc2 and Cyclin B1 were studied by western blot after 96h of treatment with chemotherapy and Chk1 inhibitor. β-actin was used to assess equal loading. **B.** Immunofluorescence staining with anti-cyclin B1 for NSCLC-CSC #1 treated with cisplatin and SB218078 for 96h. TO-PRO-3 was used to visualize nuclei. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 40x.

4.6. Mitotic catastrophe and cell death through apoptosis

The cytotoxic potential of DNA damaging agents used in anticancer therapy depends on their ability to induce growth arrest and to activate the cell death machinery. Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with or without the involvement of nucleases or proteases) and functional aspects (programmed or accidental, physiological or pathological) [115].

Apoptosis is acknowledged as the major barrier that must be circumvented by tumor cells to allow them to survive and proliferate under stressful conditions, such as chemotherapeutical treatment [143]. When apoptosis is induced by DNA damage, it is typically associated with activation of caspases, which in turn activate signaling cascades leading to biochemical and morphological apoptosis-specific changes such as cellular shrinkage, membrane blebbing and DNA fragmentation [112,114,144]. To evaluate if our NSCLC-CSCs died through apoptosis, we performed caspase assays and TUNEL staining, indicative of DNA fragmentation. Treatment with chemotherapeutic drugs and Chk1 inhibitors for 96h, did not induce either caspase activation TUNEL positive cells (data not shown).

In addition to apoptosis, tumors can be effectively eliminated following DNA damage by necrosis, mitotic catastrophe or autophagy. When we investigated the changes in cell morphology in our NSCLC-CSCs after treatment with chemotherapeutic drugs and Chk1 inhibitors, we observed a large number of giant multi-nucleated cells, suggesting that these cells were dying through mitotic catastrophe (**Figure 14A and B**). Cells dying from mitotic catastrophe are usually large, with formation of multiple micronuclei and cells might contain uncondensed chromosomes [113,114,120].

Figure 14

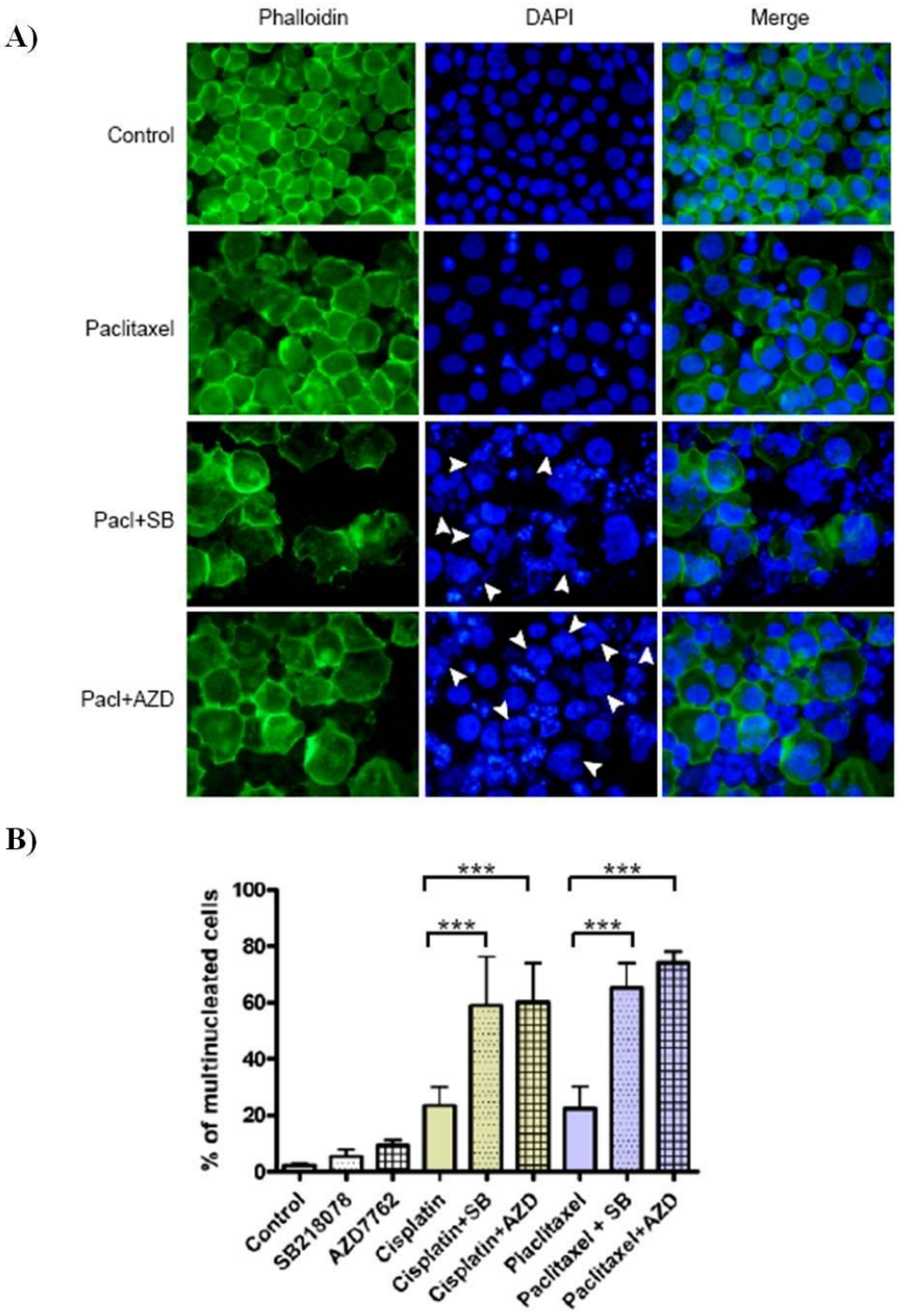


Figure 14. Chemotherapy in combination with Chk1 inhibitors induces mitotic catastrophe in NSCLC-CSCs. A. Immunofluorescence of NSCLC-CSCs treated with chemotherapy and Chk1 inhibitors for 96h. NSCLC-CSCs were stained with phalloidin and DAPI to visualize multinucleated cells and analyzed with confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 40x. One representative picture is shown. **B.** Percentage of multinucleated NSCLC-CSCs estimated by counting the nuclei in 100 representative cells on each slide. Mean value ± SD of three experiments is reported.

At present, there is unfortunately no universal definition to describe the characteristics mitotic catastrophe. In some models, mitotic catastrophe is thought to directly cause cell death that is distinct from apoptosis or, alternatively, mitotic catastrophe is associated with activation of apoptosis from the M phase [113,144]. Studies with other models, including ionizing radiation and anti-mitotic drugs, suggest that mitotic catastrophe is not a distinct form of cell death but rather an event that leads to cell death through apoptosis and/or necrosis [113,121,144].

We analyzed biochemical and morphological features of our cell model in the presence and in the absence of treatments, to study if our NSCLC-CSCs die through mitotic catastrophe or if they die through apoptosis/necrosis after going through aberrant mitosis. To distinguish between type of cell death and to investigate how severe the damage induced by the combination of chemotherapy and Chk1 inhibitor was, we performed rescue experiments (96h of treatment followed by an additional 72h in culture). Cells were stained with Annexin V and PI to distinguish early apoptotic cells (PI negative, FITC Annexin V positive) from necrotic cells (both FITC Annexin V and PI positive). We observed that the combination of cisplatin and SB218078 induced preferentially apoptosis in NSCLC-CSCs and damage was irreparable, since nearly 70% of the cells died even after withdrawal of treatment (**Figure 15A**). Giemsa staining allowed us to visualize morphological changes and although the morphological aspect of apoptosis may be incomplete, co-treated NSCLC-CSCs clearly show features associated with apoptosis (**Figure 15B**). To ascertain that the observed mitotic catastrophe eventually led to apoptosis, we examined the effects of Chk1 inhibition on the major apoptotic pathways. Caspase-3 is the common effector caspase for both the extrinsic/death receptor-induced apoptotic pathway and the intrinsic/mitochondria-triggered apoptotic pathway [36,118]. Caspase-2 is necessary for the onset of apoptosis triggered by several insults, including DNA damage and it has also been hypothesized that when Chk1 is inhibited, apoptosis induced by DNA DSBs becomes more dependent on caspase 2 [145,146]. The combination of cisplatin and SB218078 induced a clear activation of caspase-2 and caspase-3, confirming that cell death in NSCLC-CSCs treated in combination with Chk1 inhibitors finally occurs through apoptosis (**Figure 15C**).

Figure 15

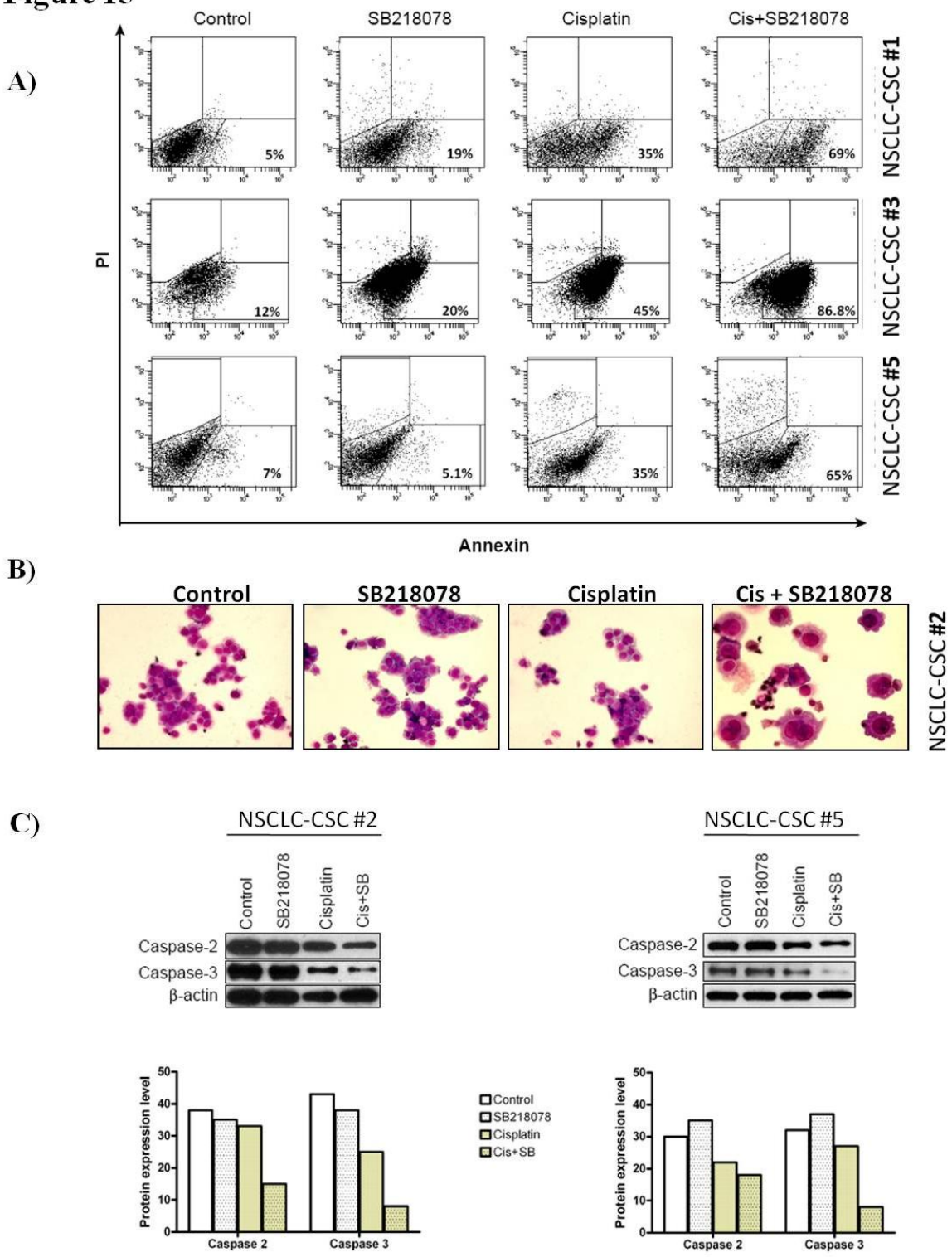


Figure 15. Mitotic catastrophe eventually leads to cell death through apoptosis. Rescue experiments. **A.** FACS analysis of PI and Annexin V Alexa Fluor 647 staining. **B.** Apoptotic cells visualized with Giemsa staining. **C.** Western blot with antibodies specific for the inactive proforms of caspase-2 and caspase-3. A β-actin antibody was used to assess equal loading. The graphs show actin-normalized levels of the proforms of caspase-2 and caspase-3.

4.7. Long term *in vitro* impact of Chk1 inhibition on NSCLC-CSCs

Our previous observations demonstrated that Chk1 abrogation potentiates the cytotoxicity of chemotherapeutic drugs in short term viability assays. To investigate the long term impact of the combination of chemotherapy with SB218078 or AZD7762 in NSCLC-CSCs, we performed soft agar assays to evaluate differences in colony forming abilities. Our results show that NSCLC-CSCs maintain the ability to form colonies after single treatment with cisplatin, paclitaxel, or inhibitors, but not after treatment with the combinations of both chemotherapy and SB218078 or AZD7762 (**Figure 16A and B**). These results confirm that the combination of chemotherapy with Chk1 inhibitors impairs both short term and long term cell viability and clonogenic activity of NSCLC-CSCs.

Figure 16

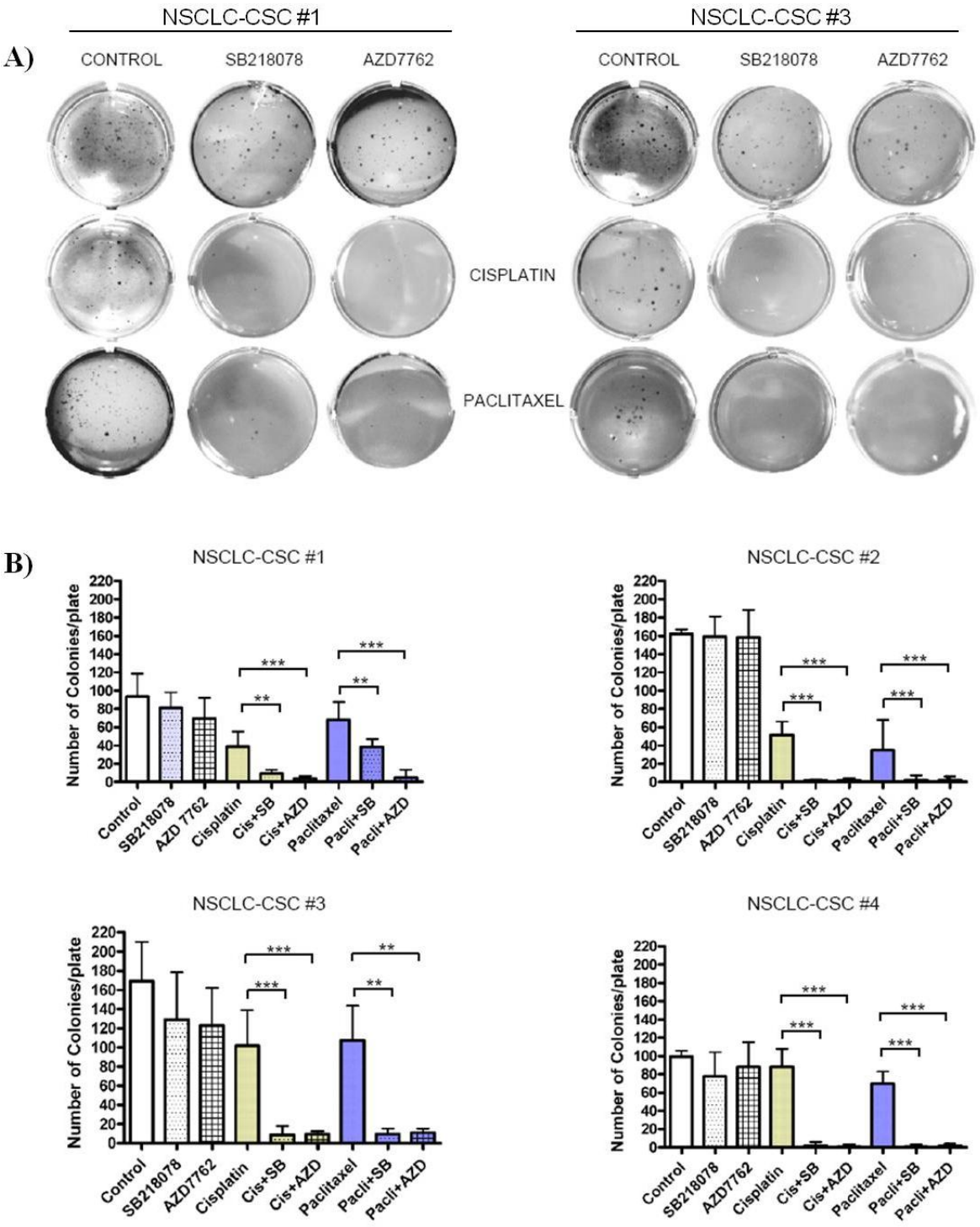


Figure 16. Chk1 inhibition reduces colony forming ability of NSCLC-CSCs *in vitro*. Representative pictures of soft agar assay for NSCLC-CSC #1 and NSCLC-CSC #3 (**A**) and the average number of colonies/well for each combination of treatment (**B**). Mean value ± SD of three experiments is presented.

4.8. Combination of chemotherapy and Chk1 inhibitors strongly affects tumor growth *in vivo*

Xenotransplantation of NSCLC-CSCs provides a solid preclinical base for the development of effective anti-cancer therapies [147]. To evaluate the ability of Chk1 inhibitors to enhance cytotoxicity of chemotherapeutic agents in lung cancer treatment *in vivo*, we assessed the effect of AZD7762 on human lung carcinoma generated by subcutaneous transplantation of NSCLC-CSCs into NOD-SCID mice. AZD7762 was chosen as the most suitable compound for *in vivo* studies since it can be reconstituted in water or PBS, while SB218078 needs to be dissolved in dimethyl sulfoxide (DMSO) that alone has toxic effects.

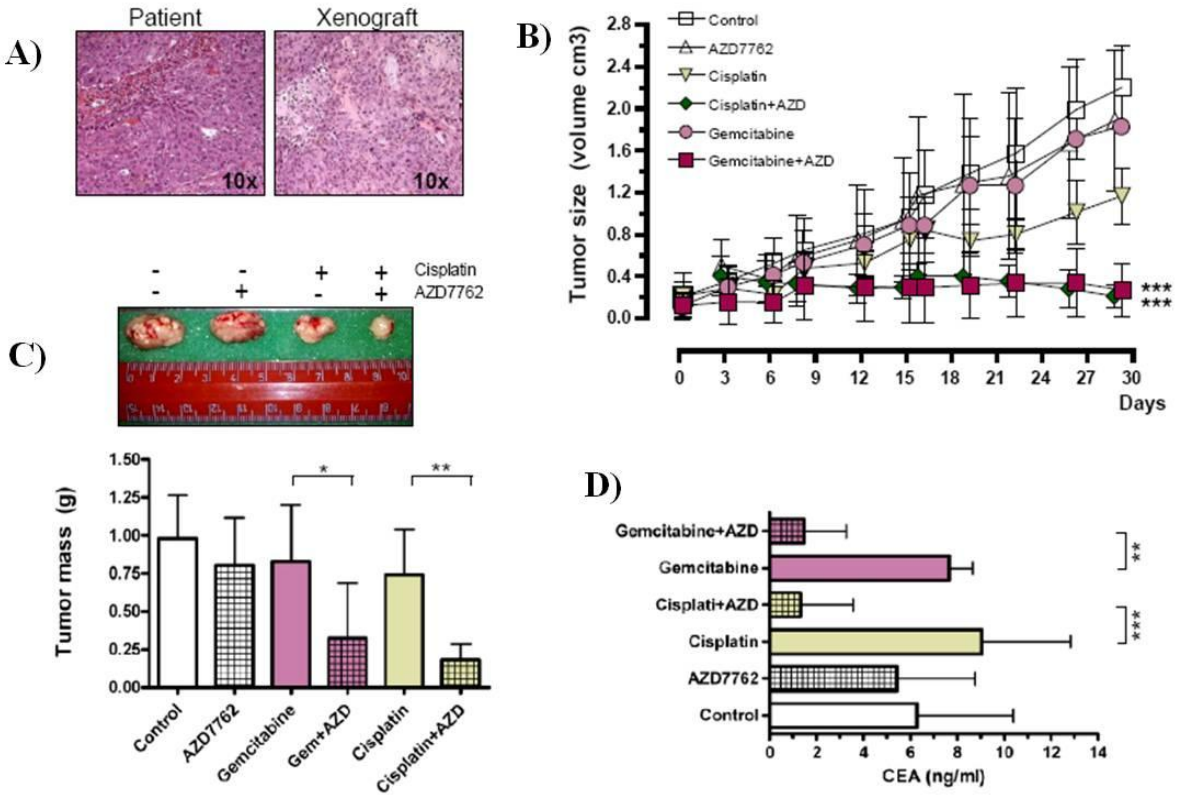
Lung tumors were allowed to grow until they reached a size of approximately 0.2 cm^3 and Hematoxylin and Eosin (H&E) staining confirmed that tumor xenografts reproduced the morphological characteristics of the human tumor of origin (**Figure 17A**). Mice were treated intraperitoneally twice a week with gemcitabine and cisplatin alone or in combination with AZD7762, injected intravenously 8 hours after chemotherapy, for a total period of 4 weeks.

As predicted by *in vitro* studies, chemotherapy had a limited anti-tumoral effect, however, the tumor response to chemotherapeutic drugs was considerably enhanced by AZD7762 administration. Specifically, AZD7762 co-treatment with gemcitabine or cisplatin significantly affected tumor size and tumor mass (**Figure 17B and C**). Additionally, we observed a significant reduction of carcinoembryonic antigen (CEA) levels in co-treated mice (**Figure 17D**). Furthermore, immunohistochemical analysis of tumor xenograft tissues showed that the combination treatment was irreparably damaging tumor cells as expression of γ-H2A.X increased considerably in mice treated with chemotherapy and Chk1 inhibitor (**Figure 17E**). Analysis of human leukocyte antigen (HLA) and murine CD45 in cells from dissociated tumors, proved the human origin of the tumors (**Figure 17F**).

Finally, to confirm the reduction of CSCs compartment *in vivo*, we performed colony forming assays with cells derived from dissociated tumor xenografts. We found a

significant reduction in the clonogenic ability of cells derived from co-treated xenografts, which should parallel the relative number of tumorigenic cells in treated lesions. (**Figure 17G**). These results strongly suggest that co-administration of chemotherapy and Chk1 inhibitors is actually targeting NSCLC-CSCs and could therefore be a potential approach for efficient lung cancer treatment.

Figure 17



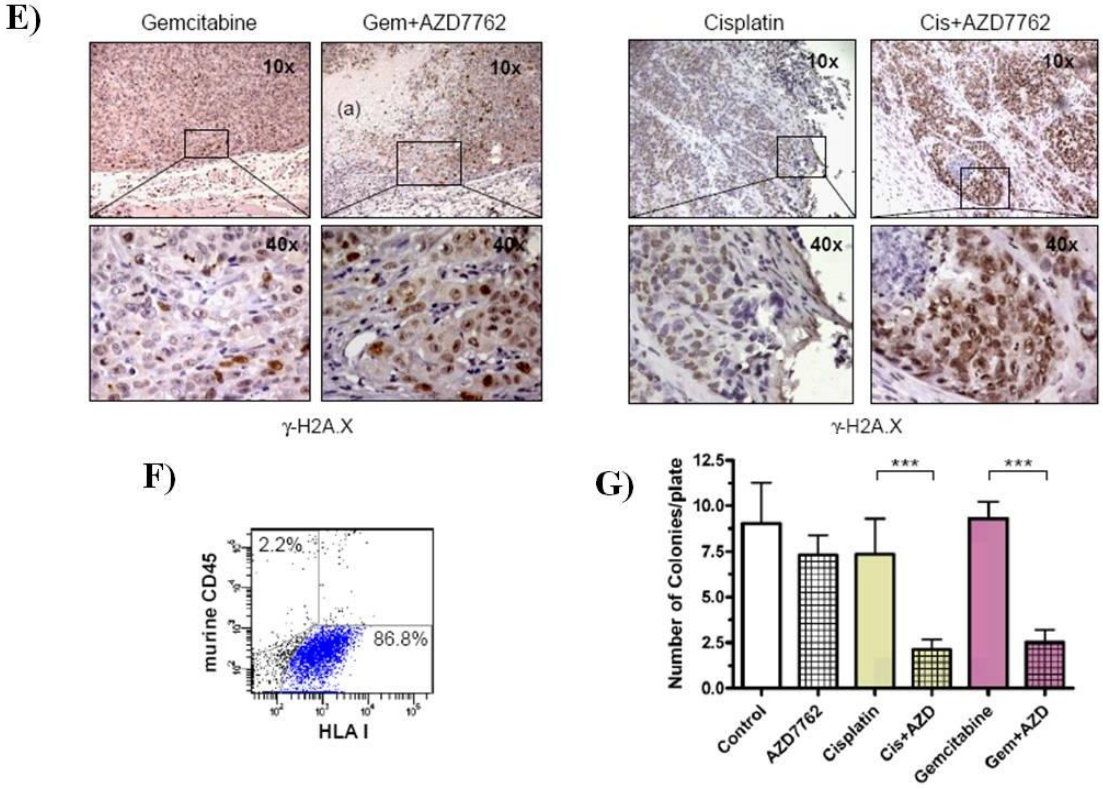


Figure 17. Combination of chemotherapy and Chk1 inhibitors strongly affects tumor growth *in vivo*. **A**. H&E staining performed on parental tumor (patient) and mouse tumor xenograft from NSCLC-CSC #3. **B.** Growth rate of mouse xenografts generated after subcutaneous injection of NSCLC-CSC #3. Tumor size was measured using an electronic caliper on the indicated days. Results of four independent experiment ± SD are reported. **C.** Representative picture of resected tumor xenografts, indicative of both size and vascularization. **D.** Tumor mass measured immediately after tumor resection. Mean value ± SD three experiments is reported. **E.** Immunoluminetric measurement of CEA in mice blood serum obtained from mice just before removal of tumors. Mean value ± SD three experiments is reported. **F.** Immunohistochemistry performed on formalin-fixed paraffin-embedded tissue for γ-H2A.X. (a) is indicating an extensive necrotic area. **G.** Flow cytometry analysis of tumor xenograft cells stained for anti-human HLA and anti-murine CD45 expression. **H.** Colony forming ability assay was performed for recovered tumor xenograft cells. The graph shows the average number of colonies/well for each combination of treatment. Mean value ± SD of three experiments is reported.

5. Discussion

Despite significant efforts in diagnosing and treating lung cancer, therapeutic resistance remains a major unresolved clinical and scientific problem. Anticancer agents that target DNA are among the most effective in clinical use, however, they have significant limitations; many patients do not respond or develop resistance to them, they are toxic and have a limited therapeutic window [24].

Recent data indicate that stem cells situated throughout the airways may initiate cancer formation and be responsible for the failure of current chemotherapeutical treatment of lung cancer [5,7]. CSCs are resistant to conventional therapies due to a high expression of anti-apoptotic proteins, drug-efflux proteins as well as an efficient DNA repair system [5,28,40,72,76]. The CSC concept has changed the view of cancer therapy, demanding development of new therapies to prevent tumor recurrence and metastasis, as well as to achieve long-term remission and survival of cancer patients. To advance the progress against cancer, the most important goal is therefore to understand the molecular basis of cancer by investigating CSCs characteristics and behaviour in response to therapy and to increase knowledge about stem cell apoptotic pathways.

In this study, we analyzed the DNA damage response induced by chemotherapy in five different LCSC clones derived from NSCLC tumors. These NSCLC-CSCs were compared with their differentiated non-tumorigenic progenies, normally comprising the bulk of the tumor. We show that NSCLC-CSCs are considerably more resistant to chemotherapeutic drugs compared to their differentiated counterparts. *In vitro* exposure of NSCLC-CSCs to cisplatin, gemcitabine or paclitaxel did not induce cell death, but resulted in a transient growth arrest that was reversible upon removal of chemotherapeutic drugs. In accordance with growth arrest, NSCLC-CSCs were blocked in S or G_2 phases of the cell cycle. This was triggered by activation of the Chk1-Cdc25-Cdc2/Cyclin B1 cascade, which kept Cdc2/Cyclin B complex inactive and prevented cells from progressing in the cell cycle, thus allowing repair of chemotherapy-damaged DNA and successful cell duplication. The ability of NSCLC-CSCs to recover after treatment with chemotherapy could, in a clinical setting, be associated with the tumor recurrence often observed in NSCLC patients.

As expected, the response to chemotherapy-induced DNA damage was increased expression of γ-H2A.X and activation of the DNA damage response through phosphorylation of both ATM and Chk1. Chk1 phosphorylation was significantly higher in NSCLC-CSCs than in the differentiated non-tumorigenic counterparts, indicating that the DNA damage machinery is more robust in NSCLC-CSCs than in their progenies. Our results are supported by a previous study demonstrating that CSCs from glioma tumors have a highly active DNA damage pathway and that activation of checkpoint proteins, like ATM, Chk1, Chk2 and Rad17, was significantly higher in the CD133+ CSC subpopulation than in CD133- cell subpopulation, indicating that CSCs have greater checkpoint activation in response to DNA damage. After gemcitabine and cisplatin treatment of NSCLC-CSCs, we observed that Chk2 phosphorylation was a late event that occurred days after Chk1 activation, suggesting that Chk1 acts as the major DNA damage checkpoint in NSCLC-CSCs. The preferential activation of Chk1 may also indicate that this checkpoint is essential for drug-resistance in NSCLC-CSCs.

These findings, combined with the fact that Chk1 plays a major role in the DNA damage response and acts as key regulator of genomic integrity, suggest that Chk1 represent a critical therapeutic target for cancer treatment. We speculated that chemical inhibition of Chk1 would increase the cytotoxic effects of chemotherapy, by abrogating the drug-induced cell cycle arrest and forcing cells to progress directly into mitosis without completing DNA reparation.

Accordingly, we observed that two different Chk1 inhibitors, SB218078 and AZD7762, increased cytotoxicity of cisplatin, gemcitabine and paclitaxel. Chk1 inhibition following chemotherapy resulted in markedly persistent γ-H2A.X expression, which indicates that these NSCLC-CSCs were unable to repair DNA damage. Chk1 inhibitors were able to alter the DNA damage response by potently reducing Cdc25 and Cdc2 phosphorylation and by promoting Cyclin B1 translocation to the nucleus. These events led to abrogation of the cell cycle arrest, mitotic catastrophe and ultimately, to cell death by apoptosis.

AZD7762 is a fairly new inhibitor of Chk1/Chk2, currently in phase I clinical trial in combination with irinotecan and gemcitabine in patients with advanced solid

malignancies. This compound has been shown to enhance the response to chemotherapy and radiotherapy in preclinical models of colorectal, lung and pancreatic cancer based on the use of cancer cell lines [130,131,134]. Although the vast majority of preclinical data obtained with such cell lines has been proven to be poorly predictive of therapeutic response in patients, the ability of Chk1 inhibitors to eradicate chemotherapy-treated NSCLC-CSCs suggests that this treatment could be used to invent more effective therapeutic approaches for NSCLC.

Unlike cancer cell lines, CSCs produce tumor xenografts that recapitulate the original tumor and provides a solid preclinical base for anti-cancer therapy development that appears to be the closest experimental system to human tumors [147]. Using NSCLC xenografts generated by NSCLC-CSCs implanted into immunocompromised mice, we found that chemotherapy had little effect on cancer progress, while the combination of chemotherapy and AZD7762 decreased tumor growth, tumor mass as well as CEA levels. The essential mechanism for these anti-tumoral effects appeared to be connected to the abrogation of Chk1 and thereby the DNA damage response by AZD7762, which is indicated by the extensive phosphorylation of H2A.X in tumor xenografts undergoing the combined treatment. Moreover, we found a significant reduction in the number of clonogenic cells in tumors from mice treated with chemotherapy in combination with AZD7762, suggesting that this treatment in fact affects the survival of NSCLC-CSCs. Since the induction of mitotic catastrophe affects proliferating cells, it is likely that a subset of slowly-proliferating tumorigenic cells sustained the residual tumor mass in mice treated with chemotherapy and AZD7762.

In conclusion, we demonstrated that proliferating NSCLC-CSCs survive during the course of chemotherapy by exploiting an efficient DNA-damage response, which can be prevented by the use of compounds that target Chk1. Due to the number of Chk1 inhibitors currently undergoing early clinical trials, our data provide valuable preclinical support in the guidance and design of future clinical trials involving combination of chemotherapy and Chk1 inhibitors for the treatment of NSCLC. Furthermore, our data suggest that γ-H2A.X, Cdc25 and Cdc2 might be useful markers for predicting activity of Chk1 inhibitors in clinical trials.

The possibility of using Chk1 inhibitors to overcome chemotherapy resistance and to eradicate NSCLC-CSCs represents a new therapeutic approach for lung cancer treatment. NSCLC-CSCs are highly resistant to chemotherapeutic drugs and combining DNA damaging agents with a cell cycle arrest abrogator could therefore be an approach to overcome the poor therapeutic effects of conventional chemotherapy on lung cancer patients. Although the combined therapy was unable to totally eradicate the tumors in our preclinical model that reproduce the tumors of NSCLC patients, the therapeutic response was significantly higher compared to only chemotherapy treatment, suggesting that Chk1 inhibition might reduce tumor recurrence and improve the 5-year survival rate of NSCLC patients. Our study was focussed on lung cancer and NSCLC-CSCs, but we believe that chemotherapy and Chk1 inhibitors can be applicable for treatment and eradication of CSCs in other types of solid tumors.

6. Publications and Posters

6.1. Publications

Bartucci M., **Svensson S.**, Ricci-Vitiani L., Dattilo R., Biffoni M., Signore M., Ferla R., De Maria R., and Surmacz E. Obesity hormone leptin induces growth and interferes with the cytotoxic effects of 5-fluorouracil in colorectal tumor stem cells. Endocr Relat Cancer. 2010; 17: 823-833.

Bartucci M., **Svensson S.**, Romania P., Dattilo R., Patrizii M., Lotti F., Pilozzi E., Duranti E., Biffoni M., Signore M., Rinaldo C., Zeuner A., Eramo A. and De Maria R. Working title: In vitro and in vivo inhibition of Chk1 sensitize lung cancer stem cells to chemotherapy. **Manuscript in preparation.**

6.2. Presentations at scientific meetings

6.2.1. Poster presentations

Bartucci M., **Svensson S.**, Dattilo R., Romania P., Biffoni M., Eramo A., Zeuner A. and De Maria R. The Chk1 inhibitor SB-218078 leads to premature Cdc2 activation and mitotic catastrophe in lung cancer stem cells. Poster presented at 6th European Workshop on Cell Death, June 1-6, 2008, Hauenstein, Germany and at ApopTrain Summer School "Advances in Cell Death Research – from Basic Principles to New Therapeutic Concepts", July 16-20, 2008, Günzburg, Germany.

Svensson S., Bartucci M., Romania P., Dattilo R., Patrizii M., Biffoni M., Lotti F., Eramo A., Zeuner A. and De Maria R. Chk1 inhibitors lead to premature Cdc2 activation and mitotic catastrophe in lung cancer stem cells. Poster presented at the 17th Euroconference on Apoptosis, 23-26 September, 2009, Paris, France.

6.2.2. Short talk

Chk1 inhibitors induce premature Cdc2 activation and mitotic catastrophe in lung cancer stem cells. Short talk was held at the 7th European Workshop on Cell Death, June 27 – July 2, 2010, Tisvildeleje, Denmark.

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