Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE BIOTECNOLOGICHE, BIOCOMPUTAZIONALI, FARMACEUTICHE E FARMACOLOGICHE

Ciclo XXXV

Settore Concorsuale: 05/G1 - FARMACOLOGIA, FARMACOLOGIA CLINICA E FARMACOGNOSIA

Settore Scientifico Disciplinare: BIO/14 – FARMACOLOGIA

Preclinical evaluation of a MYCN-specific inhibitor for the treatment of small cell lung cancer

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Esame finale anno 2023

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Abstract

Lung cancer (LC) represents the most deadly cancer worldwide, with over 1.8 million lung cancer deaths yearly. Among different types of LC, small cell lung cancer (SCLC) is the most aggressive subtype and accounts for almost 15-20% of the total lung cancer cases globally. Due to the rapid doubling time and the high metastatic potential of SCLC, the prognosis of this tumor is dismal, with a 5-year survival rate of less than 5%. Chemotherapy remains the cornerstone of SCLC treatment and only modest improvements have been achieved during the past decades, making the management of the pathology challenging. Given these premises, the search for new therapies to face this disease in order to improve patients' clinical outcomes, represents an attractive area of research. Several genetic aberrations have been identified in SCLC, among which those related to the MYC family members emerged as highly relevant. In particular, MYCN stands out as an interesting therapeutic target, due to its highly restricted pattern of expression in normal cells. In addition, MYCN amplification (MNA) defines an aggressive and immunotherapy resistant subset of SCLCs, but it is considered undruggable by traditional approaches. Thus, the development of a MYCN selective inhibitor may represent an innovative therapeutic strategy, for MYCN-expressing SCLC. Thereby, the R&D Department of Biogenera SpA has designed and synthesized an antigene peptide nucleic acid (agPNA) oligonucleotide, called BGA002, to inhibit MYCN molecular activation, acting directly at the DNA level.

In this context, my research aimed to validate BGA002, as a possible therapeutic strategy for the treatment of MYCN-related SCLC. In the first part of the project, the efficacy profile of BGA002 was studied in SCLC cells. The agPNA potently down-regulated MYCN expression, leading to cell growth inhibition and apoptosis, also overcoming multidrug-resistance. Furthermore, BGA002 showed to be a specific inhibitor, not altering cell viability in cells without target. Moreover, MYCN inhibition induced the reversion of specific pathways, in concomitance with autophagy reactivation. The second part of the project was devoted to the assessment of BGA002 efficacy *in vivo*. BGA002 treatment induced massive tumor growth reduction and increased survival in MNA-SCLC mouse models, including a multidrug-resistant one. In addition, N-Myc protein reduction was confirmed and a strong diminishment in tumor vascularization was observed. Overall, these results proved that MYCN inhibition by BGA002 is a promising approach for the treatment of MYCN-related SCLC.

1. Introduction

1.1 Small cell lung cancer: an overview

Lung cancer starts in the cells of the lungs and it is a major public health issue since it is one of the leading causes of death worldwide [1,2], representing the most deadly cancer all across the world [2–4]. Almost all lung cancers are carcinomas, they form from cells that line in the airways of the lungs, which are the bronchus, bronchioli and alveoli [5]. Lung carcinomas are histologically divided into two main types, which are Non-small cell lung cancer (NSCLC), that is the most common subtype, and small cell lung cancer (SCLC), that accounts for ~15% of all lung neoplasms [3,4,6]. SCLC belongs to pulmonary neuroendocrine tumors (Figure 1), that originate from neuroendocrine cells that line the inner airways [5,7]. Other pulmonary neuroendocrine tumors are Large cell neuroendocrine carcinomas and Carcinoid tumors, among these, SCLC is the most common form [5,8].

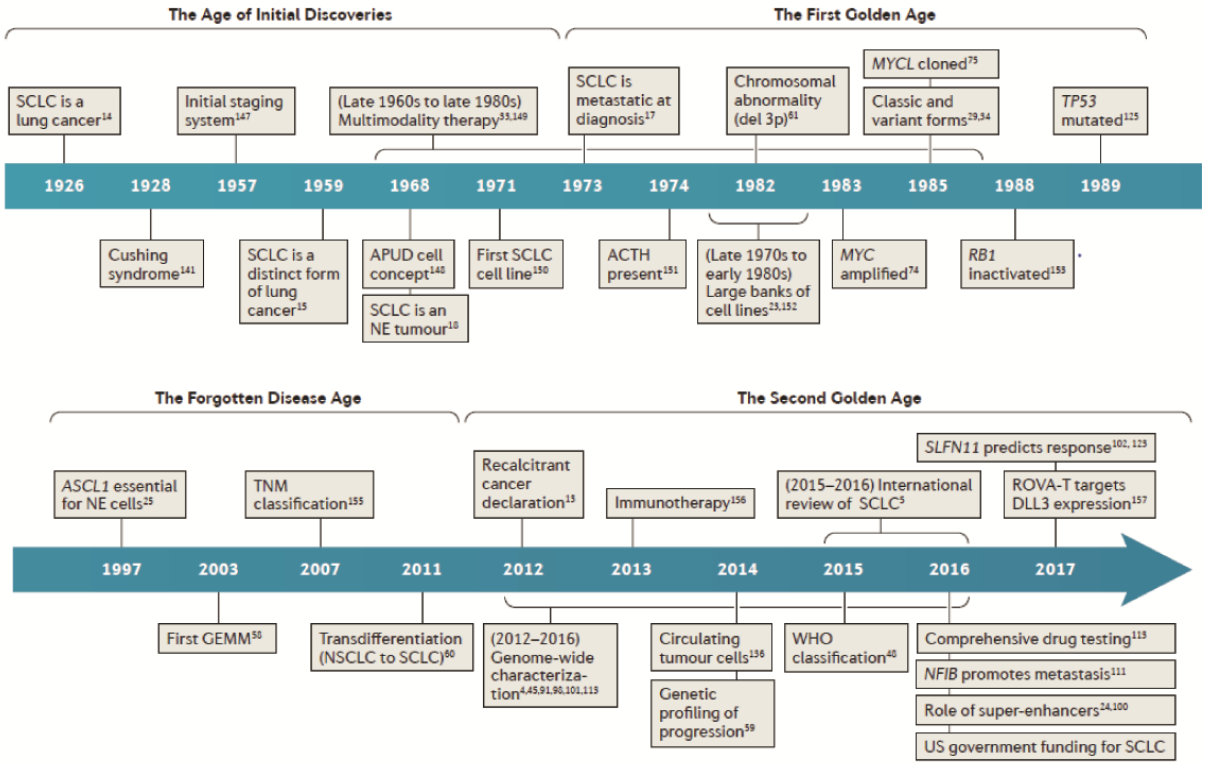

Figure 1 Discoveries and advances about SCLC over time [1]

The 2015 WHO recognizes two kinds of SCLC (Figure 1) [9,10]:

- Small cell carcinoma (formerly called oat cell carcinoma)
- Combined small and non-small cell lung carcinoma

When the tumor is composed exclusively of small cells, it is classified as small cell carcinoma [10]. Instead, combined SCLC consists of both SCLC and NSCLC histology, although no minimal percentage of NSCLC histologic elements is required for a classification of combined SCLC. The only exception is when SCLC is combined with large cell neuroendocrine carcinoma, LCNEC. In this case, at least 10% of the tumor should show LCNEC morphology, in order to get combined classification [8,10].

Among all lung malignancies, SCLC represents the most aggressive form [1,7], the main features of this tumor are rapid growth, high vascularization, genomic instability and early development of widespread metastasis, mainly in brain, liver, adrenal glands, bones and bone marrow (Figure 2) [1,11–13]. Giving the exceptional metastatic potential of SCLC, at the time of diagnosis, the majority of patients have already a extrathoracic metastasis [11,14].

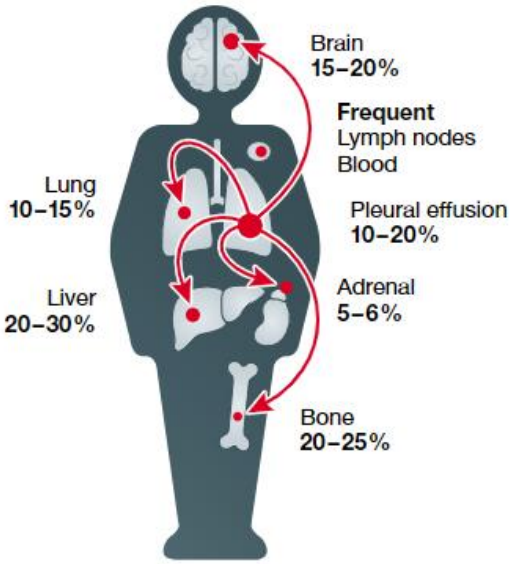

Figure 2. Representation of main metastatic sites in SCLC patients [14]

Many neurologic and endocrine paraneoplastic syndromes (PNS) are associated with SCLC [15], more than any other cancer type [1,16,17]; an estimated 3-5% of cases of SCLC will have comorbid PNS [18]. In most patients paraneoplastic syndromes occur prior to other symptoms of malignancy, so they may be helpful for early detection of SCLC [17], [18].

The most diagnosed PNS associated with SCLC is Lambert-Eaton myasthenic syndrome [1], patients with this syndrome present proximal leg weakness, due to antibodies directed against the voltage-gated calcium channels [8,19]. Other neurologic syndromes associated with SCLC are encephalomyelitis, sensory neuropathy, cerebellar degeneration and retinopathy [8,20]. There are also endocrine syndromes linked to SCLC, such as the syndrome of inappropriate antidiuretic hormone secretion (SIADH) and adrenocorticotropic hormone (ACTH)-associated Cushing syndrome [16,18,20,21].

1.1.1 Causes and risk factors

Lung cancers insurgence is strongly associated with tobacco smoking, in fact nearly all cases (~90-95%) of SCLC are registered in smokers or former smokers [5,22]; moreover risk rises with increasing duration and intensity of smoking [23]. Among males the incidence rates of SCLC is decreasing, in contrast, the incidence rates in women is increasing, and male-to-female incidence ratio is now 1:1 [2,8,24]. However, this is true only in countries with effective smoking cessation programmes, for example, USA, UK and Australia; in low-income and middle-income countries the incidence is still increasing [2,25,26]. Despite years of intensive research, the initial steps of SCLC pathogenesis remain elusive, resulting in SCLC being categorized as a recalcitrant cancer [1]. Beside smoking, other risk factors for SCLC include prior radiation therapy to the chest [5,27], age [5,13] and exposure to chemical agents such as asbestos and radon [23,27–29]. Many genetic and molecular changes are found to be related for SCLC pathogenesis. TP53 and RB1 are almost universally inactivated in SCLC [30–33], respectively in ~75-90% and in ~65-90% of SCLC cases [7,34]. These inactivating events are believed to be the essential, initiating molecular events for SCLC [1,35]. These mutations decrease proapoptotic activity during SCLC tumorigenesis, while encouraging aggressive growth and increasing the survival advantage of carcinogenic cells [36]. GEMMs have confirmed that these two events by themselves can result in a high frequency of SCLC tumour development, although the latent period is lengthy [37]. All currently available GEMMs of SCLC utilize inactivation of both these genes, usually combined with one or more additional genetic alterations [38,39].

Another of the earliest changes occurring in the pathogenesis of SCLC is the 3p deletion [40]. This frequent and non-random chromosomal abnormality is found in both SCLC cell lines and tumors [41]; and the region loss results in lesions that can damage the entire respiratory epithelium [32,42]. Genomic alterations related to SCLC, can also affect oncogenes, as in the

case of the MYC family genes (MYC, MYCL and MYCN). All three members are frequently overexpressed and/or amplified in SCLC cases [33,43–45]. Deregulation in MYC family members is linked with relapsed and poor prognosis in many type of cancers [46]. Other frequent genetic alterations include loss of PTEN [7,47], activating PI3K mutations [1], increased expression of c-kit [47], Notch inactivation [1] and telomerase activation [47].

1.1.2 Staging systems

The first staging system developed for SCLC was introduced in 1957 by the Veteran Administration Lung Cancer Study Group and characterised two clinical subgroups: "limited disease" (LD SCLC) and "extensive disease" (ED SCLC), according to the extent of disease [48–50]. Limited-stage disease is defined as tumour and nodes confined to one hemithorax and able to be encompassed within a single radiotherapy port; whereas extensive-stage disease is defined as disease beyond these bounds, including pleural or pericardial effusion or haematogenous metastases [50,51]. Approximately 30-40% of patients are diagnosed with LD SCLC, while the remaining is affected by metastatic disease [48,49,52].

In 2009, the Union for International Cancer Control TNM Classification of Malignant Tumours seventh edition recommended tumor, node, and metastasis (TNM) staging based on analysis of the International Association for the Study of Lung Cancer (IASLC) database [53]. In this staging system, the description of the anatomic extent of a tumor consists of three components [5,53]:

I. T describes the growth of the primary tumor
II. N describes involvement of lymph nodes
III. M describes distant metastases

Each component is divided into several categories and a number between 0 and 4 is associated to each letter indicating increasing gravity. Various characteristics, known as descriptors, define what is included within a T, N, or M category as summarized in Figure 3 [54].

T (Primary Tumor)		Label
T0	No primary tumor	
Tis	Carcinoma in situ (Squamous or Adenocarcinoma)	Tis
T1	Tumor ≤3 cm,	
T1a(mi)	Minimally Invasive Adenocarcinoma	T1a*(mi)*
T1a	Superficial spreading tumor in central airways[a]	T1a *SS*
T1a	Tumor ≤1 cm	T1a *≤1*
T1b	Tumor >1 but ≤2 cm	T1b *>1-2*
T1c	Tumor >2 but ≤3 cm	T1c *>2-3*
T2	Tumor >3 but ≤5 cm or tumor involving:	
	visceral pleura[b],	T2 *Visc Pl*
	main bronchus (not carina), atelectasis to hilum[b]	T2 *Centr*
T2a	Tumor >3 but ≤4 cm	T2a *>3-4*
T2b	Tumor >4 but ≤5 cm	T2b *>4-5*
T3	Tumor >5 but ≤7 cm	T3 *>5--7*
	or invading chest wall, pericardium, phrenic nerve	T3 *Inv*
	or separate tumor nodule(s) in the same lobe	T3 *Satell*
T4	Tumor >7 cm	T4 *>7*
	or tumor invading: mediastinum, diaphragm, heart, great vessels, recurrent laryngeal nerve, carina, trachea, esophagus, spine;	T4 *Inv*
	or tumor nodule(s) in a different ipsilateral lobe	T4 *Ipsi Nod*
N (Regional Lymph Nodes)		
N0	No regional node metastasis	
N1	Metastasis in ipsilateral pulmonary or hilar nodes	
N2	Metastasis in ipsilateral mediastinal/subcarinal nodes	
N3	Metastasis in contralateral mediastinal/hilar, or supraclavicular nodes	
M (Distant Metastasis)		
M0	No distant metastasis	
M1a	Malignant pleural/pericardial effusion[c] or pleural /pericardial nodules	M1a *Pl Dissem*
	or separate tumor nodule(s) in a contralateral lobe;	M1a *Contr Nod*
M1b	Single extrathoracic metastasis	M1b *Single*
M1c	Multiple extrathoracic metastases (1 or >1 organ)	M1c *Multi*

TX, NX: T or N status not able to be assessed

[a] Superficial spreading tumor of any size but confined to the tracheal or bronchial wall
[b] such tumors are classified as T2a if >3≤4 cm, T2b if >4≤5 cm.
[c] Pleural effusions are excluded that are cytologically negative, non-bloody, transudative, and clinically judged not to be due to cancer.

Figure 3. Definition for T, N, M descriptors [54]

The eighth edition of Lung Cancer Stage is now the worldwide standard for staging SCLC, starting from January 1, 2017. For this edition, the IASLC Staging and Prognostic Factors Committee assembled a new global database of 94,708 patients receiving a diagnosis between 1999 and 2010 from 35 sources and 16 countries [54].

Specific combinations of T, N, and M categories are grouped together into stage groups (Figure 4) [54].

T/M	Label	N0	N1	N2	N3
T1	T1a *MI*	IA1	IIB	IIIA	IIIB
	T1b *>1-2*	IA2	IIB	IIIA	IIIB
	T1c *>2-3*	IA3	IIB	IIIA	IIIB
T2	T2a *Cent, Visc Pl*	IB	IIB	IIIA	IIIB
	T2a *>3-4*	IB	IIB	IIIA	IIIB
	T2b *>4-5*	IIA	IIB	IIIA	IIIB
T3	T3 *>5-7*	IIB	IIIA	IIIB	IIIC
	T3 *Inv*	IIB	IIIA	IIIB	IIIC
	T3 *Satell*	IIB	IIIA	IIIB	IIIC
T4	T4 *>7*	IIIA	IIIA	IIIB	IIIC
	T4 *Inv*	IIIA	IIIA	IIIB	IIIC
	T4 *Ipsi Nod*	IIIA	IIIA	IIIB	IIIC
M1	M1a *Contr Nod*	IVA	IVA	IVA	IVA
	M1a *Pl Dissem*	IVA	IVA	IVA	IVA
	M1b *Single*	IVA	IVA	IVA	IVA
	M1c *Multi*	IVB	IVB	IVB	IVB

Figure 4. Lung Cancer Stage Grouping (Eighth Edition) [54]

In the TNM system, stages I–III correspond to LD-SCLC and stage IV corresponds to ED-SCLC (Figure 5) [51,55]. Growth in stage number, from I to IV, indicates increasing gravity and shortened survival (Figure 5) [55].

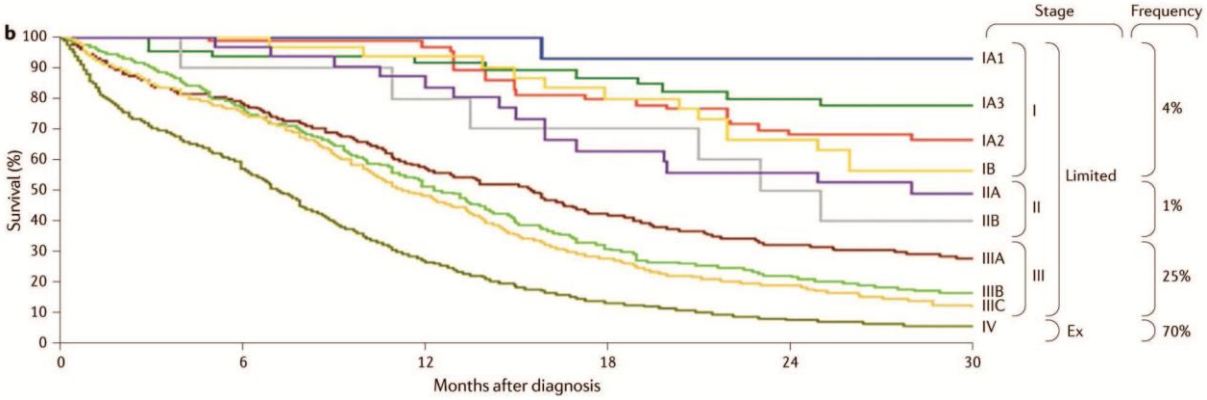

Figure 5. SCLC survival probability over time by stage at time of diagnosis [55]

A0lthough the VALSG staging system is still widely used in both designing clinical trials and presenting data from them [55], TNM classification provides better definition of risk categories, prognostic information and more precise lymph nodal staging [55,56]. The use of the TNM system is therefore beneficial in defining optimal treatment strategies in clinical trials [55].

1.1.3 Diagnosis

Common symptoms of SCLC include coughing, wheezing, difficulty breathing, loss of appetite, chest pain and coughing up blood [55].

Diagnosis is based on medical history and physical exams. When lung cancer is suspected, imaging tests (CT, PET or MRI scans) are usually performed to identify abnormalities in and around lungs [5] and to determine the extent of disease. If these initial tests identify cancer, the diagnosis is confirmed by histopathological analysis of tumour samples [5,57].

Recently, screening with low-dose computed tomography (CT) imaging, was proposed for high-risk patients for early detection of lung cancer. Unfortunately CT screening can reveal NSCLC at early stages, but not SCLC. On the basis of these results, there are currently no methods with proven efficacy for the early detection of SCLC [7,57].

1.1.4 Treatment: current therapies and future perspectives

Proper and precise staging is a key factor in both the prognostication and management of patients with lung cancer, both for non–small cell and small cell types [53]. An incorrect classification can lead to a wrong decision on treatment options, both curative and palliative ones [53,58]. In general, for patients with SCLC, the surgery is extremely rare. Therefore, the main purpose of staging is to determine whether treatment is with chemotherapy and radiation, for LD, or with chemotherapy alone, for ED [58,59]. No significant improvement has been made for patients with SCLC in the past several decades. The cornerstone of treatment for any stage is etoposide-platinum based chemotherapy [1,49].

Early-stage SCLC

A very small proportion of patients present with early-stage (T1-T2N0–N1M0) SCLC [60]. For these patients the main treatment options are: surgery, fractionated radiotherapy and stereotactic radiotherapy [55].

Surgical resection is usually limited to patients with clinical stage I or II [55,61]. After surgical treatment adjuvant chemotherapy should be given, while there is no role for adjuvant thoracic radiotherapy unless an incomplete resection was performed or pathology reveals unforeseen mediastinal nodal involvement [55,56,62]. Promising data from retrospective studies on the role of stereotactic radiotherapy have led to its inclusions as an option in

guidelines for the treatment of patients with peripheral T1-T2N0M0 disease [63,64]. Population-based data analyses estimate a 5-year survival rate of ~50%, among patients with a complete resection for T1–T2N0M0 SCLC [55].

Locally advanced SCLC

For patients with locally advanced SCLC (any T, N2–N3, M0) surgery is generally not a feasible option [55]. The current regimen for the management of locally advanced SCLC consists of four to six cycles of platinum and etoposide, with concomitant radiotherapy [7,52,55]. The standard for radiotherapy is twice-daily (45 Gy), if it cannot be delivered, once-daily (66 Gy) is a reasonable alternative [52,55,65]. Evidence from randomized controlled trials and meta-analyses favours the initiation of radiotherapy as early as is feasible in the course of CRT, preferably with the first or second cycle of chemotherapy [7,52,55,66]. Both options have a relatively favourable 3-year survival rate and similar toxicity rate [52,65]. With these multimodality treatments, up to 20% of patients will have long-term disease control [7]. Nevertheless the patients' initial response to frontline chemotherapy (60-70% of response rates) is soon countered by insurgence of resistance to second-line and subsequent therapies [7].

For patients with at least stable disease after radiochemotherapy, the use of prophylactic cranial irradiation (PCI) is useful to reduce the risk of brain metastases [52,67,68]. In patients with performance status of 0-1, PCI significantly increases overall survival in patients with non-metastatic SCLC, while the evidence supporting PCI is not as clear in patients with a performance status of 2 [55,67].

Although chemotherapy meets the objective of prolonging survival, relapse rate is high and only 5% are alive 2 years after diagnosis [49].

Metastatic SCLC

In patients with newly diagnosed metastatic SCLC, treatment is palliative, the main goals are prolong survival time, improve quality of life and minimize the risk of symptoms associated with disease [34] [69].

For over three decades, most patients received platinum-etoposide (EP) chemotherapy (with either carboplatin or cisplatin) [7,55]. Beside this combination, in a phase III clinical trial was reported superiority of cisplatin–irinotecan over cisplatin–etoposide in a Japanese population

[70], but two subsequent randomized studies in the USA failed to confirm this result [71,72]; maybe for farmacogenomic differences that may exist between Japanese and US population [72].

In recent years, multiple randomized phase III studies have demonstrated that the addition of an immune checkpoint inhibitor to first-line chemotherapy resulted in improvement of both progression-free survival and overall survival [73,74]. Giving its tolerable safety profile and the promising results, this regimen is now the new standard of care for the first-line treatment of metastatic disease [73–76].

For patients with metastatic disease the role of PCI is controversial: a European phase III study suggested that PCI diminishes brain metastases development risk and prolongs OS [77]. However, another phase III study conducted in Japan found that PCI did not offer a benefit for patients with metastatic SCLC [78]. Clinical trials to resolve this controversy are ongoing [79,80]. Giving this data, either PCI or active CNS surveillance are both reasonable options [74,81].

Recurrent SCLC

Although being initially responsive to chemotherapy, most SCLC patients relapse within a few months [8,82]. After relapse, options are limited and the prognosis is bleak [81]. If the relapse occur within 3 months of treatment, patients are considered chemoresistant and their response to further treatment is less than 10%; if the relapse occurs after 3 months, patients are defined as chemosensitive and the expected response to further treatment is 25% [82,83].

Until 2020, the only second-line drug approved by FDA, was the topoisomerase I inhibitor topotecan [7,55]. In the past 3 years, the FDA has granted accelerated approval for new drugs [55].

Lurbinectedin is an alkylating agent that binds to the minor groove of DNA. This binding affects transcription in tumor cells, resulting in DNA breaks and subsequent apoptosis [55,81]. In a single-arm phase II study of 105 patients, lurbinectedin has demonstrated a 35% response rate [84]. Due to this study, lurbinectedin monotherapy has obtained accelerated approval by the FDA, for second-line treatment of relapsed SCLC [55,81]. Currently, is also being evaluated the combination between lurbinectedin and other agents. However, in the phase III ATLANTIS trial, the combination of lurbinectedin plus doxorubicin failed to

prolong OS as compared to standard topotecan or CAV in relapsed SCLC patients, missing the primary end point of the study [81]. Ongoing studies are also evaluating the efficacy and the safety of lurbinectedin plus irinotecan [84], the combination with atezolizumab (NCT04253145) or pembrolizumab (NCT04358237) [74,81].

Immune checkpoint inhibitors have shown promising results in relapsed patients [85,86], leading to accelerated approval of both nivolumab monotherapy and pembrolizumab monotherapy as third-line treatments for patients with SCLC [74,81,82]. Unfortunately subsequent larger randomized trials failed to show meaningful benefit from PD-1 axis blockade as monotherapy or in combination with CTLA-4 blockade; after negative results obtained by nivolumab and pembrolizumab, the respective manufacturing companies withdrawn their indication as third-line therapies [81].

Other chemotherapy based options are taxanes, such as paclitaxel and docetaxel, irinotecan and temozolomide. All of these have shown a clinical activity and are included as options in treatment guidelines for recurrent SCLC [55,81], in particular they are commonly used in routine clinical practice in second or further lines of treatment [81].

Single-agent amrubicin, which has shown promising data in Japanese trials [87–89], and also superior ORR and PFS than topotecan in a randomized phase II US-European study [90], then failed to show improvement in OS in a large randomized phase III trial against topotecan, despite higher ORR in the overall population [82,91].

Target therapies

Multiple genetic abnormalities have been discovered in patient with SCLC, consistent with the effects of long-term exposure of cellular DNA to the carcinogens found in tobacco smoke [92]. Recent new insights into the biology of SCLC, have made possible to identify new targets useful for the development of innovative therapies [93]. Unfortunately, the vast majority of these targeted agents did not fulfil the expectations [15].

Angiogenesis has a relevant role in tumor growth and metastases development; in particular SCLCs exhibit increased level of vascular endothelial growth factor (VEGF), which likely enables their invasive potential. However, treatment with bevacizumab, a VEGF-neutralizing antibody, was not effective in prolonging survival [94]. Besides bevacizumab, other anti-angiogenic agents such as sunitinib, sorafenib, vandetanib, pazopanib, aflibercept and

thalidomide, have been tested in several phase I or II clinical trials with controversial results. These molecules have been studying as monotherapy in maintenance treatment after the standard schedule or in combination with chemotherapy, but they show none or minor clinical benefit and high toxicity [95–97].

Another promising target, overexpressed in SCLC, is poly(ADP-ribose) polymerase 1 (PARP) [94]. Early in vitro studies suggest that PARP inhibitors may have some activity against SCLC [15,98], but several clinical trials are needed to demonstrate these preliminary findings and to explore the activity of PARP inhibitors [99].

Delta-like 3 (DLL3) is a Notch pathway ligand which is expressed on the surface of approximately two-thirds of SCLC cells but is absent from healthy cells, making it a potential target [94]. Rovalpituzumab tesirine (Rova-T) is a novel, antibody–drug conjugate with high specificity for DLL3. Rova-T has demonstrated encouraging results in phase-1 trials, demonstrating to be effective with minimal toxicity. Unfortunately, the same could not be observed in the later trials [93,99,100].

Another interesting approach of the treatment of SCLC is the administration of aurora kinase inhibitor. Aurora kinase A is essential in mitotic spindle assembly [100,101], its inhibition induces G2/M-phase arrest and thereby arrests the proliferation of human SCLC cells [102]. Alisertib, a selective aurora kinase A inhibitor, showed promising results in a phase I/II trial-included patients with different tumor types [101,103]. Further clinical investigations are needed to better study and optimize the therapeutic utility of this compound in SCLC [101].

An interesting view of these novel agents is their combination, in particular the combination of ipilimumab and nivolumab or Rova-T plus nivolumab plus ipilimumab is under investigation [104].

1.2 MYC family of oncogenes: MYC, MYCN and MYCL

As mentioned above, alterations in oncogenes are common in SCLC. In particular all three members of the MYC family, MYC, MYCL and MYCN, are frequently amplified or overexpressed in SCLC tumours and cell lines [44,45]: approximately ~20-30% patients show amplification or transcriptional upregulation [1,35,105,106]. Interestingly, in SCLC, they are found to be affected in a mutually exclusive manner [107].

The members are situated on different chromosomes and expressed at distinct timings and locations during development, but encode proteins with similar domains and function [46]. These proteins are transcription factors that control cell proliferation, cell cycle progression, cell growth, metabolism, differentiation and tissue remodelling, as well as a variety of protective checkpoint mechanisms such as growth arrest and apoptosis [105]. The three oncogenes are paralogs with regions of structural homology, but also functional differences. They share a transactivation domain (TAD) encompassing highly conserved transcriptional regulation elements called “Myc boxes” (MB), nuclear localization sequencing (NLS) and a basic Helix-Loop-Helix Leucine Zipper (bHLHZ) domain through which they heterodimerize with MAX (Figure 6) [46,105,108]. This binding enables MYC genes to recognize the canonical E-Box CACG/ATG elements in the promoters of target genes, leading to recruitment of transcriptional cofactors and subsequent transactivation [105,109]. The conserved transcriptional activation domain (TAD) is located in the N-terminus while the bHLHZip and nuclear localization sequences (NLS) are found in the C-terminus of the proteins [110].

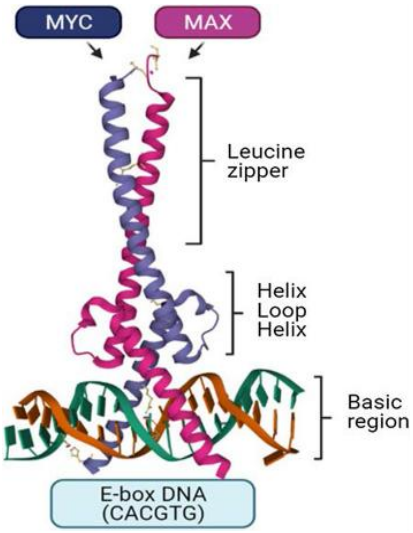

Figure 6. Crystal structure of MYC-MAX heterodimer [111]

1.2.1 MYCN regulation

The MYCN gene is located on chromosome 2 (2p24) [46]. Due to its transforming ability in case of mutations, MYCN expression is tightly regulated in normal cells at both the transcriptional and protein level [112]. The MYCN oncogene shows a particular regulation regarding both timing and tissue specificity: it is preferentially expressed in neural tissues including the forebrain and hindbrain, as well as pre-B cells, cells in the intestine, heart and kidney during embryogenesis [113].

Biologically, MYCN, like MYC, was found to promote transformation in rat embryo fibroblasts and induced proliferation and cell cycle progression in quiescent fibroblasts, confirming its role as an oncogenes [46,107,114,115]. Remarkably, MYCN plays an essential role in normal lung development [116], where it controls growth and metabolism and regulates progenitor cell proliferation and differentiation [105]. Despite their different spatiotemporal expression levels, MYCN and MYC can functionally replace each other in the appropriate context [107,117].

In normal conditions, the RNA and protein levels of all MYC family members are tightly regulated [112]. The short half-life of both MYCN mRNAs and proteins enable a close control of its function. The transport, stability and translation of MYCN mRNA is affected by multiple external factors including miRNAs [105,107]. An additional level of regulation is also present at the protein level. N-Myc is a 60-63 kDa protein with a short half-life of ~15-30 minutes, and its stability is controlled by phosphorylation of two specific residues at its N-terminal: Ser62 and Thr58 [46]. These residues are essential for protein stability during cell cycle progression: at first phosphorylation at Ser62, mediated by CDK1, leads to a transient stabilization of the protein, but also serve as the prerequisite for Thr58 phosphorylation by GSK3β. This second modification triggers ubiquitination and subsequent degradation by the proteasome [46,112,113].

1.2.2 MYCN is an oncogenic driver

MYCN plays multiple roles in malignancy, maintenance of a stem-like state and tumorigenesis in general. MYCN can activate genes involved in a variety of processes, such as metastasis, survival, proliferation, pluripotency and angiogenesis. At the same time, there are genes that promote cell cycle arrest and immune surveillance, whose expression is suppressed by MYCN [118].

During tumorigenesis, MYCN drives proliferation and cell cycle progression, by the activation of cyclins (such as cyclin D1 and D2) as well as cyclin-dependent kinase 4 (CDK4) [112,119]. At the same time, MYCN represses the expression of mediators of cell cycle arrest such as p21, leading to the failure of the cells to arrest in the G1 phase of the cell cycle [112]. MYCN sustains tumour cells growth by regulating metabolism: MYCN amplification increases glutamine transport and glutamate metabolism [46,120]; moreover another important metabolic pathway involves the synthesis of polyamines, which are organic cations that enhance transcription, translation and replication [121].

Additionally MYCN is involved in apoptotic control. Intriguingly, MYCN can have a dual role in the regulation of apoptosis: it has been shown that MYCN up-regulates NOXA, a pro-apoptotic regulator [46]; on the other hand MCYN levels strongly correlates with H-TWIST, that is an anti-apoptotic oncoprotein [119,122].

Misexpression of MYCN also has a positive effect on angiogenesis, inducing the expression of proangiogenic factors including angiogenin and vascular endothelial growth factor (VEGF) via the PI3K/mTOR pathway [118].

Another gene whose expression strongly correlates with MYCN is MRP1 (the multidrug resistance-associated protein), a glycoprotein that belongs to the superfamily of ATP-binding cassette (ABC) transmembrane transporters [112]. This transporter is able to confer resistance to a broad range of structurally unrelated chemotherapeutic drugs, and numerous studies have shown its up-regulation in many solid tumors such as those of the lung, breast and prostate [112,123]. MRP1 expression is a predictive hallmark of poor response to chemotherapy in SCLC, and its expression is increased in SCLC metastases detected at relapsed, indicating its involvement in cell survival [124–126].

MYCN interferes with levels of chromatin acetylation and methylation, markers of transcriptional activation and repression, through HATs and HDACs [127].

The MYCN oncogene has been shown to be implicated in the oncogenesis of several primary tumours, including SCLC [118,128]. The expression levels of MYCN correlates with invasive and metastatic behaviour, resulting in more aggressive phenotype of SCLC [128]. In fact, MYCN is associated to tumour progression, contributing to adhesion, invasion and degradation of surrounding matrices [118,129]. Specifically, MCYN down-regulates integrins

α1 and β1, promoting detachment from the extracellular matrix and allowing cells to migrate and invade [118].

Giving all these data, genomic amplification of MYCN positively correlates with poor response to radiotherapy and chemotherapy [128,130], leading to significantly shorter survival and poor prognosis for patients [105,130,131].

1.2.3 Therapeutic strategies to target MYCN in cancer

As mentioned above, MYCN plays a key role in driving and maintaining SCLC tumorigenesis, moreover the fact that regulates all hallmarks of cancer, make it an attractive target for tumour-specific therapy. Furthermore, its tightly regulation regarding physiologic expression in terms of lineage and timing, suggests a wide therapeutic index for MYCN-specific drugs [105,132,133].

Both direct and indirect strategies have been developed for MYCN targeting, but despite intensive efforts, they have largely failed [134]. Failure of direct approaches is predominantly related to MYCN biology: like many transcription factors, MYCN is mainly localized in the nucleus, a compartment still impenetrable for the vast majority of drugs [105,132]. Beyond that, N-terminal of MYCN is intrinsically disordered in its monomeric form [135], while in the C-terminal lack the defined hydrophobic pockets, typically targeted by small molecules [132,136]. Lastly MYCN inhibition could interfere with its highly homologous MYC, that is ubiquitously expressed, also in normal cells [105,132].

A recently developed therapeutic strategy, interesting for undruggable target, involves Proteolysis targeting chimeras (PROTACs) [113]. PROTACs are heterobifunctional molecules composed by a recognition moiety for a protein of interest, connected via chemical linker to an E3 ubiquitin ligase ligand [137,138]. These molecules induce specific protein degradation by recruitment of ubiquitin-proteasome system [113,137]. Small molecules 10058-F4 and 10074-G5, known to bind to the c-MYC and inhibiting the c-MYC/MAX interaction, have been shown to bind MYCN as well [113,139]. These molecules may be useful for the development of PROTACs for MYCN, thus providing a new strategy for the inhibition of the oncogene.

Because strategies to directly target MYCN have not achieved their goal yet, researchers have studied and developed approaches for its indirect inhibition, acting on MYCN transcription

and mRNA stability or N-Myc protein stability and degradation [133,134,140,141]. Strategies proposed for indirectly targeting MYCN, summarized in Figure 7, include different types of inhibitors [142]:

- Bromodomain and extraterminal (BET) proteins inhibitors
- Histone deacetylase (HDACs) inhibitors
- PI3K/mTOR inhibitors
- Aurora Kinases inhibitors
- Mouse double minute 2 homolog (MDM2) inhibitors

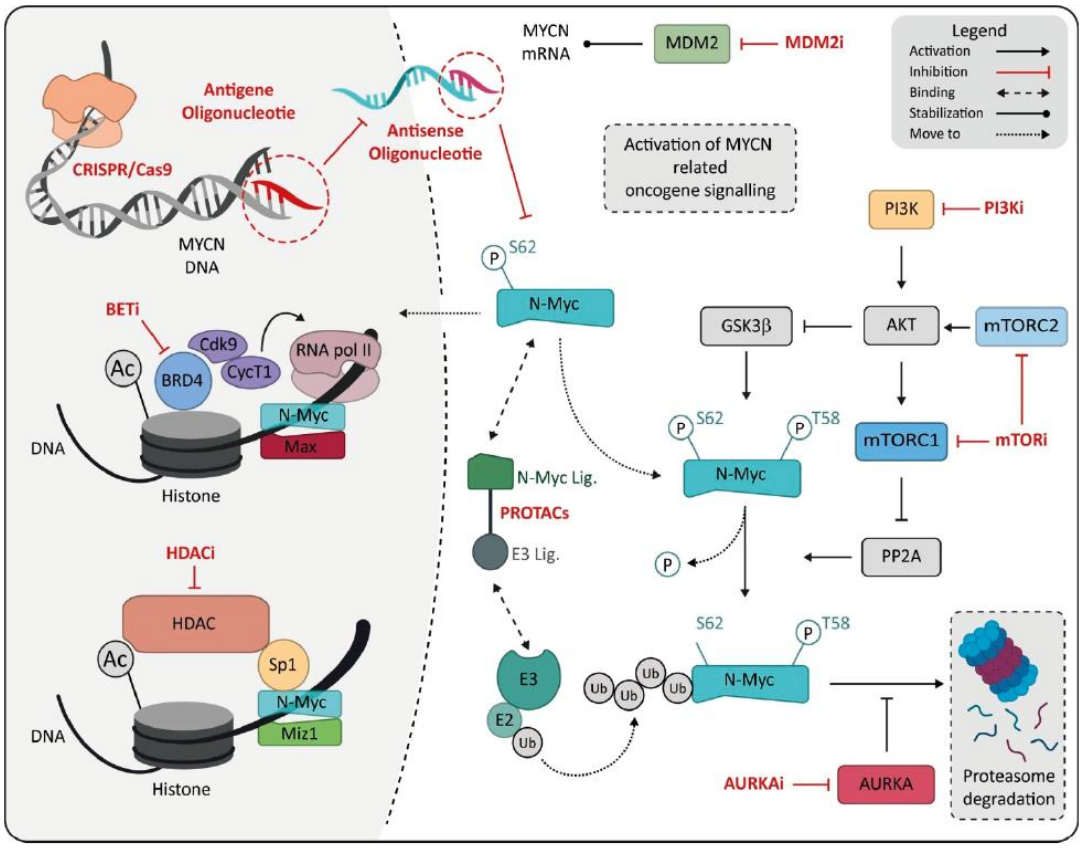

Figure 7. Schematic representation of drugs that target MYCN directly or indirectly [142]

BET inhibitors (BETi)

Bromodomain and extraterminal domain family (BET) is a group of four proteins: BRD2, BRD3, BRD4, and BRDT. Through their binding to the DNA, BET proteins recruit complexes to promote transcription initiation and elongation [142,143]. Interestingly these factors, are required for both MYCN transcription and, MYCN-driven transcription so that

their inhibition can have a double effect [46,113,142]. Many BET inhibitors showed their capacity in the inhibition of MYC oncogenes in vitro [106,144,145], in particular SCLC was found to be sensitive to BET inhibition in preclinical models [146,147], unfortunately their application in clinical setting was not successful. Importantly, new other improved BET inhibitors such as GSK525762, ZEN003694 and OTX015/MK-8628 were developed and their safety, pharmacokinetics and activity are currently under assessment in Phase I and Phase I/II clinical trials for different condition, including SCLC [133].

HDACs inhibitors

As mentioned above, MYCN is capable of silencing suppressor genes through the binding of MIZ1 and SP1 and therefore recruiting histone deacetylases (HDACs), which have a pivotal role in both chromatin packaging. For this reason, it may represent a viable path to target MCYN-amplified SCLC [113,118]. FDA has still approved different HDACs inhibitors (vorinostat, belinostat and panobinostat), mainly for haematological tumors [148–150]. CUDC-907 and FK228, dual of HDACs and PI3K signalling inhibitors, display a significant anticancer activity, in SCLC [151,152]. FK228 also enhances the therapeutic effects of radiotherapy, mainly through the induction of chromatin decondensation [152]. These encouraging results provide a rational for further clinical investigation. In the meantime, JBI-802 (NCT05268666) and entinostat (NCT04631029) are already in clinical trials for the treatment of solid tumours, including SCLC.

PI3K/mTOR Inhibitors

MYC oncogenes are very unstable, with a short half-life in normal cells, however, in many cancer types occur aberrant protein stabilization [134]. Tight control of N-Myc expression is essential and a major pathway involved in the regulation of MYCN stability is PI3K, through AKT and GSK3ß [113,130,153]. Briefly the regulation occur via ubiquitin-mediated degradation by the proteasome: CDK1 phosphorylates MYCN at S62, subsequently GSK3ß phosphorylates it at T58 [113]; upon this phosphorylation MYCN is polyubiquitinated by the E3 ligase FBW7 and degraded by the proteasome [105]. Activation of the mTOR complex can lead to over-expression and stabilization of MYC family members [105]: AKT, under mTORC2 control, phosphorylates GSK3ß, suppressing its kinase activity and thus, stabilizing N-Myc protein [113,130]. Moreover, mTORC1 also directly inhibits PP2A, decreasing MYCN phosphorylation and enabling its accumulation [154]. In a similar way, N-Myc is also indirectly regulated upstream by mTOR complex. Not surprisingly, PI3K inhibitors reduce

and destabilize N-Myc [130,154]. These inhibitors may be relevant for N-Myc driven tumors [130,155]. In a recent work of Polley and colleagues, 65 SCLC cell lines were screened for many drugs and inhibitors [156]. This study demonstrated SCLC sensitivity to mTORC1, mTORC2, and PI3K/mTOR inhibitors [156]. mTOR inhibition decreases cells growth, promotes apoptosis and enhances sensitivity to cisplatin/etoposide in both SCLC cells and PDX models [156–158].

Despite promising preclinical results, mTOR inhibitors trials in SCLC have demonstrated limited efficacy in clinical trial, failing to improve survival. A phase II trial conducted with temsirolimus did not improve PFS in patients with extensive-stage disease, both when combined with chemotherapy or as monotherapy [159]. Another phase II study, carried out with everolimus in relapsed SCLC, showed limited activity [160]. A new phase I/II trial is ongoing, testing the combination of sirolimus and auranofin in patients with advanced or recurrent SCLC or NSCLC (NCT01737502) [156].

Aurora Kinases (AURKs) Inhibitors

Aurora kinases family is composed by three serine/threonine kinases, essential for cell cycle regulation: AURKA, AURKB, and AURKC [161,162]. AURKA and AURKB have key roles in mitosis, whereas AURKC has unique role in meiosis [140,161,163]. AURKs deregulation was found in several cancer types, making them attractive candidates for therapies [162,163]. AURKA directly regulates N-Myc protein stability, by the formation of a complex, which protect the N-Myc from proteasomal degradation [140]. The knockdown of Aurora A arrests cell proliferation and promotes the arrest in G2/M phase of cell cycle, in SCLC [164]. Another AURKA inhibitor, CD532, induces allosteric conformation change in its kinase domain, impairing AURKA interaction with N-Myc and allowing its proteasomal degradation [130,165]. AURKB inhibitor, Barasertib (AZD1152), has also shown to be effective in SCLC, inhibiting growth both in vitro and in vivo [166]. Alisertib (MLN8237) and Erbumine (LY3295668), two selective inhibitors for Aurora-A, were evaluated in clinical trials for advance solid tumors [167–169] (NCT01045421, NCT03898791). Many trials with AURK inhibitors are still ongoing, for example those with Chiauranib, now in phase III, for patients with SCLC (NCT05371899, NCT04830813).

MDM2 Inhibitors

Tumor suppressor p53 is a master regulator of cell cycle and cell death, activating apoptosis in response to DNA damages or stress conditions [46]. This protein has been reported to be mutated in almost 50% of human cancer [170]. Levels of p53 are controlled by the proto-oncogene MDM2, which is a ubiquitin ligase that promotes proteasomal degradation of different tumor suppressors, including p53 [46]. In human lung tissue, MDM2 levels are frequently elevated [171]. Interestingly MDM2 also interacts with MYCN: through the binding of AU-rich elements of the 3′ UTR of MYCN mRNA, MDM2 increases MYCN mRNA stability and translation [172]. For these reasons p53-MDM2 axis results to be an attractive target, in a broad range of tumors [171]. In SCLC cells, upon MDM2 inhibition, p53 is restored and this leads to down-regulation of prosurvival proteins and upregulation of proapoptotic Bim protein [171]. There is an ongoing clinical trial, evaluating KRT-232, a small MDM2 inhibition, in subjects with relapsed or refractory SCLC (NCT05027867).

1.3 Peptide nucleic acids (PNAs)

The term PNA, originally standing for “Polyamide nucleic acid”, was introduced in 1991 by Nielsen et al. [173,174], to describe a new “third generation” oligonucleotide, able to target DNA major groove. The same term nowadays is used with the meaning of Peptide Nucleic Acid, describing a class of molecules widely employed in different fields, such as chemistry, biochemistry, medicine, nanotechnology [175].

The procedure of PNA synthesis is similar to peptides, which are tipically synthetized via solid-phase techniques, either by manual or automated [175]. There are several protected monomers used for the synthesis, the most common are tBoc- or Fmoc- monomers, of which the four natural nucleobase are commercially available [175,176]. Usually PNA oligomers are deprotected and cleaved off the resin using TFMSA/TFA or TFA, depending on which protective group was used in the previous step [175,177].
After synthesis, PNA molecules are purified by reversed-phase high-pressure (performance) liquid chromatography (HPLC); finally oligomers are characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or electrospray ionization mass spectrometry (ESI-MS) [175].

PNAs are lipophilic molecules, so they tend to selfaggregate both on the solid support during synthesis, leading to low yield of the desidered product, as well as in solution leading to precipitation and non sequence-specific interactions [178]. To reduce the risks of these phenomena occurring, usually PNA oligomers synthesized on a solid support do not exceed 16 bases [176].

1.3.1 Structures and properties

PNAs are synthetic analogues of nucleic acids, in which the entire sugar-phosphate backbone is replaced by a peptide-like skeleton consisting of repeated units of N-(2-aminoethyl)-glycine, connected to nucleobases through a methylene-carbonyl bond [174] (Figure 8).

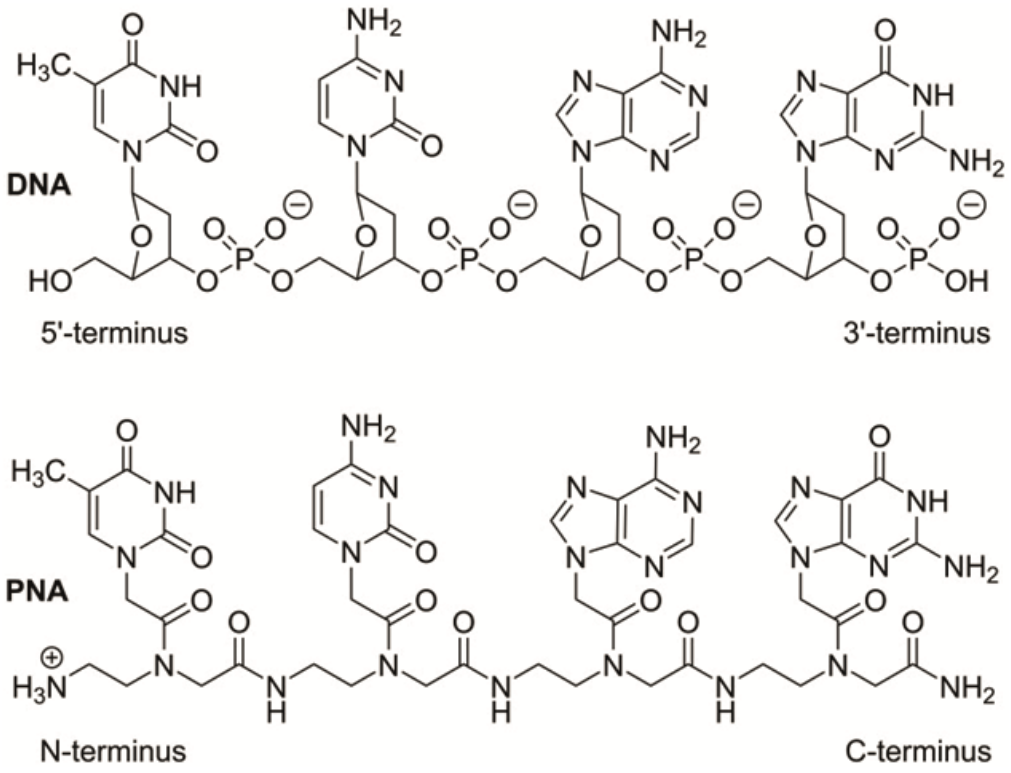

Figure 8. Structural comparison of PNA and DNA [179]

PNAs retain the ability to recognize DNA or RNA following Watson-Crick base pairing rules [179], therefore forming very stable complex, through the formation of hydrogen bonds [174,175]. Due to their neutral backbone there is no electrostatic repulsion and consequently they easily destabilize DNA-DNA duplexes [176,180] and bind complementary sequences in both parallel (N-terminus of PNA binds to 5'-end of target) and antiparallel orientation (C-terminus of PNA binds to 3'-end of target), although the latter mode is preferred [181,182]. PNA molecules have some advantages when compared to DNA and RNA: for example they are generally more chemically stable, in fact they do not degradate in acid or basic conditions, as well as at elevated temperatures [176,183]. Furthermore PNAs are biochemically stable, they are not substrate for enzymes such as proteases, peptidases and nucleases [183,184]. PNA molecules are also stable over a wide range of pH [176]. These features facilitate synthesis, purification, storage and make them a good candidate for in-vivo studies [176,183].

1.3.2 Binding modalities

PNA can bind dsDNA in several ways, as reported in Figure 9 [174,175,185–187].

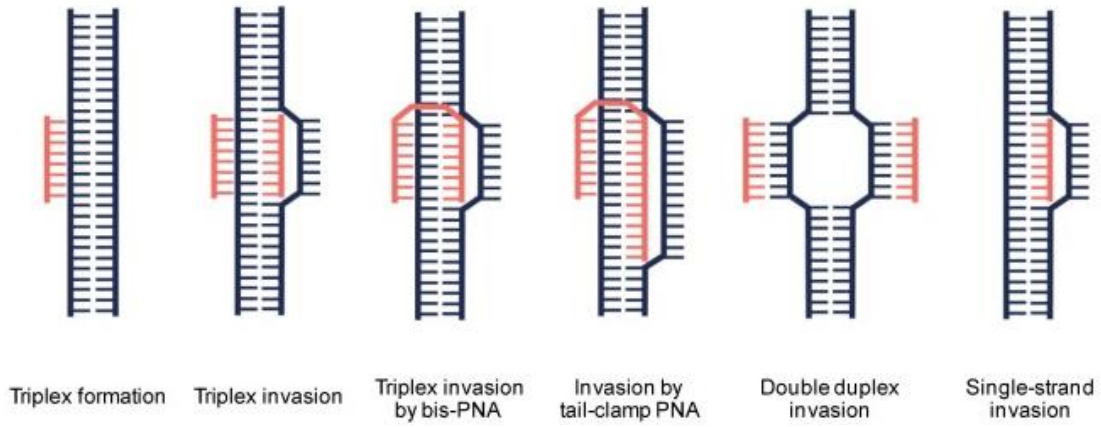

Figure 9. Different modes of targeting dsDNA (blue) by PNA oligomers (pink) [174]

PNAs were originally designed to target the major grove of dsDNA, via Hodgsteen bonds formation [173]. Remarkably, it quickly became clear that PNA oligomers bound their complementary sequence on dsDNA, not exploiting the triplex formation, but they preferred triplex invasion modality [186,187]. For both triplex and triplex invasion strategies, a homopurine/homopyrimidine sequence on DNA target is required [187,188].

PNAs are also capable of invading DNA double helix, leading to the formation of a sufficiently stable duplex, to compensate the slow kinetics of formation [187]. This mechanism appears to be limited to homopurine PNAs, which can form extremely stable PNA-DNA complexes [175,187].

Another binding mode is the double duplex invasion, that is facilitated by the use of Pseudo-Complementary PNA (pcPNA), in which adenine and thymine are respectively substituted with diaminopurine and thiouracil [185,186,189]. This modality allows the formation of very stable complex at mixed purine-pyrimidine targets, as long as they mantain approximately 50% of A/T content [175].

Finally, the tail-clamp PNA is made by two homopyrimidine strands of PNA connected through a flexible ethylene glycol-type linker, that recognize homopurine sequences on the target [190–192]. Thanks to the Watson-Crick and Hoogsteen bonds, there is the formation of form thermally-stable PNA/PNA/DNA triplex structures [190].

1.3.3 Limitations of PNA technology

While PNAs hold tremendous therapeutic potential, as demonstrated by many *in vitro* and *in vivo* studies, there are fundamental challenges that still need to be faced before they are broadly used in clinical setting [193,194]. First, their hydrophobicity promotes PNA aggregation: when they are in solution, PNA oligomers tend to fold into complex globular structures, presumably due to both the intra- and inter-molecular affinity between their hydrophobic nucleobases [193]. In addition to that, the hydrophobicity of PNAs also seems to promote relatively nonspecific adherence to both other macromolecules and larger surface, causing numerous problems in handling PNAs in complex applications [194]. To overcome poor water solubility of PNA oligomers, several strategies are developed, including the addition of amino acid residues at their termini [195], the conjugation to negatively charged DNA molecules [193,196], and the conjugation to high molecular weight polymers such as polyethylene glycol (PEG) [197].

The second significant challenge of PNAs is the poor cellular uptake. If they do not cross the cell membrane, there is no interaction with their target sequence and this results in no biological activity [193,194]. To overcome this limitation a large variety of systems have been proposed. These methods can be divided into two main categories: those involving unmodified PNAs and those involving modification of PNAs, to improve their cellular delivery [185].

The delivery of unmodified PNAs can be achieved with artificial techniques for penetrating the cell membrane, such as microinjection, or with other techniques that physically disrupt the membrane, for example electroporation or streptolysin O treatment [175,193,194]. Microinjection is an excellent tool for assessing PNA activity, but at the same time it is a laborious technique applicable only for small-scale experimental set-ups [185]. This is probably the reason why other researchers have not chosen this strategy for PNAs delivery. On the other hand, electroporation represents a more feasible methods for transfer of PNAs to cells, as demonstrated by many studies [175,185]. For example, electroporation has been used for transfection of anti-telomerase PNA into SV40 transformed fibroblast resulting in more than 60% inhibition of the telomerase activity, while a mismatch PNA, used as control, was shown not to inhibit telomerase activity [198]. In another study, electroporation of PNA to human erythroleukemia cells has been used to demonstrate the ability of PNA to induce the transcription of γ-globin gene [199].

Unmodified PNAs have also been successfully delivered directly to cells: in *Escherichia coli* mutant strain AS19, which have a cell wall/membrane defect, direct addition of PNA in micromolar concentrations is sufficient for specific antisense inhibition of β-galactosidase and β-lactamase [200,201]. Also in eukaryotes, simple addition of PNA to the culture medium, can in some cases result in positive cellular uptake [202].

As mentioned above, several modifications have been introduced in PNAs, to improve their delivery in the target site. One such modification is the conjugation of PNA to a liphophilic moieties, such as adamantyl acetic acid or phosphonium cation, to promote the encapsulation of PNA into liposomes [203–205]. In an unpredictable way, this approach was only partially successful, as it turned out that the efficiency of the transfection depends on the employed cell type and the PNA sequence [185,205].

Currently, the most common approach to improve the cellular uptake is the covalently conjugation of cell-penetrating peptides (CPPs) to PNA [174,175,185,194]. These peptides, which can transport molecules (oligonucleotides and peptides) across biological membranes, in a receptor independent way, comprise penetratin, transportan, Tat peptide and nuclear localization signal (NLS) [175,185,194]. The addition of CPPs to PNAs is an extremely efficient delivery methods, has no addition cellular toxicity and can also reduce the physiological clearance rates of these molecules therapeutic [174,193,194,206]. This latter consideration is particularly important since PNAs are hydrophilic molecules, which are excreted very rapidly through the kidneys [187,207,208]. The half-life of these compounds is approximately less than one hour, and this represents a challenge in terms of using these molecules *in vivo* [185,193,207,208]. Although conjugation of PNA with CPPs has many advantages, also contradictory observation have been raised [174,194]: the carrier, in addition to carrying PNA into the cytoplasm or nucleus, should also allow its release [174,193]. But, in some cases, PNAs were permanently trapped inside endosomes, and therefore they could not exert their activity [209]. Limited endosomal escape of CPP-PNA conjugates, often requires co-administration of chloroquine or Ca2+ to facilitate release into the cytosol; simultaneously limits the therapeutic efficiency of CPP-PNAs [194,210].

In conclusion, to date there is no general and easy to perform method, that can be universally used for cellular delivery of PNAs. In fact, it is necessary to evaluate from time to time several factors such as the nature and size of the molecule to be transported, the target cell and the site of action.

1.3.4 PNA-based strategy for gene modulation

The physico-chemical properties of PNAs make them a powerful tool with a wide range of applications, in many different fields [183,193,194,211]. Due to their unique properties, including resistance to enzymatic digestion, higher biostability combined with great hybridization affinity toward DNA and RNA, they are a great interest in medicinal and biotechnological areas [183,193,194,211,212].

PNAs are highly promising as potentially therapeutic agent, initially as candidate for antisense and antigene therapy, for inhibition of transcription and translation of target genes (Figure 10) [193,212]. More recently, other applications to produce specific genome modifications, such as splicing modulation and gene editing, have been developed [194,211,213].

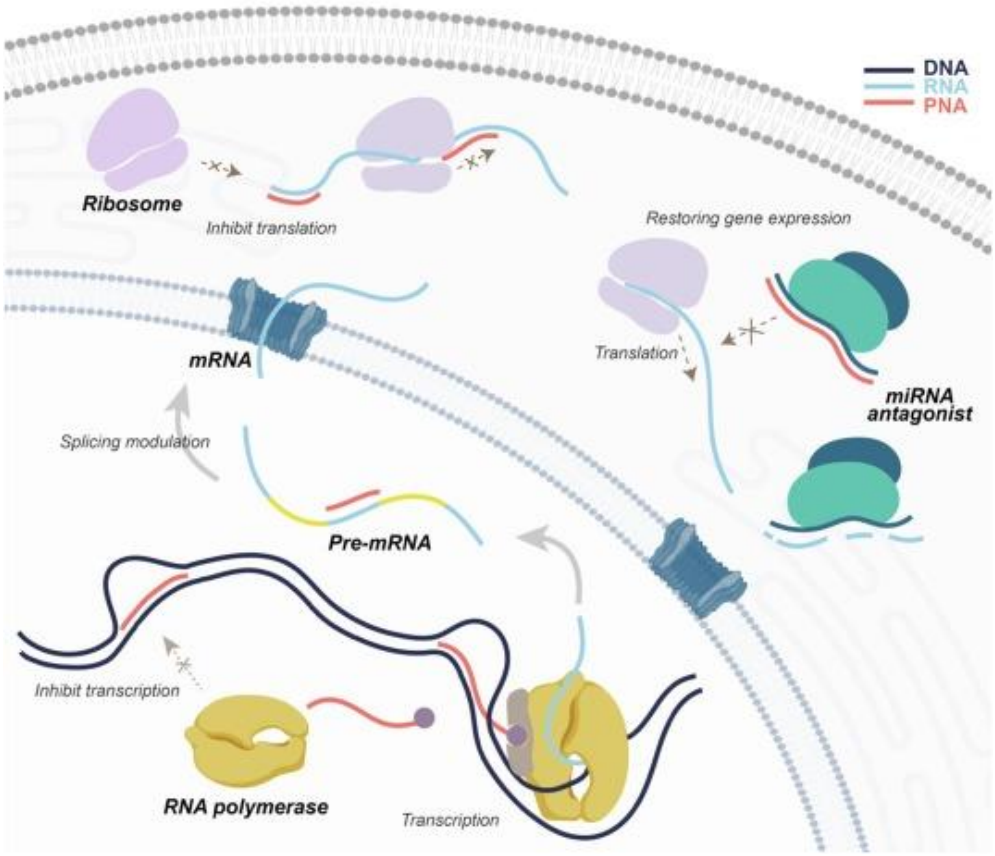

Figure 10. Antigene and antisense approaches [174]

Antigene strategy

PNAs used as antigene agents (agPNA) are designed to recognize and bind a complementary sequence of DNA in a target gene, interfering with its transcription and preventing the synthesis of the corrensponding mRNA (Figure 10) [194,212]. PNAs are capable of arresting

trascriptional processes thanks to their ability to form stable complexes with dsDNA (Figure 9). These different complexes can prevent the unwind of dsDNA, can hamper the binding of trascription factor to the promoter region or still can stop the elongation of RNA polymerase, leading to the formation of truncated transcripts [193]. This approach is advantageous over antisense mechanism because only one or two copies of the target sequence are evoked over many mRNAs in the cell [214]. The potential of agPNAs has been confirmed in many studies: as examples Alagpulinsa and colleagues have highlighted how a PNA targeting RAD51 gene's transcription start site, conjugated with a NLS to improve nuclear delivery, significantly reduced RAD51 expression, in multiple myeloma cells [215]; another PNA, designed to bind the second exon of c-Myc oncogene; reduced its expression in Burkitt's lymphoma cell line [216]. To explore the clinical applications in fibro-proliferative disorders, an agPNA was developed to target COL1A1 gene, resulting in a decrease of mRNA level of this gene, thereby reducing type I collagene production by fibroblast cells [217].

Antisense strategy

Antisense PNAs bind to complementary mRNA sequence of the target gene thereby blocking the translation processes and the synthesis of the associated protein, through the steric blockage (Figure 10) [194,208,212]. It has been observed that duplex-forming PNAs show the highest potency when targeted to 5' UTR of mRNA or to the translational starting codon, where they prevent ribosomal assembly. On the other hand, triplex forming PNAs can target the coding region, leading to the inhibition of the elongation process [193].

Beyond the regulation of protein-coding RNAs, there is a growing interest in the modulation of non-coding RNAs, such as micro-RNA (miR) [193,211,212]. Micro-RNA are short regulatory RNA (20-24 nucleotides), whose expression dysregulation has been implicated with various disease, including cardiavascular disease, hepatitis and cancer [212,218].

It has been reported that in a mouse model of lymphoma, a PNA encapsulated in unique polymer nanoparticles can efficently inhibit miR-155, which regulates various pathways associated with immune regulation and cell division [219,220]. Additionally, it has been shown that a PNA can be used to inhibit a cystic fibrosis transmembrane regulating miRNA, miR509-3p [221].

Modulation of splicing

Antisense PNAs are able to modulate splicing patterns, blocking specific intron-exon junctions in pre-mRNA [222–224]. Upon targeting intron–exon junction sites, spliceosome

either skips one exon (or more) or may retains intron sequence (Figure 11). Antisense-mediated exon skipping is similar to alternative splicing procedure, the results is the synthesis of a different protein [193,225]. On the contrary, intron retention leads to the production of nonfunctional mRNA containing an intron, which is susceptible to destabilization [193,226].

Since several diseases result from shifting in the splicing patterns of pre-mRNA, the employment of PNAs to block specific spicing sites, offers novel possibilities for therapeutic intervention [193,227]. Furthermore impairing the splicing mechanism can be usefull in various disorder-related genes such as tau, c-myc, IL-5R, bcl-x, dystrophin, and ß-globin to obtain therapeutically favorable results [193,228].

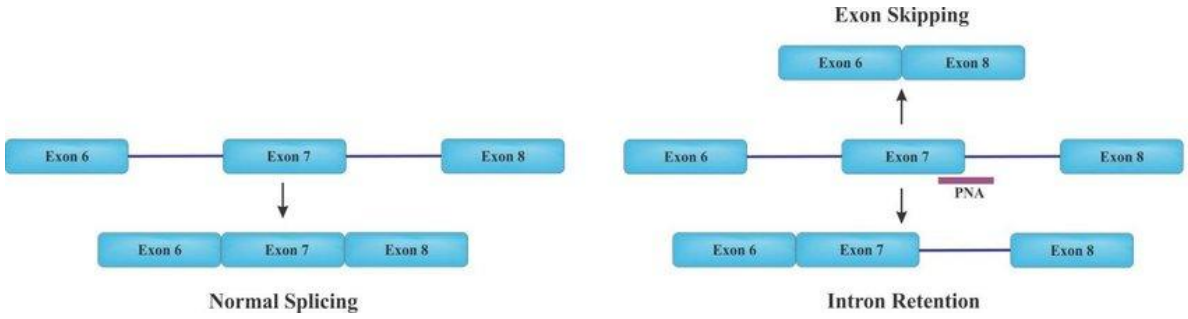

Figure 11. Splicing pattern of pre-mRNA. Left: Normal. Right: Modification of splicing by antisense PNAs [193]

Gene editing approaches

Two PNA oligomers are attached together by a linker, forming a bis-PNA: one strand binds in the anti-parallel orientation based on Watson-Crick rule, while the other strand is available for binding through Hoogsteen base pairing in the parallel orientation to dsDNA; thus leading to triplex formation. This structure results in a modified DNA helical, that is recognized by DNA repair system and leads to the recombination of "donor" DNA into the desired target site, close to the PNA binding site (Figure 12A) [193,194,211].

The combination of pcPNA and Ce(IV)-EDTA complex as an artificial DNA cutter (ARCUT) was found to promote homologous recombination in human cells by Katada et al. (Figure 12B) [229,230]. Its scission site in the genome is determined by Watson-Crick rule [231]. In the presence of donor dsDNA, the targeted gene repairs by homologous recombination have been achieved with high efficiency (> 50%) and with sufficiently high specificity to target a single site in the human genome in cultured cells [174,230].

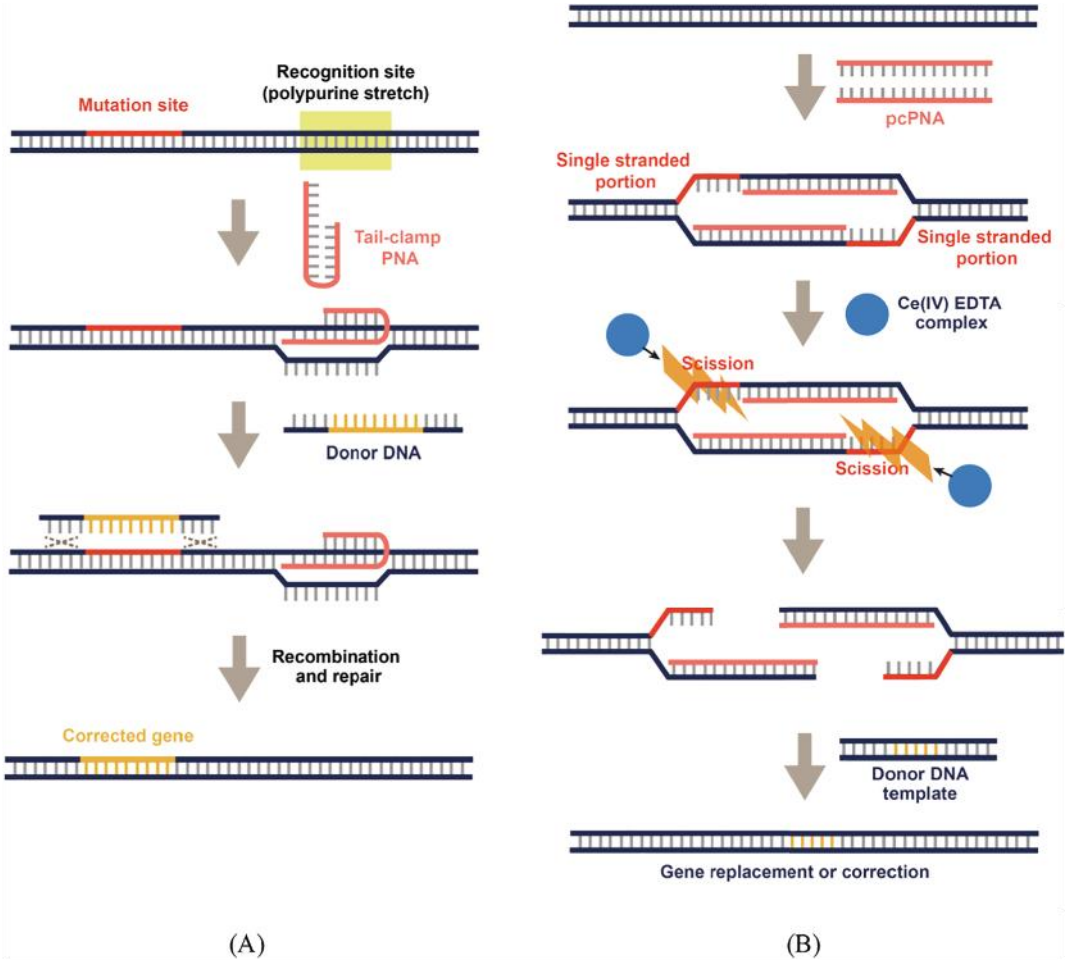

Figure 12. Gene editing mediated by PNA invasion (A) and by PNA-based artificial cutter (B) [174]

PNA-based gene editing technology was used to correct a base-pair mutation in the β-globin gene, responsible for β-thalassemia [232].

In addition to correcting disease-causing mutations, this technology was exploited to introduce stop codon in the CCR5 gene, to prevent HIV-1 infection. Hematopoietic stem cells (HSCs) were edited and subsequently injected into mice. Gene modification persisted in vivo for longer than four months post-engraftment and led to decreased viral load [233,234].

2. Aim of the research

Lung cancer is the leading cause of cancer related deaths worldwide and SCLC accounts for 15-20% of all lung neoplasms [6]. It is an extremely aggressive tumor characterized by poor prognosis and low survival rates, in fact it has been designated as a recalcitrant malignancy [235]. The treatment of this tumor continues to be a challenge and still relies on cytotoxic chemotherapy, that has not considerably changed for the past decades [1]. Immunotherapies have transformed the treatment of many cancers and recently, immune checkpoint inhibitors have been combined with chemotherapy in SCLC; however, the clinical benefits were limited, and only a small number of patients have benefited from these immune-based therapies [236–240]. Indeed, it is important to develop new strategies to overcome acquired resistance phenomena and to improve patients' clinical outcomes.

Numerous studies have reported that SCLC presents a high number of genetic alterations, including extensive chromosomal rearrangements, loss-of-function mutations of several tumor suppressor genes, copy number gains and other somatic mutations in oncogenes [35,241]. Among these genomic aberrations, MYC family members and in particular MYCN are altered in a majority of cases, representing the most prominent activating oncogene alteration in SCLC [43]. Since many evidences indicate that MYCN aberrant expression is related to tumor progression and poor outcome, it represents an attractive therapeutic target for MYCN-driven tumors [34,46,242]. Despite numerous strategies have been proposed to inhibit MYCN expression and reduce its molecular activation, they have largely failed and it is still considered undruggable [113,130]. In this scenario, the agPNA BGA002 was designed and developed by Biogenera SpA, as a novel inhibitor for the oncogene. In previous studies, BGA002 has demonstrated its efficacy in silencing MYCN expression in neuroblastoma (NB) [243,244], thus representing a novel precision medicine approach, also for MYCN-related SCLC.

In this context, my PhD project aims to study the efficacy and the safety profiles of BGA002 *in vitro*, through cellular and molecular experiments. The efficacy profile of the compound will be studied also *in vivo*, using MNA-SCLC xenograft ectotopic mouse models.

3. Materials and Methods

3.1 Cell lines and cell culture

For the experiments different SCLC cell lines are used. NCI-H69, H69AR, NCI-H526, DMS 79 and NCI-H510A were purchased from ATCC, GLC-14 was kindly provided by Professor E.G.E. de Vries, while NCI-N592 was kindly provided by Doctor S. Ferrini. HEK293T (Human Embryonic Kidney) cell line was purchased from DSMZ.

Cell lines are cultured in RPMI-1640 (Lonza, Verviers, Belgium) and supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Carlsbad, CA, USA) and 2 mM of L-Glutamine (Euroclone, Milan, Italy). All cell lines are incubated at 37°C in a 5% CO_2 atmosphere. Culture medium is renewed as needed depending on cell line.

Cell line	Site	MYCN status
NCI-H69	Pleural effusion	Amplified
H69AR	Pleural effusion	Amplified
NCI-N592	Bone marrow	Amplified
NCI-H526	Bone marrow	Amplified
GLC-14	Supraclavicular lymph node	Amplified
DMS 79	Pleural effusion	Overexpressing
NCI-H510A	Adrenal metastasis	Not Expressing
HEK293T	Kidney	Not Expressing

Table 1. List of cell lines used in this study

3.2 Cell line treatments

Cells were removed from flask and counted using a Burker's chamber. Then they were centrifuged and resuspended with RPMI-1640 supplemented with 0.5% FBS. Cell lines were seeded at a concentration of 3.5 x 10^5 cells/mL for 24-well plate or 1 x 10^5 cells/mL for 6-well plate and 96-well flat-bottom plate and then subjected to treatment. After 6 hours of treatment, 9.5% of FBS was added to cells.

PNA anti-MYCN (BGA002) was designed and prepared according to previously published studies [245–247]. The antigene PNA oligonucleotide was prepared by the chemistry department of Biogenera SpA and provided to biology departement as a powder resuspended

in NaCl 0.9%. The agPNA was freshly produced and used or stored at 4°C. siRNA for CNTFR was mixed with Lipofectamine (Invitrogen) and then diluited in RPMI-1640.

3.3 Quantitative real-time PCR

After 12 hours of treatment, cells were transferred to a 1.5 ml Eppendorf tube and centrifuged. The pellet was lysed and RNA was extracted using the RNAspin Mini RNA isolation Kit (GE Healthcare). Each sample was quantified with NanoDrop Spectrophotometer ND-100 (Thermo Fisher Scientific) and then retrotranscribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem).

The cDNA obtained was used to perform Real Time PCR: 10 ng of each sample were loaded in duplicates then real Time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and CFX Connect Real-Time PCR Detection System (Bio-Rad).

Crossing points (Cp) from each analyte were calculated using the second derivative maximum method, and the expression level was quantified by comparison with the BIRC4 gene.

The list of primers used in this study in reported in the following Table 2.

Gene	Forward primer	Reverse primer
ACACA	CAGAGGGAACATCCCTACGC	AAGAGACCATTCCGCCCATC
AKT1	GCACAAACGAGGGGAGTACA	AAGGTGCGTTCGATGACAGT
AKT1S1	TGAGCCCACAGAGACAGAGA	CGGGGTCTGACTCACAGAAG
AKT2	TGATGGAGTATGCCAACGGG	GTCCTCCAGCACCTTGATGT
ATXN2	GGAACGTGGTCATCAGTGGT	CAGCTTGGGGAGAAGCAAGA
ATXN2L	CAGCCATTGCCATGAACTCG	GCTCTCGCTGACGAAACTCT
BIRC4	ACAAGGAGCAGCTTGCAAGA	AGCATGTTGTTCCCAAGGGT
CTRC2	GAGGGACGGGGAAGGAAGAT	TGTGTATGCCAGTCGCAGTT
EIF4EBP1	ACCTGTGACCAAAACACCCC	GGTAGTGCTCCACACGATGG
EIF4G1	ACCTGTGTGACGAGCAGAAG	ACGGAGCCACTTGAAGAAGG
FASN	AGCAGTTCACGGACATGGAG	ATGGTACTTGGCCTTGGGTG
G6PC3	GGGTCCATGAGTCTGGTTACTAC	CTGGTGAGGGAAATGTGCTAAGAT
G6PD	CCTTCCATCAGTCGGATACACA	ATAGCCCACGATGAAGGTGTTT
IGF2	TCGTTGAGGAGTGCTGTTTC	GTATCTGGGGAAGTTGTCCG
MDH2	GTTCAACACCAATGCCACGATT	AAACTTCTGCTGTGATGGGGATG
MLST8	TGGCAGCTGTCAATAGCACC	TCTTGATGCTCAGCTCCGTC

MYCN I	ACCCGGAGACACCCGCGCAGAATC	GTAGAAGCAGGGCTGTAGCGAGTC
NANOS1	TGTTGGGGGTCCTTCATGTG	TGAGGGTGGGAGGGTAAAGT
NBAS	CAGTACCGAAGACACTGGGATT	CATTCCCCTTGTTGCTTCAGAG
NFKB1	ATCTGCACTGTAACTGCTGGA	TGGCGGATTAGCTCTTTTTCC
RELA	TGTATTTCACGGGACCAGGC	GGTCCGCTGAAAGGACTCTT
RPTOR	GGTGCTGTTAAGCCAAGTGC	TAGGGGAAGATGCCGACAGA
SAMD4B	CCCCAGGCCATTCTCATGTT	GTCTGTCCCATCACCCAAGG
SLC1A5	ACCATATCTCCTTGATCCTGGC	TACGGTCCACGTAATTTTGGAG
SLC7A5	CTCTTCCTGATCGCCGTCTC	GACCACCTGCATGAGCTTCT
TNFAIP3	ATCAAAATGGCTTCCACAGAC	TGGAGAGGCAAGTAAATTCCAC
TP73	TCCGCGTGGAAGGCAATAAT	ATGGTGGTGAATTCCGTCCC
WDTC1	GGACATGGGCTCCTGAAGTT	GGGGTCAGTGACATGGTAGC

Table 2. List of primers used in this study

3.3 Cell viability assay

Cell viability was evaluated through CellTiter-Glo® Luminescent Cell Viability Assay developed by Promega. Cells were seeded and treated as previously described. After 72 hours 50 µl CellTiter-Glo reactive were directly added to each well. The plate was incubated for 10 min at room temperature, to induce cell lysis and then luminescence was measured using Tecan Infinite F200.

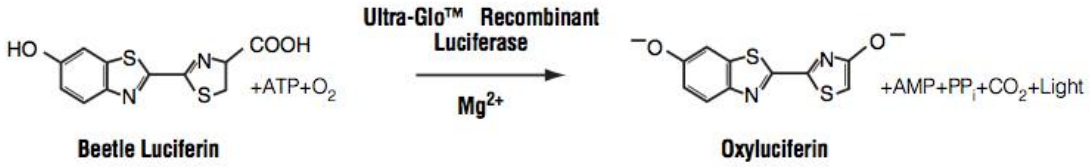

Figure 13. CellTiter-Glo mechanism of action (image from CellTiter-Glo product manual)

3.4 Western Blot analysis

Western blot analyses were performed by standard methods. After 24 hours of treatment, cells were lysed in RIPA lysis buffer with protease inhibitor (Thermo Fisher Scientific), and then

sonicated. Protein fraction were collected by centrifugation at 13000 g, 4 °C, for 10 min.
A total of 25 μg of proteins were loaded and separated by SDS–PAGE using Criterion TGX polyacrylamide gels (Bio-Rad) and blotted into a nitrocellulose membrane (Bio-Rad). The following antibodies from Cell Signaling Technology were employed: anti-Phospho-Akt (Ser473) (#4060) 1:1000; anti-Akt (#9272) 1:1000; anti-Phospho-p70 S6 Kinase (Thr389) (#9206) 1:1000; anti-p70 S6 Kinase (#9202) 1:1000; anti-Phospho-S6 Ribosomal Protein (Ser235/236) (#4858) 1:1000; anti-S6 Ribosomal Protein (#2217) 1:1000; anti-Phospho-4E-BP1 (Thr37/46) (#2855) 1:1000; anti-4E-BP1 (#9452) 1:1000; and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#5174) 1:1000. To analyze N-Myc protein expression sc-53993, 1:800 (Santa Cruz Biotechnology) was used. Bands were detected using the Cyanagen Westar ECL western blotting detection reagent. Images were captured by ChemiDoc-It2 Imaging System and analyzed with the Vision Works LS Software (UVP).

3.5 Apoptosis analysis

NCI-H69, H69AR, NCI-N592 and NCI-H510A cells were treated as described previously. After 48 hours of treatment cells were stained with Annexin V/FLUOS Staining Kit (Roche) according to manifacturere's instructions. All samples were analyzed through the CytoFLEX Flow Cytometer (Beckman Coulter). The data were analyzed using FCS Express 7 (De Novo Software).

3.6 Transmission electron microscopy

NCI-H69, H69AR, NCI-N592 cells were seeded in a 6-well plate. After 24 hours, cells were treated with with NaCl 0.9%, BGA002 10 μM in low FBS culture medium. After 6 hours, up to 10% of FBS was added. After 48 hours of treatment cells were fixed. Cells were removed from well, washed in warmed PBS then fixed with 2.5% (v/v) glutaraldehyde in cacodylate buffer 0.1mol/L for 2 h at 4°C. Cells were then post fixed with a solution of 1% osmium tetroxide in 0.1 mol/L cacodylate buffer and embedded in epoxy resins after a graded-aceton serial dehydration step. Ultrathin slices of 100 nm were stained by uranyl acetate solution and lead citrate, and then observed with transmission electron microscope CM100 Philips (FEI Company) at an accelerating voltage of 80 kV. Images were recorded by Megaview III digital camera (FEI Company).

3.7 Lysosomes distribution analysis

The NCI-H69, H69AR and NCI-N592 cell lines were seeded in a Nunc Lab-Tek Flask on Slide for live staining. Treatment was administered 48 hours before acquisition. Lysosome Staining Kit (Green Fluorescence, Abnova) was added and the cells were incubated for 45 min at 37°C, at 5% CO_2. For each condition, z-stacks (at a 200 nm interplane distance) were acquired using a Nikon Ti2-E microscope (Nikon). Images were elaborated using the Fiji distribution of ImageJ software. Images containing lysosome were modified for background reduction. Each images was duplicated then maximum with radius 3 is applied, then gaussian blur was applied with sigma 100 μm in one image replicates. In the other replicates subtract backgrouns was applied with rolling ball methods with radius 100 μm. The first image was then subtracted to the second one using image calculator then subtracting, then gussian blur of 1 μm was applied. Images were then bynarized using threshold, as method was selected minum and finally were watershedded. Lysosomes were then analyzed using Analyze > Analyze Particles, with the lower value size set to 0.1 μm^2.

3.8 SCLC luminescent cells

The luciferase retroviral espression plasmid pMMP-Lucneo (kindly provided by Professor Andrew Kung) was transfected with Lipofectamine 2000 (Invitrogen) into Phoenix cells to generate the amphotropic retrovirus. The viral particles were collected at 48 and 72 hours after transfection. The NCI-H69 and H69AR cell lines were spinoculated with the viral particles and polybrene (hexadimethrine bromide, Sigma). Cells were incubated for 48 hours in growth medium and then subjected to selection for 15 days with 1 mg/mL of G418 (Calbiochem). The best cell clones were selected and their luminescence was measured. The resulting cell lines were named respectively NCI-H69-Luc and H69AR-Luc.

3.9 Xenograft ectotopic SCLC mouse models

Six-week-old severe combined immunodeficient mice (NOD.CB17-Prkdc^{scid}/NCrCrl) were purchased from Charles River Laboratories. Mice of both sexes were separated into different groups for each type of cell line and then inoculated with NCI-H69-Luc or H69AR-Luc cells. A total of 10 x 10^6 cells growing at logarithmic phase were centrifuged and resuspended in

100 µL of physiologic solution. Mice were sedated with isoflurane and then cell suspensions were injected subcutaneously in the dorso–posterior–lateral position with an insulin syringe.
The engraftment of the tumor was evaluated by luminescence acquisition. D-Luciferin was administered by intraperitoneal injection. Luminescence was acquired by the UviTec Imaging System (Uvitec). Treatment was performed after the tumor reached the predefined starting point in the bioluminescent acquisition. Mice were treated daily for 28 days with vehicle, BGA002 (50 mg/kg/day) or BGA002 (100 mg/kg/day). Animals were monitored once every other day, using a caliper, for tumor mass assesment. An endpoint of at least 20 mm tumor diameter and a total tumor volume of 4188 mm^3 were established. Animals were sacrified when they reached the predefined endpoint or 60 days post treatment. Tumors were removed, measured, weigthed, and fixed in 4% formalin.

3.10 Histological and IHC analyses

After fixation, tumors were washed under running water and kept in 70% ethanol solution. The tumors were dehydrated, embedded in paraffin, and cut into 4 mm sections. Paraffin removal was accomplished by incubating histologic slides in toluene followed by incubation in ethanol. The slides were incubated in 2% H_2O_2/methanol for inhibition of endogenous peroxidase activity. Hydration was performed by serial incubation with 96% ethanol, 70% ethanol, and distilled water.
For the histological analysis the slides were stained with haematoxylin, dehydrated, and mounted.
For the immunochemistry analysis, after hydration, antigen retrieval was performed by heat processing in 1 mmol/L EDTA, pH 8, both for N-Myc antibody and Ki-67 antibodies. The slides were blocked with 10% BSA in PBS, stained with the N-Myc (OP13, Calbiochem) and Ki-67 (MIB1, Dako) antibodies and subsequently treated with secondary antibody (peroxidase conjugated anti-mouse, Dako). The peroxidase coloration reaction was performed using the Dako DAB Kit. Images were acquired with the Leitz Diaplan microscope.

3.11 Statistical analyses

All data were analyzed using GraphPad Prism 8 software or with R software version 3.5 or Python software version 3.7. The significance of difference between two experimental groups was determined by a Student’s t-test. Survival curves were estimated using the Kaplan–Meier method and compared with a log-rank test (Mantel-Cox). The different analyses and tests

were specifically designed for each experiment; p values ≤ 0.05 were considered statistically significant.

3.12 Data availability

Expression data analysed in this study were obtained from Cancer Cell Line Encyclopedia, from the European Genome-phenome Archive, under accession number EGAS00001000925 and from EMBL-EB, using accession number E-MTAB-1781.

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4. Results

4.1 BGA002 treatment inhibits expression of both MYCN mRNA and protein in SCLC

A panel of six SCLC cell lines was selected, to determine BGA002 in vitro activity: five cell lines were MYCN amplified (MNA) while one was characterized by MYCN overexpression, but not amplification (not-MNA). All cells expressing high level of MYCN mRNA (Figure 15A), showed a dose-dependent decrease in MYCN transcript, following 12 hours of treatment (Figure 14A). Thanks to dose-response curves (Figure 15C), EC_{50} values reported in Figure 15B were calculated.

In order to verify that the reduction of mRNA, corresponded to a decrease in N-Myc protein levels, western blot analysis was conducted on different MNA-SCLC cells. Experiments carried out in collaboration with the group of Prof. Martelli, showed a diminishment in N-Myc protein in all cells analysed; interestingly the trend of reduction was confirmed also in H69AR, that is a multidrug-resistant clone of NCI-H69 (Figure 14C).

4.2 MYCN reduction is accompanied by cell growth inhibition in SCLC cells

Cell viability inhibition was tested after 72 hours of treatment in all SCLC cells, with the same doses used in previous qPCR experiments, from 0.6 μM to 20 μM of BGA002. The observed reduction in MYCN activity was followed by a decrease in cell growth, in all cell lines analysed (Figure 14B). Also for these experiments, dose-response curves (Figure 15C) exhibited low GI_{50} values, in fact, they ranged approximately from 2.50 μM to 5.50 μM (Figure 15B).

To further investigate the mechanism behind the reduction in cell proliferation, flow cytometry analysis was performed on MNA-SCLC cells. These experiments showed an inducement of apoptosis, following 48 hours of treatment (Figure 14D). Also in this scenario, NCI-H69 and its resistant clone H69AR, showed a comparable behaviour; whereas in NCI-N592 dose-dependent apoptosis activation was more evident (Figure 14D). The induction of apoptosis was also confirmed by TEM analysis, conducted on NCI-H69 and H69AR cells (Figure 16). After treatment, can be observed cells in early apoptosis, which showed the first signs of nuclear chromatin condensation; at the same time there were also cells in late

apoptosis, in which semilunar chromatin condensation were evident, and there were no more recognizable cytoplasmatic structures (Figure 16).

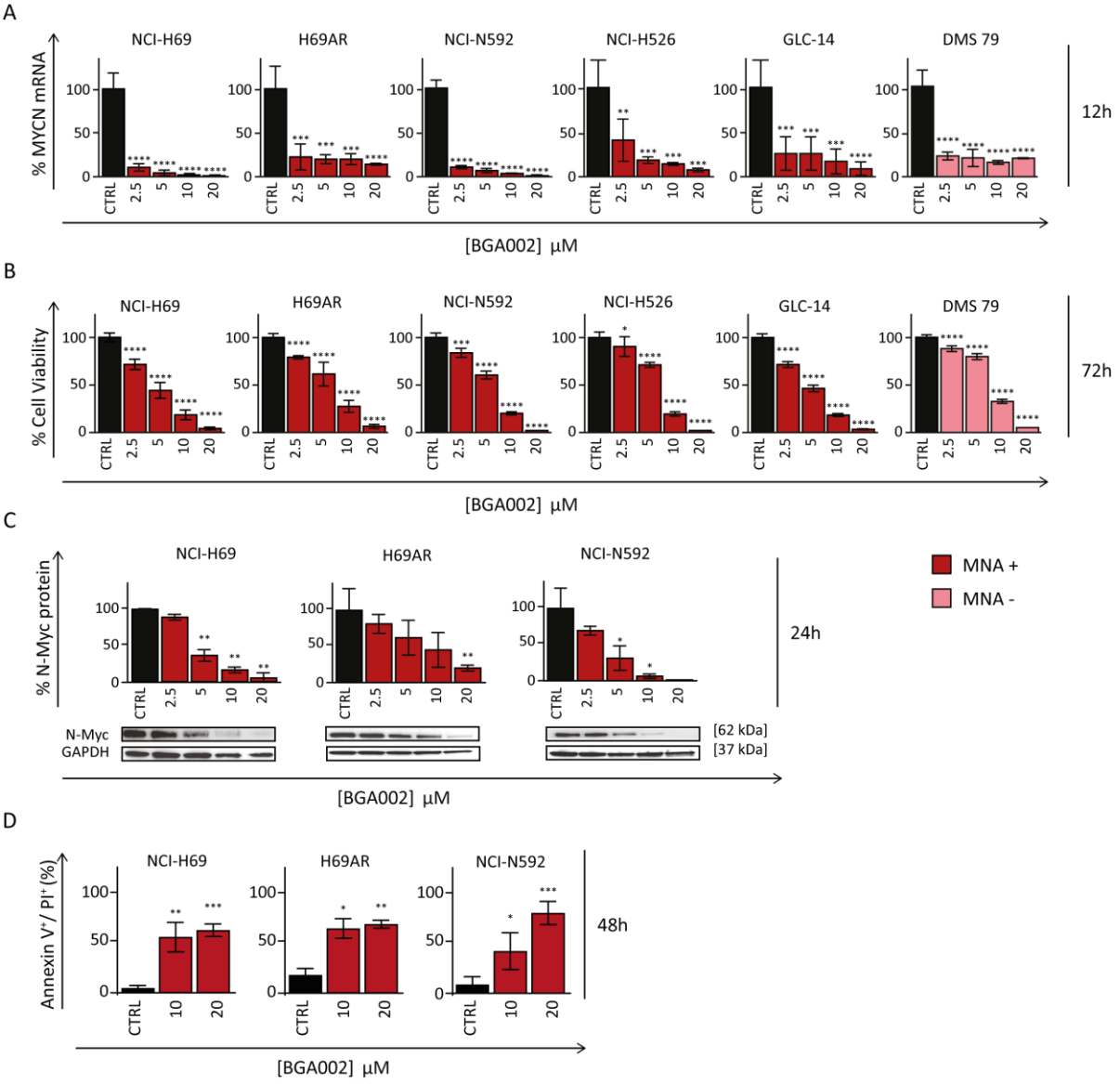
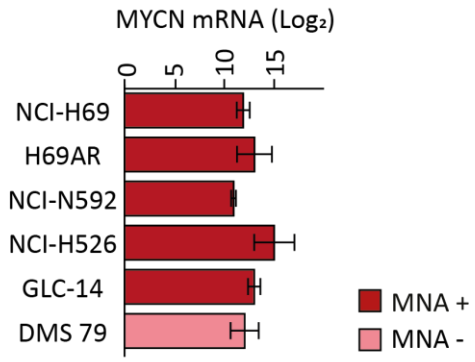


Figure 14. A: MYCN mRNA expression inhibition through RT-PCR after 12 hours of treatment in SCLC cell lines (n=3, biological replicates for each cell line). **B:** Cell viability assay after 72 hours of treatment in SCLC cells (n=3, biological replicates for each cell line). **C:** Western blot analysis after 24 hours of treatment. Representative staining for N-Myc (top) and associated GAPDH staining (bottom) is shown. Bar plot indicating the quantification of N-Myc expression, normalized to GAPDH signal (n=3, biological replicates for each cell line). **D:** Apoptosis measured after 48 hours of treatment for NCI-69 (left), H69AR (middle) and NCI-N592 (right) cell lines. Bar graph representing the percentage of apoptotic cells (Annexin V^+/PI^+) (n=3, biological replicates for each cell line). Data are analyzed with two-tailed Student's t-test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; where not indicated p -value > 0.05.

A



B

	MYCN mRNA EC_{50} [µM]	Cell Viability EC_{50} [µM]
NCI-H69	0.27	3.54
H69AR	0.41	4.98
NCI-N592	0.24	3.92
NCI-H526	0.80	5.01
GLC-14	0.60	2.58
DMS 79	0.72	5.49

C

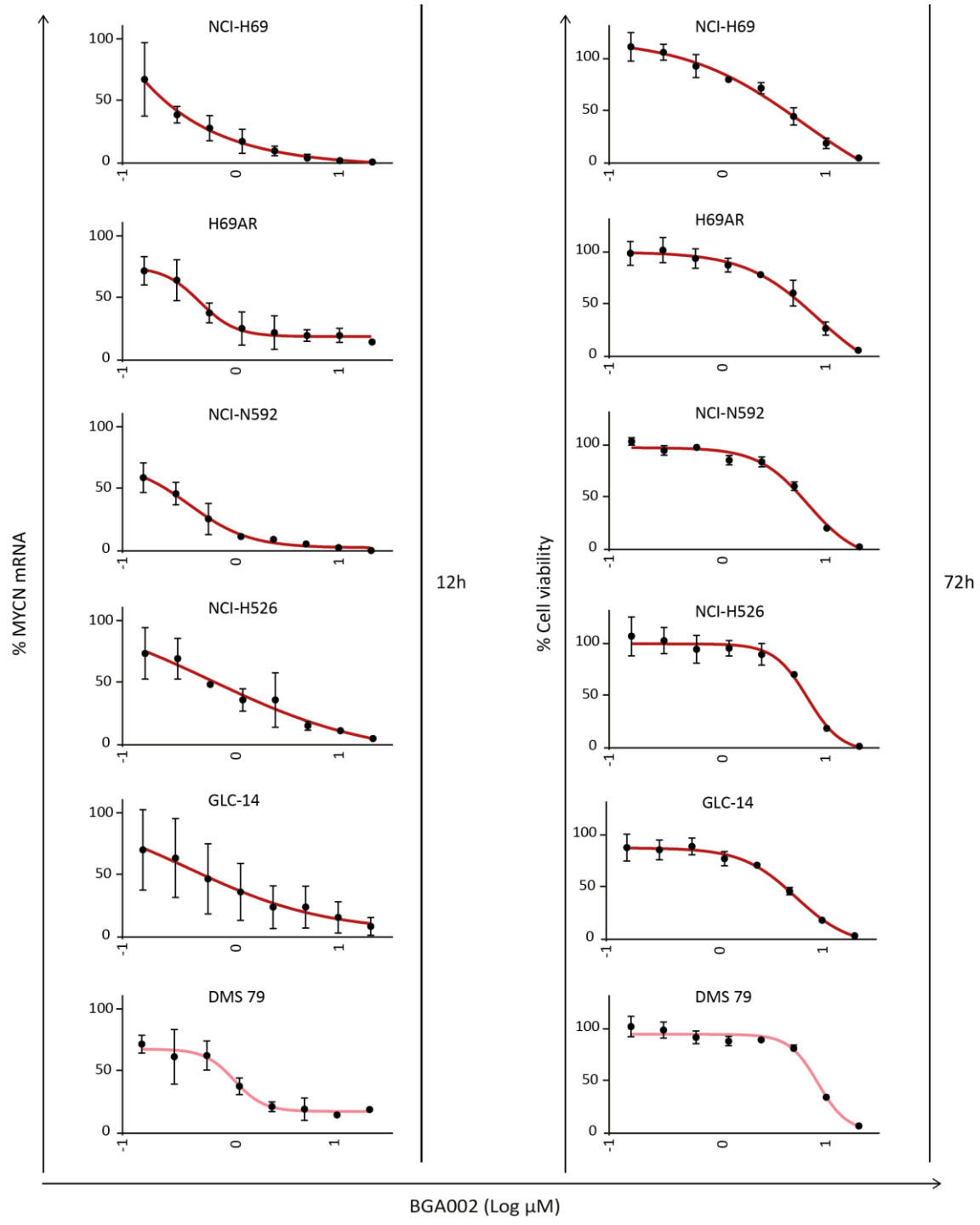


Figure 15. A: MYCN mRNA basal expression across different SCLC cell lines. Values normalized to MYCN expression in the NCI-H1694 (negative control). **B:** EC_{50} values for both mRNA expression and viability, for each SCLC cell line. **C:** Non-linear regression analysis in order to evaluate EC_{50} values. On the left, dose-response curves regarding MYCN mRNA expression inhibition by RT-PCR (n=3, biological replicates for each cell line). The curves on the right are referred to cell viability inhibition (n=3, biological replicates for each cell line).

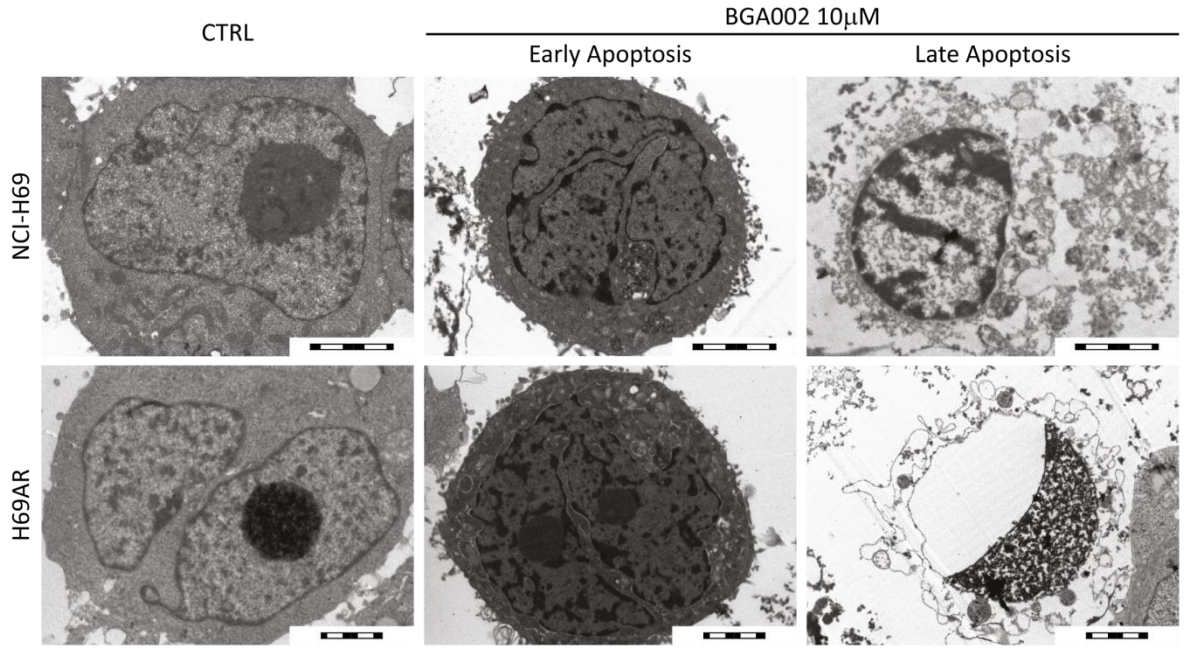


Figure 16. Transmission electron microscopy images of cells untreated or treated with BGA002 (10μM) for 48 hours. NCI-H69 (top) and H69AR (down) representative images for each condition are presented (n=2 experiments for each cell line). Signatures structures appeared for both early and late apoptosis.

4.3 BGA002 is a specific antigene oligonucleotide anti-MYCN oncogene

To verify if BGA002 is a specific inhibitor, different experiments on cells that do not express MYCN, were performed. BGA002 did not alter cell viability in the non tumorigenic MYCN-unexpressed HEK293 cell line (Figure 17A), except for the concentration of 20 µM. In addition, the antigene oligonucleotide did not induce apoptosis in NCI-H510A, a SCLC cell line that do not express the target (Figure 17B). In all conditions cell population was predominantly alive.

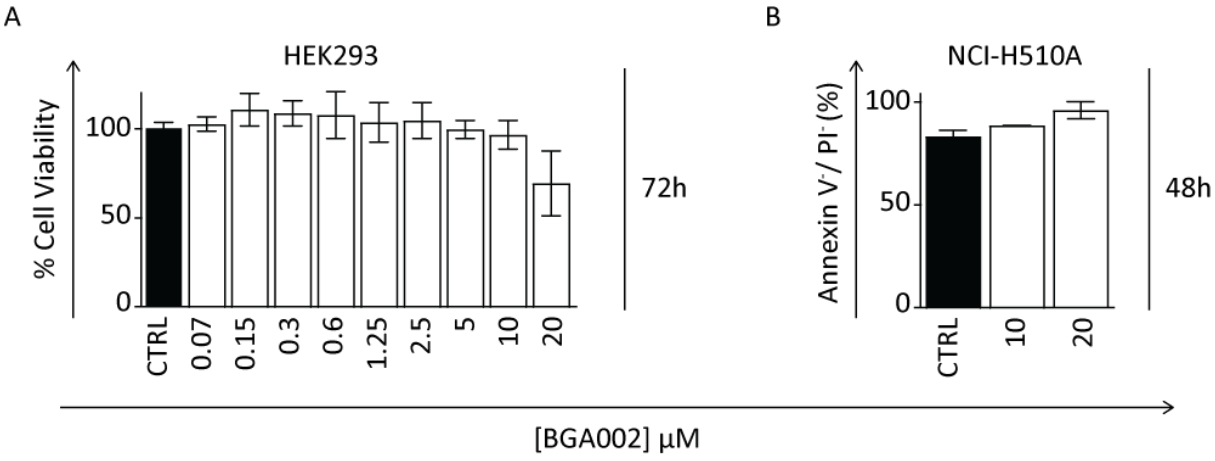


Figure 17. A: Viability inhibition in HEK293 cell line after 72 hours. Cells treated with increasing doses of BGA002 (n=3, biological replicates); values are normalized to the control. **B:** Apoptosis measured after 48 hours of treatment for NCI-H510A (negative control). Bar graph represent the percentage of live cell population stained by Annexin V^-/PI^- (n=2, biological replicates). Data are analyzed with two-tailed Student's t-test, where not indicated *p*-value > 0.05.

4.4 Blocking of MYCN leads to specific gene expression signature in SCLC cell lines

The three members belonging to the MYC family of oncogenes are also known as "super-transcription factors", since they regulate at least 15% of the human genome, promoting cell growth and division, angiogenesis, cell differentiation, as well as increasing cell metabolism [248]. In fact, their deregulation, which often occurs in cancer, consequently up- and down-regulates a subset of genes responsible for uncontrolled cell growth, genomic instability and immune surveillance escape, leading to tumor progression and development [46,120,248].
Thus, was performed a gene expression profile analysis in order to identify genes up- or down-regulated following MYCN inhibition, in SCLC cells. Genes analysed through qPCR experiments and reported in the heatmap (Figure 18A) are involved in different pathways, such as glucose metabolism (e.g. G6PD, G6PC3) [249,250], apoptosis (TP73) [251] and protein synthesis (EIF4G1) [252]; all of them are linked to tumor maintance and progression [253–255]. Interestingly, the expression of these genes was down-regulated following BGA002 treatment, in all SCLC cell lines (Figure 18A).

Among all different genes analysed, CLCF1-CNTFR signaling is emerging as a target for specific therapies for NSCLC [256–260]. MYCN inhibition by BGA002, resulted in a strong down-regulation of CNTFR in all SCLC cells tested, independently by the MNA status or multidrug-resistance (Figure 18A). Moreover, CNTFR showed low expression in the majority of tumor types, while in NB and SCLC it had the highest expression (Figure 18B). These premises suggested that BGA002 may exert its role against MYCN-related SCLC, through CNTFR inhibition. Silecing of CNTFR heavily reduced cell viability in MNA-SCLC (NCI-N592), accounting for a large percentage of the effect mediated by BGA002, underlying the importance of CNTFR in this context (Figure 18C).
To further extend these findings, was evaluated if MYCN inhibition can affect CNTFR expression in NB. CNTFR expression in MNA-NB cells (Kelly) was reduced after BGA002 administration, extending the relationship between MYCN and CNTFR also in another MYCN-driven tumor (Figure 18D).

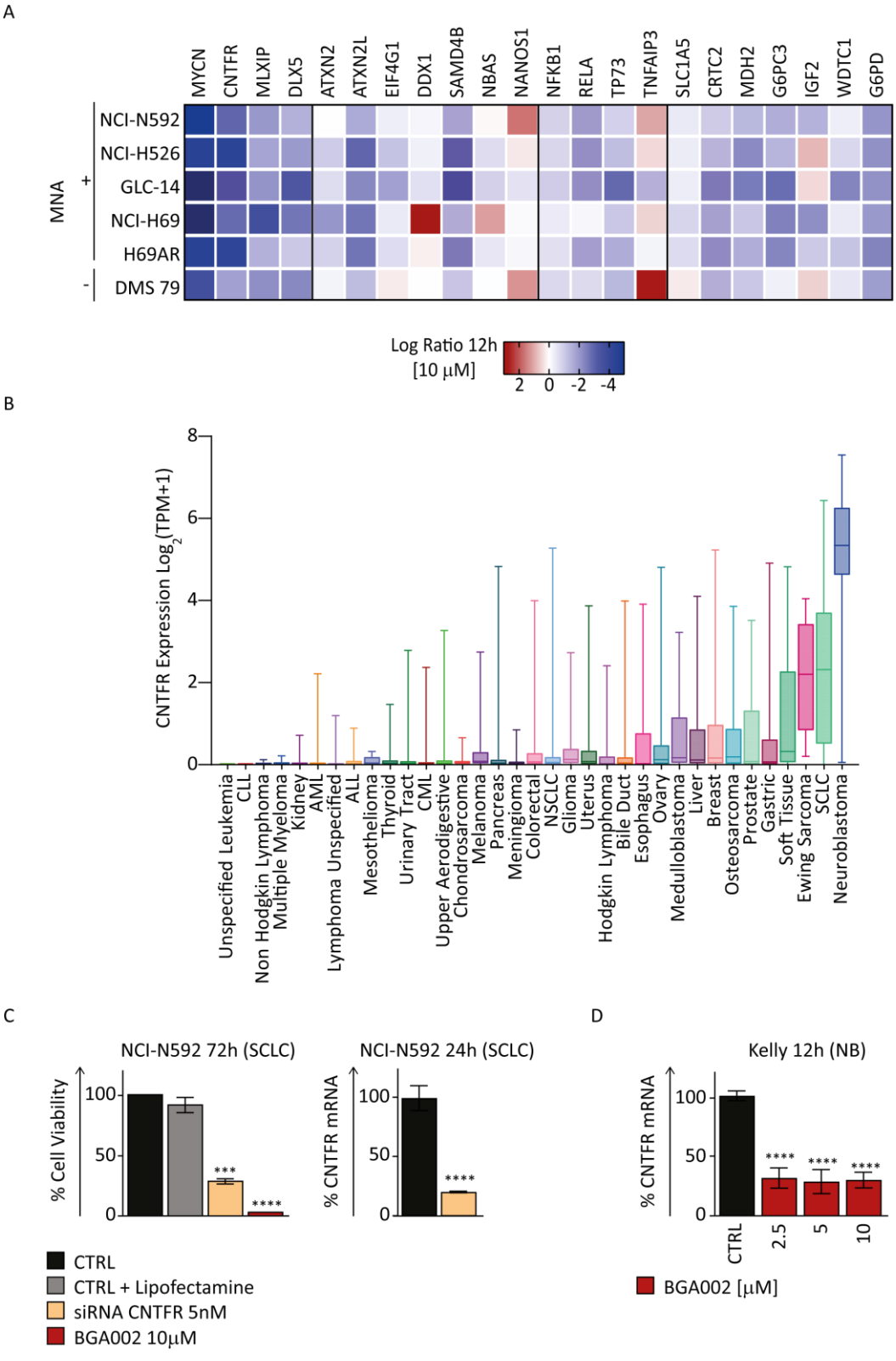


Figure 18. A: Heatmap of cancer related gene expression variation after 12 hours of BGA002 treatment (10µM) in SCLC cells. **B:** CNTFR expression in tumor cell lines as reported in CCLE. SCLC showed the second higher value of CNTFR expression among other tumor types. Middle line indicates the mean, the whiskers represent the standard deviation. **C:** Cell viability assay after treatment with BGA002 or CNTFR siRNA in NCI-N592 cell line (left). Gene expression inhibition in NCI-N592 cell line after treatment with the same CNTFR siRNA used for viability assessment (right). **D:** CNTFR expression inhibition after treatment with BGA002 for 12 hours at different concentrations, in MNA-NB cell line (Kelly). Data are analyzed with two-tailed Student's t-test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; where not indicated p - value > 0.05.

DLX5 is another gene significantly down-regulated upon MYCN inhibition by BGA002, in all SCLC cells (Figure 18A). The DLX5 homeodomain is associated with lymphomas, breast cancer and lung cancer; in particular it promotes cancer development and progression by binding the promoter region of MYC [261,262]. In addition DLX5 expression was correlated with poor prognosis in lung cancer [263,264]. Among different tumor types, DLX5 showed the highest expression in SCLC (Figure 19A). Gene expression analysis conducted in SCLC and NB patients confirmed the correlation between MYCN and DLX5. Overexpression of DLX5 was an exclusive features of MYCN overexpressing patients in SCLC, in comparison to MYC and MYCL expressing patients (Figure 19B).
In a similar way, DLX5 expression was influenced by MYCN amplification status in NB, where was also an indicator of poor prognosis (Figure 19C). Furthermore, MYCN inhibition led to a consistent reduction of DLX5 in MNA-NB (Figure 19D).

BGA002 was able to induce up-regulation of genes in MYCN-related SCLC, as in the case of TNFAIP3 (Figure 18A). TNFAIP3 is a potent anti-inflammatory protein and a crucial gatekeeper of inflammation homeostatis, mainly through the regulation of NF-κB pathway [265,266]. Indeed, also two relevent genes (NFKB1 and RELA) involved in this pathway, were down-regulated by BGA002 in SCLC cells (Figure 18A).
The implication of TNFAIP3 in SCLC was confirmed by the analysis of its expression in a panel of different tumors, where TNFAIP3 showed the second lowest expression in SCLC (the lowest expression was reported in NB) (Figure 20A).
Within the MYC family, gene expression analysis in SCLC patients, showed a specific association between MYCN and TNFAIP3, while did not with MYC or MYCL (Figure 20B). In particular, high expression of MYCN was correlated to low levels of TNFAIP3 (Figure 20B). Gene expression analysis performed in NB patients confirmed this correlation: MNA-NB patients showed lower level of TNFAIP3, compared to non-MNA patients and survival probability appeared higher when TNFAIP3 expression was maintained (Figure 20C).
Interestingly, BGA002 was able to restore TNFAIP3 expression also in MNA-NB cells (Figure 20D).
In conclusion, BGA002 was capable of modulating different genes, via MYCN inhibition. In particular, it was demonstrated that the expression of CNTFR, DLX5 and TNFAIP3 was affected following BGA002 treatment, in both SCLC and NB.

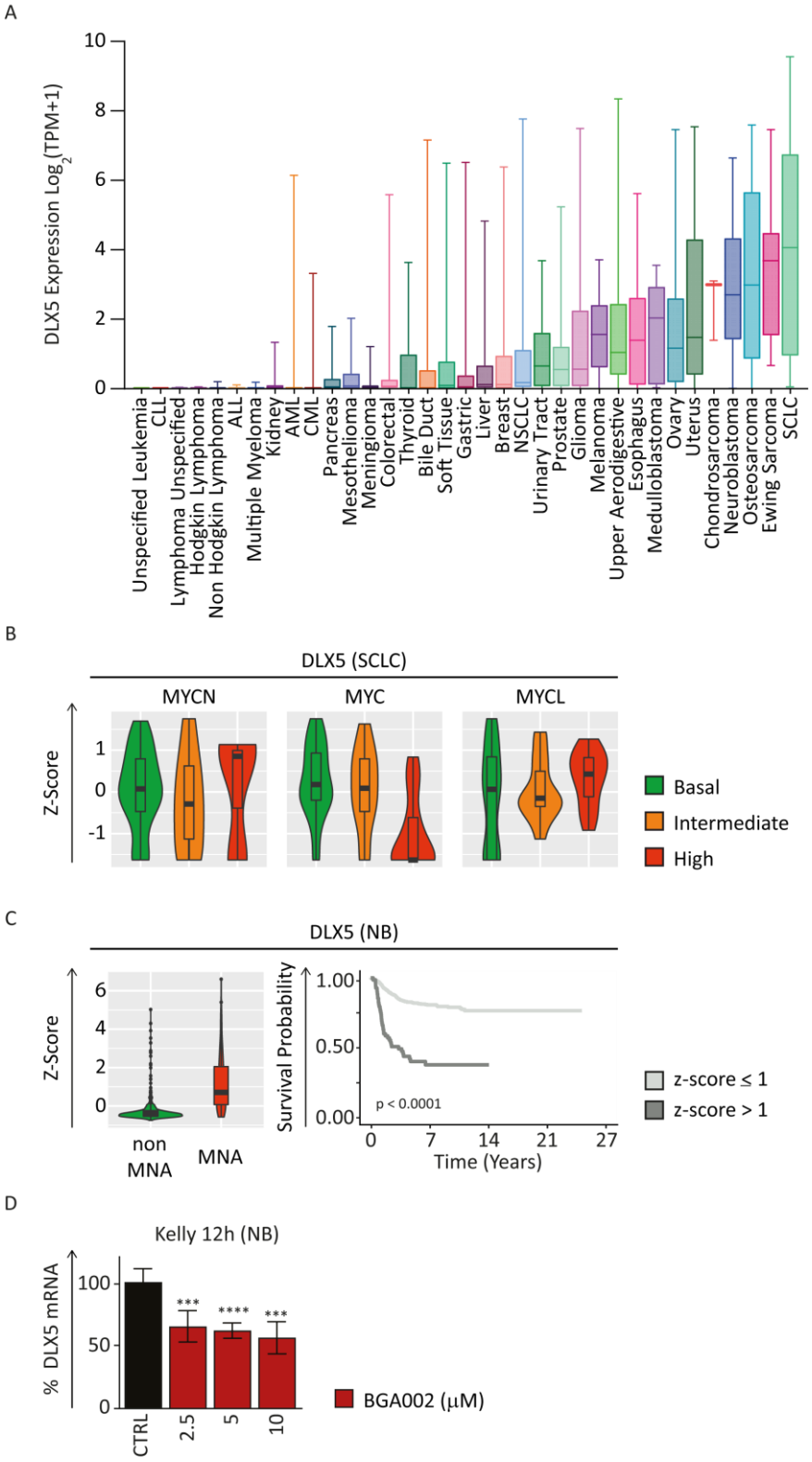


Figure 19. A: DLX5 expression in tumor cell lines as reported in CCLE. SCLC showed the highest value of DLX5 expression among the other tumor types. Middle line indicates the mean, the whiskers represent the standard deviation. **B:** Expression of DLX5 presented as Z-score according to MYCN (left), MYC (middle) or MYCL (right) level of expression in SCLC patients. Middle line indicates the median; the whiskers indicate samples within 1.5 times the interquartile range. **C:** Expression of DLX5 in MNA and non-MNA NB patients presented as Z-score (left). Each dot represents an individual sample. Kaplan–Meier plot for the probability of overall survival over time for NB patients (right). The dark gray line represents a z-score > 1. *p*-value (log-rank test) is shown at the bottom left of the plot. **D:** DLX5 expression inhibition after treatment with BGA002 for 12 hours at different concentrations, in MNA-NB cell line (Kelly). Data are analyzed with a two-tailed Student's t-test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; where not indicated *p* -value > 0.05.

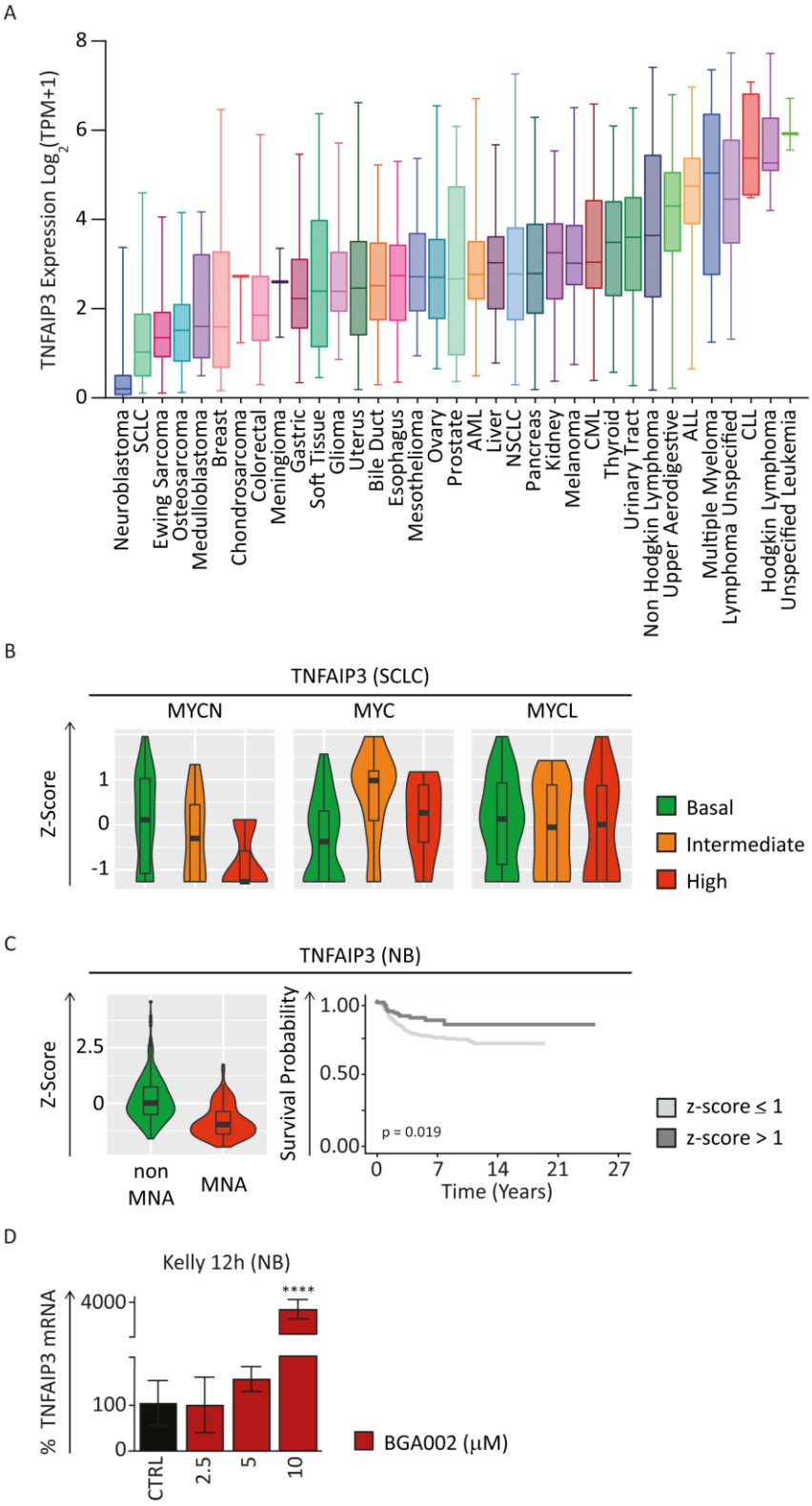


Figure 20. A: TNFAIP3 expression in tumor cell lines as reported in CCLE. SCLC showed the lower value of TNFAIP3 expression among the other tumor types. Middle line indicates the mean, the whiskers represent the standard deviation. **B:** Expression of TNFAIP3 presented as Z-score according to MYCN (left), MYC (middle) or MYCL (right) level of expression in SCLC patients. Middle line indicates the median; the whiskers indicate samples within 1.5 times the interquartile range. **C:** Expression of TNFAIP3 in MNA and non-MNA patients presented as Z-score (left). Each dot represents an individual sample. Kaplan–Meier plot for the probability of overall survival over time for NB patients (right). The dark gray line represents a z-score > 1. *p*-value (log-rank test) is shown at the bottom left of the plot. **D:** TNFAIP3 expression upregulation after treatment with BGA002 for 12 hours at different concentrations, in MNA-NB cell line (Kelly). Data are analyzed with two-tailed Student's t-test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; where not indicated p -value > 0.05.

4.5 MYCN inhibition affects mTOR complex in SCLC cells

Data in literature have demonstrated that MYCN amplification led to the activation of many downstream pathways including PI3K/AKT/mTOR, that is a master regulator of cell growth, proliferation and metabolism [267,268]. Genetic alterations in mTOR signalling were detected in 36% of patients with SCLC, where have been linked to radiation and chemotherapy resistance [269,270]. The PI3K/Akt/mTOR pathway has become a hot therapeutic target for SCLC since several studies have shown that its inhibition resulted in a reduced growth, promotion of apoptosis and enhanced sensitivity to cisplatin/etoposide in both SCLC cells and PDX models [158,269,271,272]. Despite promising preclinical results, mTOR inhibitors that reached clinical trials, failed to improve survival of SCLC patients [158,271].

Data from SCLC patients clearly indicated how the expression of key genes belonging to the mTOR pathaway (SLC7A5, MLST8 and EIF4EBP1) correlated with MYCN expression (Figure 21A).The same correlation was not observed for MYC or MYCL (Figure 22).
Thus, I hypothesized to downregulate mTOR complex via MYCN inhibition. Since BGA002 activity was proven to be specific, this approach may be beneficial over classic mTOR inhibitors, known for their dangerous side effects [273,274].
BGA002 treatment induced the inhibit of mTOR genes analyzed, in all MYCN-related SCLC cell lines (Figure 21B). To further confirm mTOR complex inhibition, the pathway activity was evaluated through protein phosphorylation, conducted in collaboration with the group of Prof. Martelli. Western blot analysis on MNA-SCLC cells (NCI-N592) showed a reduction of Akt, P70S6K, S6RP and 4E-BP1 phosphorylation after treatment (Figure 21C-D).
Taken together these findings demonstrate that MYCN inhibition strongly suppress mTOR pathway, in MYCN-expressing SCLC.

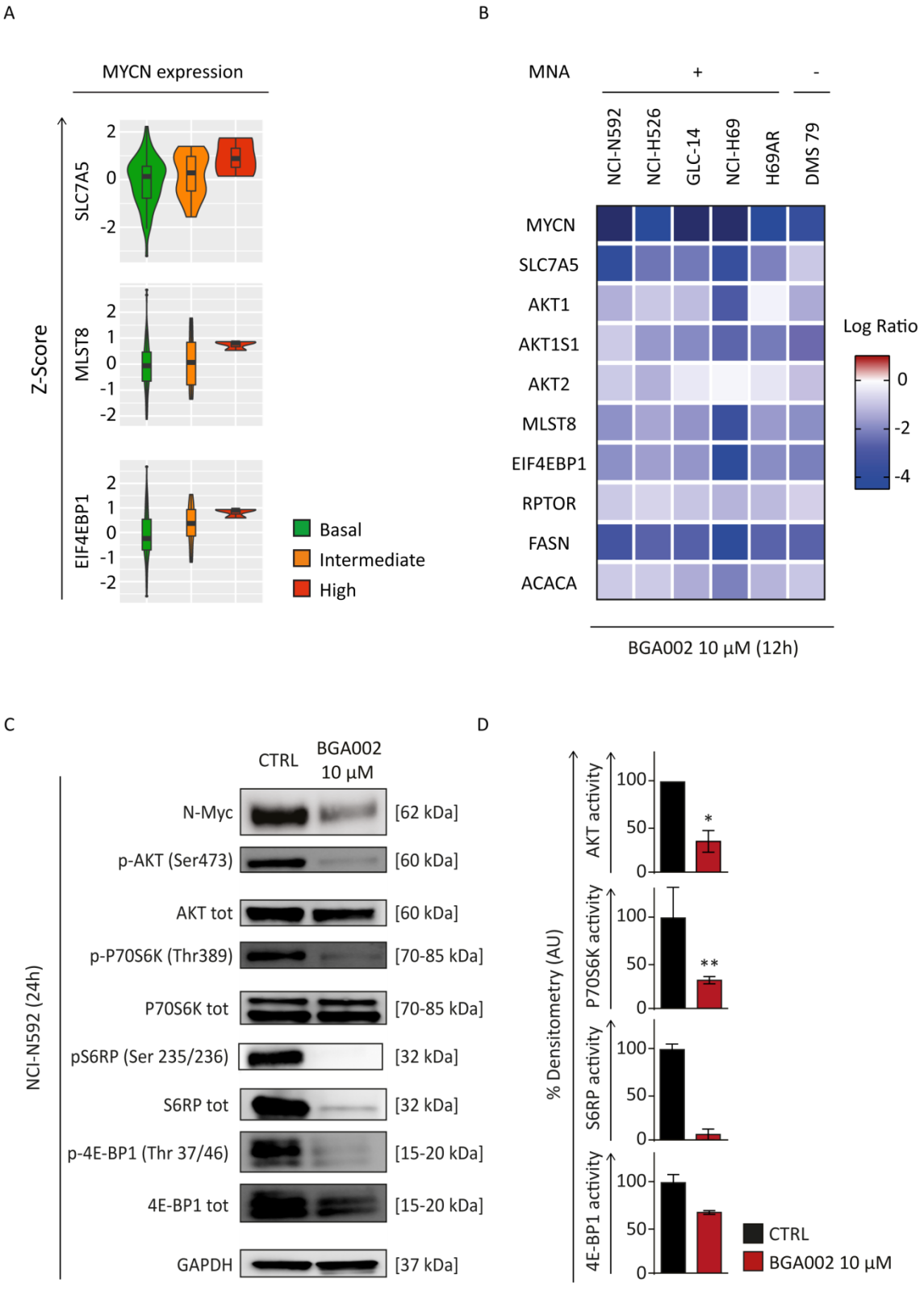


Figure 21. A: Expression of different mTOR genes presented as Z-Score, according to MYCN expression in SCLC patients. Middle line indicates the median; the whiskers indicate samples within 1.5 times the interquartile range. **B:** Heatmap of the genes expression variation after 12 hours of BGA002 treatment (10µM) in SCLC cells. Columns represent cell lines, rows represent genes belonging to the mTOR pathway and the color scale represents the $\log_2$-fold change in comparison with the untreated cells (n=3 for each cell line). **C:** Western blot analysis for mTOR pathway activity in NCI-N592 cell line after 24 hours of treatment (representative image of 1 out 2 biological replicates). **D:** mTOR pathway activity quantification, normalized over the control (n=2 experiments). Bars represent the mean and the whiskers are the standard deviation. Data are analyzed with a two-tailed Student's t-test: *, $p \leq 0.05$; **, $p \leq 0.01$; where not indicated p -value > 0.05.

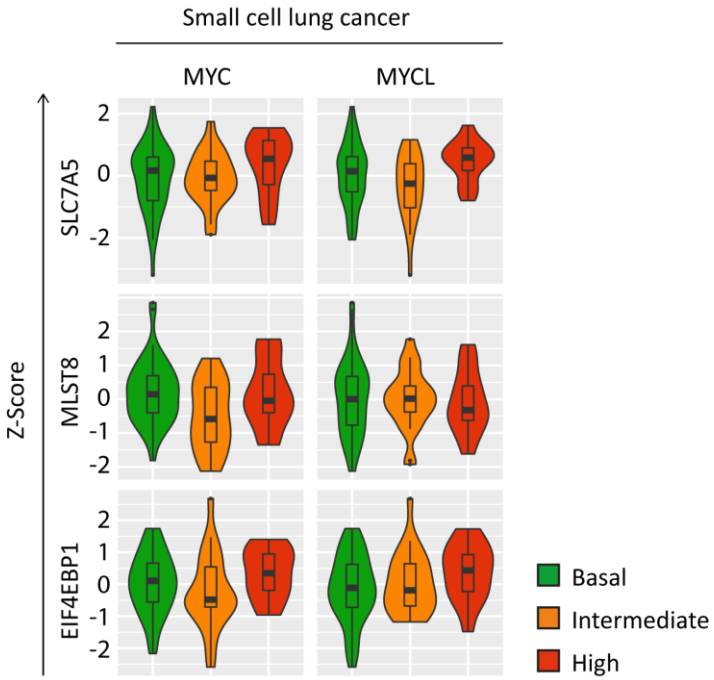


Figure 22. Expression of different mTOR related genes (SLC7A5, MLST8 and EIF4EBP1) presented as Z-Score, according to MYC (left) or MYCL (right) expression (from basal to high) in SCLC patients. Middle line indicates the median; the whiskers indicate samples within 1.5 times the interquartile range.

4.6 BGA002 treatment induces ultrastructural changes in MNA-SCLC cell lines

To better understand the biological processes that occur following MYCN reduction, morphological observations were performed by electron microscopy analisys (TEM). NCI-H69, H69AR and NCI-N592 cell lines were treated with BGA002 at concentration of 10 μM and after 48 hours were fixed and processed for electron microscoy analysis, conducted in collaboration with the group of Prof. Teti.

Control samples, from all cell lines tested, appeared normal and had both a regular nucleus and cytoplasm. In contrast, treated cells showed structures deputed to lipid and protein degradation, such as phagosomes, along with signs of chromatin condensation attributable to apoptotic phenomena (Figure 23A). Data that emerged from TEM analysis suggested that BGA002 had an effect on lysosomes and lysosomal related structures, which have been found in cells after treatment, while they were not evident in controls as well (Figure 23A).

To further investigate the modulation of lysosome physiology and organization, confocal microscopy analysis was performed. Following treatment, all MNA-SCLC cells, marked for lysosome identification, showed a significant increase in the number of lysosomes, as well as a modified distribution (Figure 23B). While mean diameters were unaltered, treated cells displayed a particular shift from lower diameters (<1 μm) to higher, highlighting a major lysosomal activity [275] and suggesting the possible activation of autophagy phenomena (Figure 23B). Interestingly this modulatory effect, induced by MYCN downregulation appeared clear also in H69AR cells, highlighting again the ability of BGA002 to stimulate anti cancer mechanisms, also in the context of acquired chemoresistance.

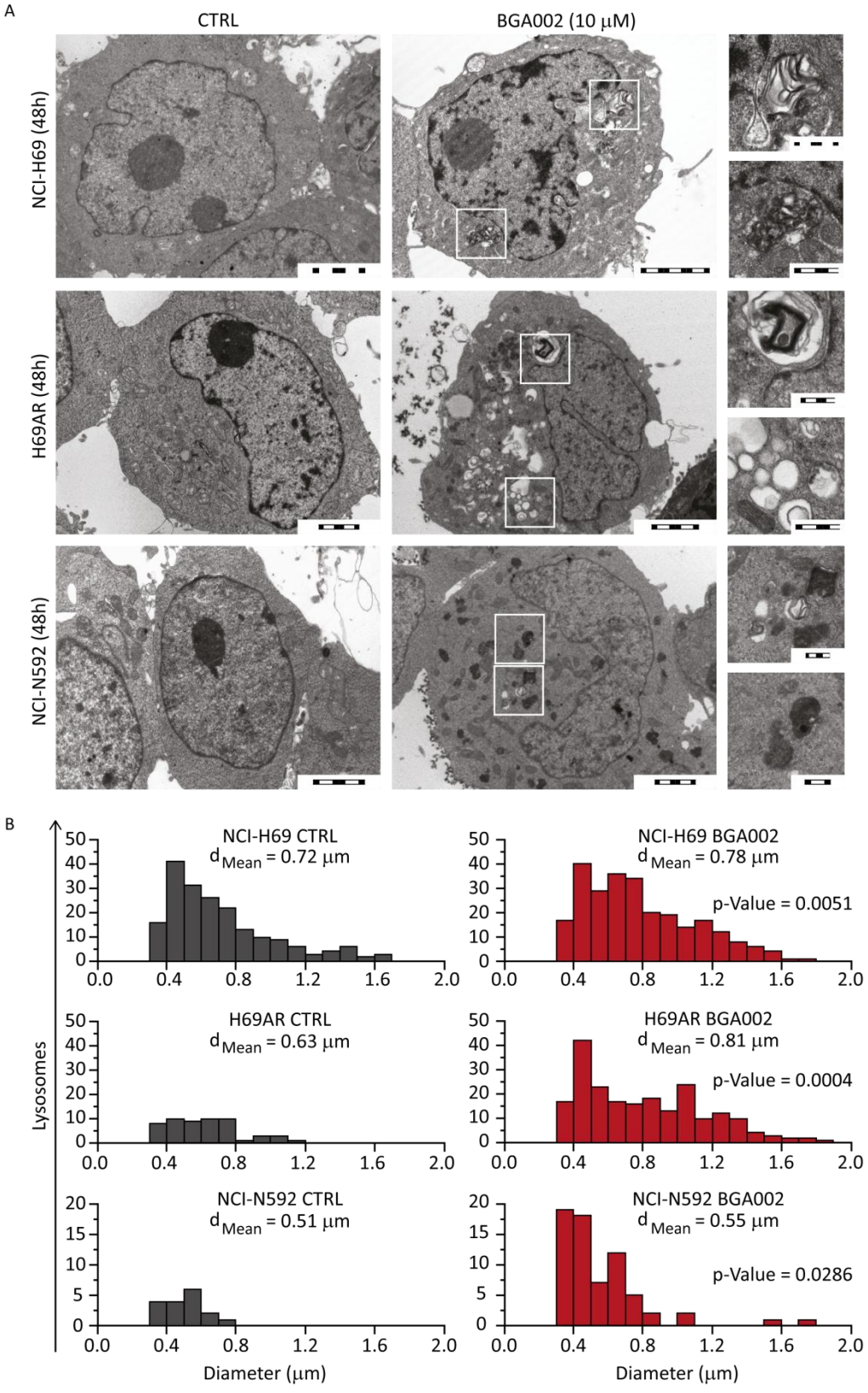


Figure 23. A: Transmission electron microscopy images of cells untreated or treated with BGA002 (10μM) for 48 hours. Representative images for each condition are presented (n=2 experiments for each cell line). Magnifications highlighting lipid degradation structures and vesicles formation. Scale bar in the whole cell images correspond to 2 μm; scale bar on the magnifications correspond to 500 nm. **B:** Distribution of lysosome diameters in NCI-H69 cell line (top), H69AR (middle) and NCI-N592 (down). In all plots n=40 cells were analyzed. For each cell line mean lysosome diameter is reported. Data are analyzed with two tailed, paired Student's t-test performed between control and treated group; for each cell line, *p* -value is shown.

4.7 N-Myc inhibition leads to tumor growth reduction and increases survival in MNA-SCLC xenograft mouse models

Finally, was evaluated the *in vivo* antitumor activity of BGA002 in two different models of MNA-SCLC: NCI-H69-Luc and multidrug-resistant H69AR-Luc. When animals reached a predefined value of tumor luminescence, they were randomly divided in different experimental groups.

Animals inoculated with NCI-H69-Luc were divided in two experimental groups:

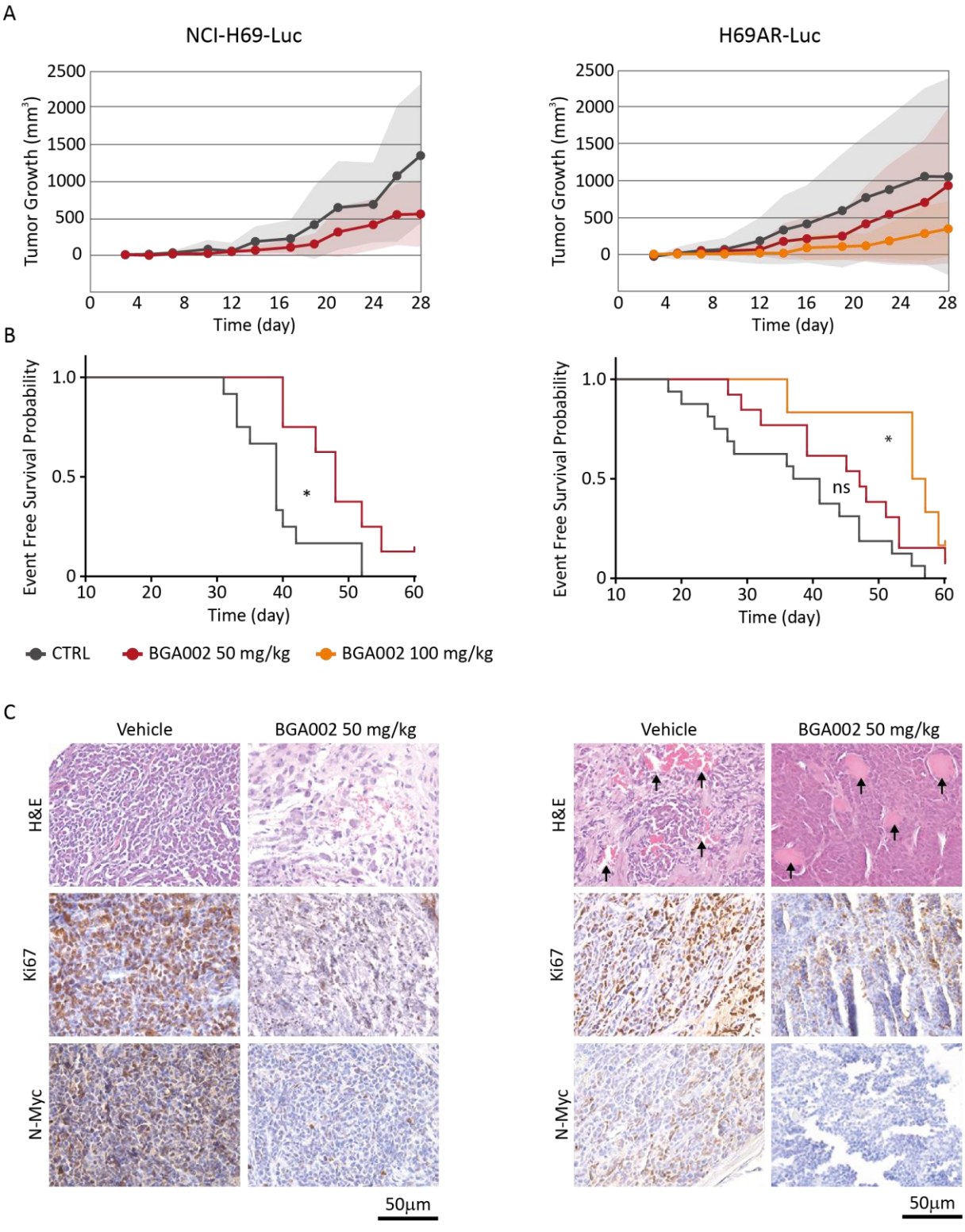
I. Vehicle (NaCl 0.9%)
II. Treated with BGA002 (50 mg/kg/day)

On the other hand, animals inoculated with H69AR-Luc were divided in three experimental groups:

I. Vehicle (NaCl 0.9%)
II. Treated with BGA002 (50 mg/kg/day)
III. Treated with BGA002 (100 mg/kg/day)

The systemic administration of BGA002 (daily for 28 days) was able to reduce tumor growth in comparison to the vehicle, in both models (Figure 24A). Furthermore, BGA002 treatment resulted also in a significant survival augmentation in both MNA-SCLC models (Figure 24B). Strikingly, although at the cellular level there seemed to be no differences between the two cell lines, either in the response to PNA treatment or from a molecular point of view; treatment at 50 mg/kg/day induced a significant increase in survival only in NCI-H69-Luc model (Figure 24B). However, increasing the dosage of BGA002 up to 100 mg/kg/day, it was possible to overcome this phenomenon, leading to significant survival augmentation also in the multidrug-resistant model (Figure 24B).

To further analyze BGA002 activity *in vivo*, tumors were extracted and histological and immunohistochemistry (IHC) analyses were performed. Histological analysis of tumors, comparing animals treated with vehicles or with 50 mg/kg/day, revealed a relevant tumor vascularization in the multidrug-resistant model, that was eliminated after treatment (Figure 24C). Moreover, IHC analysis demonstrated a consistent reduction in N-Myc protein staining after treatment, confirming the ability of BGA002 to target MYCN in vivo as well as in vitro. The oncoprotein reduction was accompanied by a significant decrease in the protein levels of Ki-67, a well known marker of cell proliferation (Figure 24C).



A

Figure 24. A: Evaluation of tumor growth in SCLC xenograft mouse models (NCI-H69-Luc on the left, H69AR-Luc on the right) treated with vehicle (respectively n=12 and n=16), BGA002 50 mg/kg/day (respectively n=8 and n=13) and BGA002 100 mg/kg/day (n=6). **B:** Kaplan–Meier plots for the probability of event-free survival over time for mice (NCI-H69-Luc and H69AR-Luc xenograft) treated with vehicle (black; respectively n=12 and n=16), BGA002 50mg/kg/day (red; respectively n=8 and n=13) and BGA002 100 mg/kg/day (orange; n=6). Data were analyzed with log-rank test: *, $p \leq 0.05$. **C:** Histological and IHC analyses of NCI-H69-Luc and H69AR-Luc mice untreated or treated with BGA002 50 mg/kg/day. Images of sections are shown stained with haematoxylin and eosin (H&E; first row), Ki-67 antibody (second row), N-Myc antibody (third row). Similar results were obtained from four independent mice. Black arrows indicate vascular structures.

5. Conclusions and discussion

The present work focused on the evaluation of BGA002, as a possible therapeutic strategy for the treatment of MYCN-related SCLC. Specifically, the study aimed to investigate at cellular and molecular levels, the changes occurring after drug administration, in the context of SCLC. BGA002 is an antigene peptide nucleic acid, belonging to the third generation of oligonucleotides [185]. PNAs are synthetic analogs of nucleic acids, which hold tremendous therapeutic potential [183,184], being more chemically and biochemically stable than DNA and RNA, but still maintaining the ability to recognize their target sequences, following Watson-Crick base pairing rules [175,176,179]. BGA002 was specifically developed to inhibit MYCN oncogene, binding a unique sequence in the exon 2 of MYCN DNA.

Lung cancer still represents a major health issue, since it is the first cause of cancer deaths worldwide [2]. SCLC, which comprises 15-20% of LC diagnosis [2,8], is initially sensitive to cytotoxic treatments, but most patients rapidly develop drug resistance, and therefore survival rates are low [83,93,276]. Unlike other types of lung cancer, the absence of real advances in the management of SCLC is unambiguous, and highlights the unmet need of novel therapeutic approaches.

Genomic analysis of SCLC has indicated that it is often associated with a high mutational burden and extensive chromosomal rearrangments [35,241]. Besides inactivating mutations of TP53 and RB1, mutually exclusive amplification and overexpression in one of the MYC family of oncogenes, is found in 20-40% of SCLC patients [107]. In contrast to MYC and MYCL, MYCN is mainly amplified in embryonic and neuroendocrine tumors [277], where its aberrant expression is related to drug resistance and poor outcome [105,118,128–130]. Although numerous efforts have been made to find a viable way to inhibit the oncogene, a strategy to effictively reduce its aberrant activation is still missing.

We have previously demonstrated the efficacy of BGA002 in NB, that is, similarly to SCLC, an aggressive neuroendocrine tumor [243,244]. This opens up the possibility of employing the same approach in other MYCN-driven mallignancies, such as SCLC.

First was confirmed the ability of BGA002 to effictively reduce the target expression in both MNA and non-MNA SCLC cells, regardless of the basal level of MYCN expression. The diminishment in mRNA was followed by a corresponding reduction of the oncoprotein in MNA-SCLC cells. Furthermore, BGA002 treatment induced a decrease in cell viability in all

SCLC cells analysed. This reduction in cell proliferation was further confirmed by the observation of apoptosis activation. Interestingly, data in literature have highlighted that MYCN is differentially expressed between chemosensitive and chemoresistant SCLC cells, being sharply upregulated in chemoresistant cell lines [277]. Functionally, MYCN promotes the chemoresistance through the inhibition of drug-induced apoptosis [277]. Among the cell lines used in this study, H69AR cells are indeed a chemoresistant clone, derived from NCI-H69 cells. BGA002, through MYCN inhibition, has overcome the resistance phenomenon, inducing apoptosis also in H69AR cells.

Moreover, BGA002 action was specific and did not influence cell viability in the non tumorigenic MYCN-unexpressed HEK293 cell line. The *in vitro* specificity of agPNA was further endorsed on NCI-H510A, a SCLC cell line characterized by MYCL amplification. Also in this case, high doses of BGA002 did not result in apoptosis induction, despite the high sequence homology between MYCN and MYCL.

We have previously reported that autophagy triggered by lysosome activation seemed to be a major pathway involved in NB cell death [244]. Interestingly, also in LC lysosomes were reported as an important features of autophagy and drug-resistance related processes [278,279]; in addition their modulation was able to reactivate tumor response to therapy in NSCLC and SCLC [280–283]. Given this background, the ability of BGA002 to regulate lysosome organization was explored, in three MNA-SCLC cells: NCI-H69, H69AR and NCI-N592. Following treatment, all cell lines evidenced an increased lysosomes number. The mean diameters were unaltered between control and respective treated sample, but there was an interesting shift in the lysosomal distribution: in treated MNA cells lysosomes moved from lower diameters to higher diameters, suggesting an increase in their activity [275]. Electron microscopy analysis confirmed the effect of BGA002 on lysosome physiology and organization. In fact, lysosomal related structures were found in cells after treatment but were not evident in control samples as well; thus reinforcing the hypothesis that following BGA002 treatment, there was a reactivation of autophagy processes.

SCLC was long considered as a single disease and this is one of the reasons why multiple clinical trials have failed in improving patients' survival. Also immune checkpoint inhibitors, recently introduced in first-line treatment for patients with metastatic SCLC, showed limited improvement in survival and only a small subset of them seemed to derive clinical benefits

[238–240]. Recently, several studies have identified four different SCLC molecular subtypes, based on the differential expression of transcriptional factors: ASCL1 (SCLC-A), NEUROD1 (SCLC-N), and POU2F3 (SCLC-P) [284–286]. A fourth subtype is characterized by low expression of all three transcription factors and presents a high infiltration of inflammatory cells, for this reason is called SCLC-I [284,286]. This new classification is a clear hint of SCLC inter- and intra-tumoral heterogeneity, and has allowed identifying specific therapeutic vulnerabilities across the subtypes, with important implications regarding response to treatments as well as chemoresistance onset [284,287].

Given the relevance of the gene expression profile, was performed an analysis to identify genes of interest whose expression was affected by MYCN inhibition, induced by BGA002. Gene profile analysis on SCLC cell lines showed a down-regulation of mTOR complex. Previous studies have demonstrated that genetic alterations in mTOR pathway has been detected in 36% of patients with SCLC and has been related to radiation and chemotherapy resistance [269,270]. PI3K/AKT/mTOR is a master regulator of cell growth, proliferation and metabolism [267,268], and its aberrant activation has been implicated in metabolic disorders, neurodegeneration, ageing and in various cancer types [268,270]. In the context of SCLC, mTORC2 was linked to tumor progression and metastasis formation, beside that, expression of Rictor and Akt is a negative prognostic factor for overall survival [288]. Several studies have shown that the inhibition of the mTOR signaling resulted in a reduced growth, promotion of apoptosis and enhanced sensitivity to cisplatin/etoposide in both SCLC cells and PDX models [158,271,272]. Thus, this signaling emerged as a hot therapeutic target for the development of new therapies for SCLC [158,269,288], nevertheless the clinical trials reached disappointing results, failing to improve survival [158,271]. In addition, inhibitors of mTOR, such as rapamycin and its derivatives (rapalogs) are not selective for cancer cells, being poorly tolerated due to their toxicity and making clinical transition difficult [273,274,289]. Hence, BGA002 may represent a more specific and less toxic approach to block mTOR pathway.

Data from patients indicated that SLC7A5, MLST8 and EIF4EBP1 were correlated with MYCN expression, in both SCLC and NB [244]; while there was no clear correlation depending on MYC and MYCL status. SLC7A5 (Solute Carrier Family 7 Member 5) acts upstream of mTOR pathway, MLST8 (MTOR Associated Protein, LST8 Homolog) is involved in mTOR activation, whereas EIF4EBP1 (Eukaryotic Translation Initiation Factor 4E Binding Protein 1) is an mTOR target. BGA002 was able to markedly downregulate genes

involved in all the phases of mTOR signaling. Moreover, WB analysis conducted on NCI-N592 cells, confirmed an overall reduction in the activity of mTOR complex. So, the indirect inhibition of mTOR complex, mediated by BGA002, occurs only in cancer cells, leaving healthy cells unaffected and avoiding toxicity phenomena, in contrast to what happen with classic mTOR inhibitors.

Since mTORC1 is implicated in a large number of processes that control the metabolic state of the cell, and in particular suppresses autophagy [290,291]; the inhibition of the complex following BGA002 treatment, further supports the possible reactivation of phagocytosis.

Besides mTOR signaling, gene analysis on SCLC cells highlighted other genes involved in tumorigenesis and potentially regulated by BGA002 inhibition of MYCN.

CNTFR plays a critical role in tumor cell survival and growth and is emerging as target for specific therapy in LC [256–260]. This gene showed low expression in many tumor types, while it is upregulated in NB and SCLC. For this reason, I have analyzed if BGA002 can impact tumor progression through this signature gene.

The silencing of CNTFR suppressed SCLC cells proliferation, confirming its role in SCLC maintenance, in addition BGA002 strongly reduced CNTFR in all SCLC cells and was able to further increase cell growth reduction in NCI-N592 cells, highlighting how MYCN regulation may impact at higher level cancer progression.

To further expand this consideration, was evaluated if MYCN inhibition can affect CNTFR expression in a different pathological context. Also in NB, after BGA002 administration, CNTFR expression was reduced, consolidating our findings about the relationship between MYCN and CNTFR, as a crucial target gene.

A recent study from Lv and colleagues suggested that CNTFR may function as an oncogene, and more interestingly its inhibition has been related with suppression of M2 macrophage polarization in the tumor microenvironment (TME) [292].

TME is a complex and continuously evolving entity and is emerging as a tremendously influent factor in tumor progression, metastasis formation, and response to therapies [293–297]. The balance of polarization between macrophages M1 and M2, driven respectively by Th1 cells and Th2 cells, can be either suppress tumor formation or promote tumorigenesis [293,294,297,298].

This is of particular interest since we have previously found that MYCN, and not its high homologous MYC, is associated with immune repression and a Th2-lymphocytes/M2 macrophages axis upregulation, driving a tumor immunosuppressive microenvironment in

different MYCN-expressing cancers such as NB, SCLC, acute myeloid leukemia, and Wilms' tumour [299].

Gene expression analysis has also identified TNFAIP3 as an extremely relevant gene in SCLC, specifically considering its correlation with immune system activation and inflammation homeostasis, mainly through the regulation of NF-κB pathway [265,266]. As emerged from CCLE analysis, TNFAIP3 is downregulated in SCLC and in NB, where it can be easily restored by BGA002. Considering its association with the innate immune response and the pro-inflammatory cytokines induction, it may resume M1 macrophages activation [300].

Similar analysis were conducted on DLX5 gene, which, through the binding to the promoter region of MYC, can stimulate cancer progression [262] and was reported as a negative prognostic factor in LC [264] and in NB [244]. DLX5 was strongly down-regulated by BGA002 in both SCLC and NB cells.

From the bioinformatics analysis emerged that CNTFR, TNFAIP3 and DLX5 are modulated by MYCN status in SCLC, while the same is not true for MYC and MYCL. This, in combination with the same trend observed in MNA-NB patients, suggested that these genes could represent a distinct expression signature of a subset of MYCN-driven SCLCs.

In fact, a prior study conducted on SCLC by Kim and colleagues, has identified different sets of genes whose expression was correlated with one of the MYC family members, furthermore finding a little overlap among the different groups [301]. This was not the only difference found between distinct subsets of SCLC, driven by one of the MYC genes. For example, MYC-driven SCLC was found metabolically distinct from the other subtypes, in particular MYC SCLCs were more sensitive to arginine depletion [302]. Furthermore MYC-, but not MYCN- or MYCL-driven SCLCs, was responsive to Aurora Kinase Inhibitors, indicating a specific therapeutic vulnerabilities [107,303,304].

Studies regarding the mutually exclusive way of amplifications of MYC genes, represented the first example of discoveries regarding SCLC heterogeneity [129,301,305–307].

The recent stratification of SCLC, depending on differential expression of lineage-defining transcription factors ASCL1, NEUROD1, and POU2F3, highlighted a crucial role for MYC family members [284,285,308]. The most abundant subtype is SCLC-A, a neuroendocrine (NE) subtype characterized by MYCL expression; on the contrary MYC-driven SCLCs correspond with ASCL-1 low samples [309,310]. Intriguingly, recent studies have shown how MYC, through the activation of Notch signaling, can induce the conversion from SCLC-A, to SCLC-N, to SCLC-I, which has the most mesenchymal phenotype [286,309,310]. The

switching from SCLC-A to SCLC-I was observed after first line chemotherapy, and has been implicated in SCLC plasticity and acquired resistance phenomena [284]. Despite the subsequent negative response to chemotherapy, SCLC-I demonstrated to be the most responsive to immune checkpoint blockade in the IMpower133 trial (NCT02763579) [284,286]; consequently MYC expression was associated with clinical benefits to immunotherapy, with respect to MYCN and MYCL [310]. In particular, MYCN is strongly related with SCLC-N, which resulted to be the subtype most unresponsive to immunotherapies [284].

As last part of my research project, was evaluated the *in vivo* antitumor activity of BGA002 in two MNA-SCLC xenograft models: NCI-H69-Luc and multidrug-resistant H69AR-Luc.
The animals, treated for 4 weeks and then sacrificed when reached the predefined end-point, were used to build a Kaplan-Meyer plot for the probability of event-free survival over time. In both models, BGA002 treatment induced a significant survival augmentation and concurrently reduced tumor growth.
Following BGA002 administration, the tumor vascularization widely present in the multidrug-resistant model was profoundly reduced, in accordance with the observed decrease of N-Myc protein expression.
Furthermore, BGA002 has received the Orphan Drug designation for SCLC treatment, from the Food and Drug Administration (FDA), certifying its potential as a novel therapeutic approach in the management of SCLC, also opening the possibility to combine it with immunotherapy and other drugs.
In the end, alterations of MYCN define a wide range of tumors such as rhabdomyosarcoma, medulloblastoma, Wilms tumor and retinoblastoma. Therefore, the use of BGA002 could be evaluated and extended to other MYCN-driven tumors.

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