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DOTTORATO DI RICERCA IN
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ACTIVITY AND MECHANISMS OF ACTION OF NOVEL ORGANOSULFUR DERIVATIVES OF THE HDAC INHIBITOR VALPROIC ACID IN HUMAN EXPERIMENTAL MODELS OF NON-SMALL-CELL LUNG CANCER

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Summary

1. Introduction

1.1 Non-small-cell lung cancer (NSCLC)

1.1.1 Incidence and mortality

Lung cancer represents the second malignancy in men in occidental world, accounting for 21% of new cancer cases and 30% of total death for cancer. In the United States 200.000 new cases are roistered every year, with about 160.000 death/year. In the European Union one lung cancer is registered every 100.000 habitants, with 50 deaths.

It has been calculated that one man of 9, and 1 woman of 36 develop a lung cancer in his lifespan. In our country, lung cancer in men, from 2006 to 2009, were more incident in the north regions (73,0 cases/100.000 habitants) respect to the central (64,7 cases/100.000 habitants) and the south one (65,7 cases/100.000 habitants). In women, the difference in incidence between north and south regions is more marked, with 22,0 cases/100.000 habitants in north regions, 18,4/100.000 habitants in middle regions and 13,8/100.000 habitants in the south. A modest decrease in lung cancer incidence was observed in men, in relation to a parallel decrease in the smoking habits (-2,0%/year from 1996 to 2010). As contrary an increase was observed in women (+2,5%/year from 1996 to 2010).

Lung cancer represents the first cause of death in men (26% of total death) and the third cause in women (11% of total death). Also for mortality, from 1996 to 2010 a decrease in men (-2,0%/year) and a constant increase in women (+1,8%/year) were observed. 5% survival of lung cancer patients is unmodified in the last years, and is about 14% in men and 18% in women.

1.1.2 Risk factors and prevention

Cigarette smoke represents the most important risk factor for lung cancer, and it is considered to be responsible for 85% of observed cases.

The relative risk is in tight relation with the number of smoked cigarette/die, with the duration of smoking (years) and the content of tar (1-6). The relative risk of

smokers with respect to non smokers is 14, whereas that of heavy smokers (more than 20 cigarette/die) is 20 times. For smokers who stopped smoking, the risk decrease in the follow 10-15 years. Regarding passive smoke, it has been stimated an increase of about 20-50% with respect to non smokers.

Some substances are recognized as lung carcinogens (asbestos, chrome, arsenic, beryllium, vinyl cloride, radon, etc) and they can potentiate their effect in presence of tobacco smoke.

Air pollution represents another risk for lung cancer.

1.1.3 Staging and TNM classification

Staging is one of the most important components in the management of lung cancer. Accurate staging is important because it allows the clinician to predict prognosis and assign appropriate therapy and also provides a system that allows clinicians and researchers to stratify patients into reasonably homogenous groups so that treatment outcomes can be appropriately compared. Tumor staging is broadly broken down into clinical staging and pathologic staging. Clinical stage refers to the best prediction of lung cancer stage prior to the commencement of therapy. Pathologic stage refers to the best prediction of stage following pathologic analysis of the patient's tumor, lymph nodes, and/or metastases and is usually applied following surgical resection or exploration. In common with most other solid malignancies, lung cancer staging is defined by the local extent of the primary tumor (T), involvement of associated lymph nodes (N), and whether or not metastases (M) exist. The TNM classification for lung cancer was originally proposed by Mountain in the early 1970s based on an analysis of 2,155 surgically resected patients at the MD Anderson Cancer Center (MDACC) (7) . The International Association for the Study of Lung Cancer (IASLC) convened a lung cancer staging workgroup in 1998 and collected data on a total of 100,869 patients from multiple institutions worldwide (8) . The last updated staging system is the 7th edition of the American Joint Commission on Cancer (AJCC) and the International Union Against Cancer (UICC) Staging Manual. (Tables 1 and 2).

Table 1. TNM Classification of NSCLC

Primary tumor (T)	
TX	Primary tumor cannot be assessed, or the tumor is proven by the presence of malignant cells in sputum or bronchial washing but is not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤ 3 cm in greatest dimension, surrounded by lung or visceral pleura, no bronchoscopic evidence of invasion more proximal than the lobar bronchus (not in the main bronchus); superficial spreading of tumor in the central airways (confined to the bronchial wall)
T1a	Tumor ≤ 2 cm in the greatest dimension
T1b	Tumor > 2 cm but ≤ 3 cm in the greatest dimension
T2	Tumor > 3 cm but ≤ 7 cm or tumor with any of the following: • Invades visceral pleura • Involves the main bronchus ≥ 2 cm distal to the carina • Associated with atelectasis/obstructive pneumonitis extending to hilar region but not involving the entire lung
T2a	Tumor > 3 cm but ≤ 5 cm in the greatest dimension
T2b	Tumor > 5 cm but ≤ 7 cm in the greatest dimension
T3	Tumor > 7 cm or one that directly invades any of the following: • Chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, or parietal pericardium; Or tumor in the main bronchus < 2 cm distal to the carina but without involvement of the carina

	Or associated atelectasis/obstructive pneumonitis of the entire lung or separate tumor nodule(s) in the same lobe
T4	Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, or carina; or separate tumor nodule(s) in a different ipsilateral lobe
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in the ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in the contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or malignant pleural (or pericardial) effusion
M1b	Distant metastasis

Table 2. Stages classification

Stage	T	N	M
Ia	T1a	N0	M0
	T1b	N0	M0
Ib	T2a	N0	M0
IIa	T1a	N1	M0
	T1b	N1	M0
	T2a	N1	M0
	T2b	N0	M0
IIb	T2b	N1	M0
	T3	N0	M0
IIIa	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
IIIb	T4	N2	M0
	T1	N3	M0
	T2	N3	M0
	T3	N3	M0
	T4	N3	M0
IV	T Any	N Any	M1a or 1

1.1.4 Cellular classification of NSCLC

Malignant non-small cell epithelial tumors of the lung are classified by the World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC). There are three main subtypes of non-small cell lung cancer (NSCLC), including the following:

- Squamous cell carcinoma (25% of lung cancers).
- Adenocarcinoma (40% of lung cancers).
- Large cell carcinoma (10% of lung cancers).

There are numerous additional subtypes of decreasing frequency.

WHO/IASLC Histologic Classification of NSCLC

1. Squamous cell carcinoma.
    1. Papillary.
    2. Clear cell.
    3. Small cell.
    4. Basaloid.
2. Adenocarcinoma.
    1. Acinar.
    2. Papillary.
    3. Bronchioloalveolar carcinoma.
        1. Nonmucinous.
        2. Mucinous.
        3. Mixed mucinous and nonmucinous or indeterminate cell type.
    4. Solid adenocarcinoma with mucin.
    5. Adenocarcinoma with mixed subtypes.
    6. Variants.
        1. Well-differentiated fetal adenocarcinoma.
        2. Mucinous (colloid) adenocarcinoma.
        3. Mucinous cystadenocarcinoma.
        4. Signet ring adenocarcinoma.
        5. Clear cell adenocarcinoma.

3. Large cell carcinoma.
    1. Variants.
        1. Large cell neuroendocrine carcinoma (LCNEC).
        2. Combined LCNEC.
        3. Basaloid carcinoma.
        4. Lymphoepithelioma-like carcinoma.
        5. Clear cell carcinoma.
        6. Large cell carcinoma with rhabdoid phenotype.
4. Adenosquamous carcinoma.
5. Carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements.
    1. Carcinomas with spindle and/or giant cells.
    2. Spindle cell carcinoma.
    3. Giant cell carcinoma.
    4. Carcinosarcoma.
    5. Pulmonary blastoma.
6. Carcinoid tumor.
    1. Typical carcinoid.
    2. Atypical carcinoid.
7. Carcinomas of salivary gland type.
    1. Mucoepidermoid carcinoma.
    2. Adenoid cystic carcinoma.
    3. Others.
8. Unclassified carcinoma.

Squamous cell carcinoma

Most squamous cell carcinomas of the lung are located centrally, in the larger bronchi of the lung. Squamous cell carcinomas are linked more strongly with smoking than other forms of NSCLC. The incidence of squamous cell carcinoma of the lung has been decreasing in recent years.

Adenocarcinoma

Adenocarcinoma is now the most common histologic subtype in many countries, and subclassification of adenocarcinoma is important. One of the biggest problems

with lung adenocarcinomas is the frequent histologic heterogeneity. In fact, mixtures of adenocarcinoma histologic subtypes are more common than tumors consisting purely of a single pattern of acinar, papillary, bronchioloalveolar, and solid adenocarcinoma with mucin formation.

Criteria for the diagnosis of bronchioloalveolar carcinoma have varied widely in the past. The current WHO/IASLC definition is much more restrictive than that previously used by many pathologists because it is limited to only noninvasive tumors.

If stromal, vascular, or pleural invasion are identified in an adenocarcinoma that has an extensive bronchioloalveolar carcinoma component, the classification would be an adenocarcinoma of mixed subtype with predominant bronchioloalveolar pattern and a focal acinar, solid, or papillary pattern, depending on which pattern is seen in the invasive component. However, the future of bronchioloalveolar carcinoma as a distinct clinical entity is unclear; a multidisciplinary expert panel representing the IASLC, the American Thoracic Society, and the European Respiratory Society proposed a major revision of the classification of adenocarcinomas in 2011 that entails a reclassification of what was called bronchioloalveolar carcinoma into newly defined histologic subgroups.

The following variants of adenocarcinoma are recognized in the WHO/IASLC classification:

- Well-differentiated fetal adenocarcinoma.
- Mucinous (colloid) adenocarcinoma.
- Mucinous cystadenocarcinoma.
- Signet ring adenocarcinoma.
- Clear cell adenocarcinoma.

Large cell carcinoma

In addition to the general category of large cell carcinoma, several uncommon variants are recognized in the WHO/IASLC classification, including the following:

- LCNEC.

- Basaloid carcinoma.
- Lymphoepithelioma-like carcinoma.
- Clear cell carcinoma.
- Large cell carcinoma with rhabdoid phenotype.

Basaloid carcinoma is also recognized as a variant of squamous cell carcinoma, and rarely, adenocarcinomas may have a basaloid pattern; however, in tumors without either of these features, they are regarded as a variant of large cell carcinoma.

Neuroendocrine tumors

LCNEC is recognized as a histologically high-grade non-small cell carcinoma. It has a very poor prognosis similar to that of small cell lung cancer (SCLC). Atypical carcinoid is recognized as an intermediate-grade neuroendocrine tumor with a prognosis that falls between typical carcinoid and high-grade SCLC and LCNEC.

Neuroendocrine differentiation can be demonstrated by immunohistochemistry or electron microscopy in 10% to 20% of common NSCLCs that do not have any neuroendocrine morphology. These tumors are not formally recognized within the WHO/IASLC classification scheme because the clinical and therapeutic significance of neuroendocrine differentiation in NSCLC is not firmly established. These tumors are referred to collectively as NSCLC with neuroendocrine differentiation.

Carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements

This is a group of rare tumors. Spindle cell carcinomas and giant cell carcinomas comprise only 0.4% of all lung malignancies, and carcinosarcomas comprise only 0.1% of all lung malignancies. In addition, this group of tumors reflects a continuum in histologic heterogeneity as well as epithelial and mesenchymal differentiation. On the basis of clinical and molecular data, biphasic pulmonary blastoma is regarded as part of the spectrum of carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements. (9)

1.1.5 Biomolecular characterization and target therapy

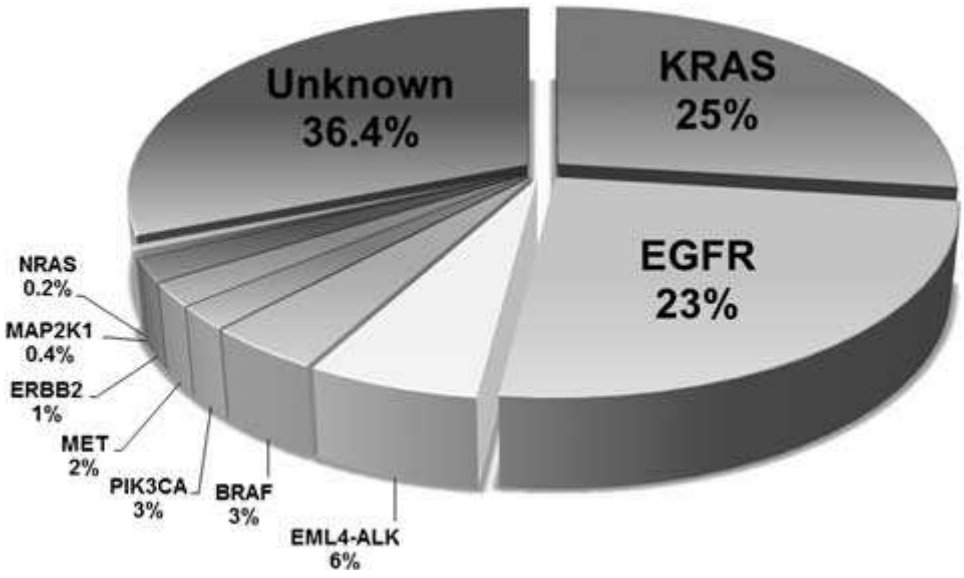
In recent years, attention has been paid to the role that 'driver mutations,' such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), have in the tumorigenesis of adenocarcinomas, and their potential use as targets for therapy (10-12). Recent data suggest EGFR may also serve as a prognostic factor, in addition to its role as a predictive factor, as patients-bearing EGFR mutations have shown favorable clinical outcomes even with conventional chemotherapy (13). EGFR mutations, which are associated with objective responses to single-agent TKI therapy in lung adenocarcinomas, are preferentially observed in a specific subset of patients: females of East Asian ethnicity who have never smoked and who have adenocarcinoma with lepidic growth pattern (formerly bronchioloalveolar carcinoma) (13). In adenocarcinomas, the majority of mutations have been identified in exons 18–21 of the EGFR gene. These mutations can be roughly classified into three major categories: in-frame deletions in exon 19, insertion mutations in exon 20, and missense mutations in exons 18–21.

Different EGFR mutations have different signaling properties, but most mutations affect the ATP binding cleft, where targeting TKIs compete for binding. The most frequent mutations were located at exon 19 and exon 21. There are over 20 variant types of exon 19 deletions, with the most common including delE746-A750, delL747- T751insS, and delL747-P753insS. L858R, in exon 21, is the second most frequent mutation. Additional mutations are located at exon 18 including G719C, G719S, G719A, and S720F and mutations found in exon 21 including L861Q and L861R.

The exon 20 insertions frequently associated with EGFR-TKI non-responsiveness, including D770- N771insNPG, D770-N771insSVQ, D770-N771insG, and point mutations, including T790M, V769L, and N771T. The most important mutation in exon 20 is T790M, which is associated with a small fraction of adenocarcinomas with primary resistance to EGFR TKI and over one-half of the patients with acquired resistance to EGFR TKI. The ability to detect multiple driver mutations in lung adenocarcinoma has revolutionized the medical management of this disease and multiplexed testing for all common driver mutations will provide physicians with a more precise guide for therapy (14). Other driver mutations have been

identified in NSCLC (in about 60% of cases) and in Figure 1 are represented the frequency of the founded alterations (15).
It is noteworthy that 95% of molecular lesions were mutually exclusive.

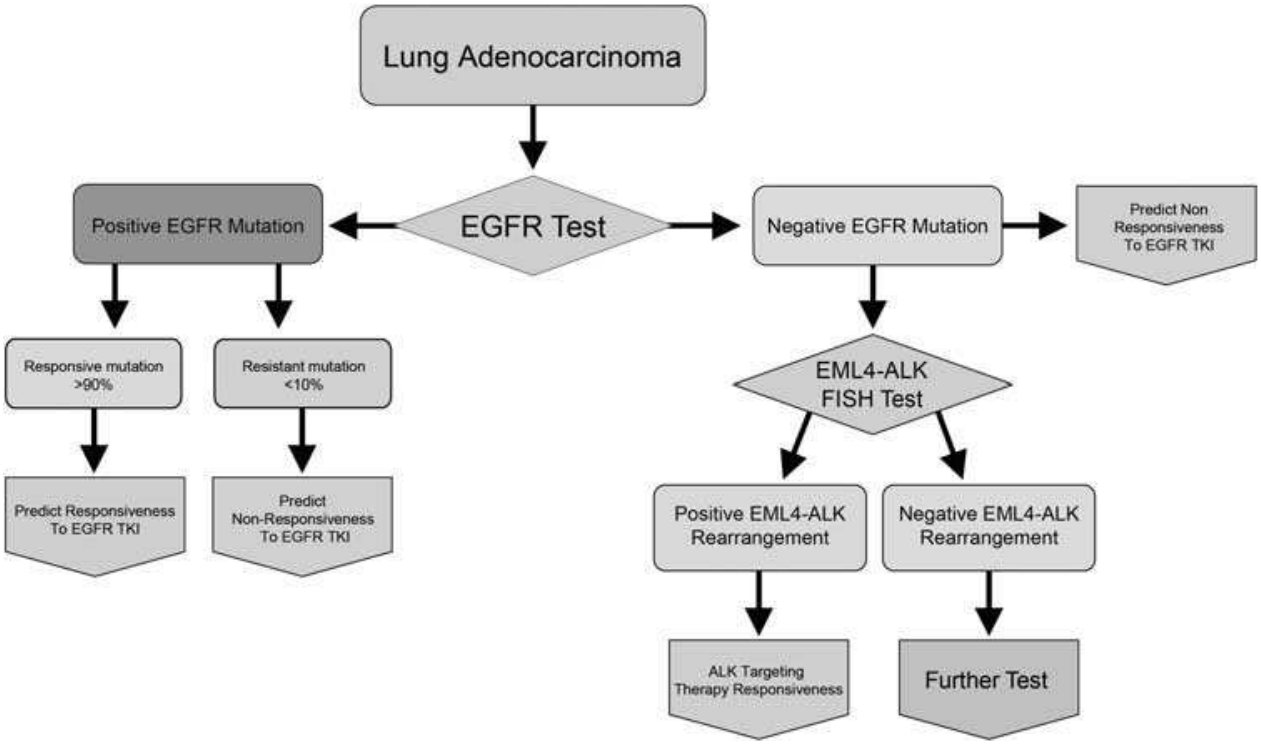
Figure 1. Frequency of major driver mutations in signaling molecules in lung adenocarcinomas (16).



The ALK gene encodes a receptor tyrosine kinase found in a number of fusion proteins consisting of the intracellular kinase domain of ALK and the amino terminal portions of different genes. The EML4–ALK fusion is a rare abnormality detected in 3–13% of patients with adenocarcinomas (17). It has been reported that although ALK-fusion positive lung cancers are resistant to the EGFR TKIs, gefitinib, and erlotinib, they are sensitive to small molecule TKIs against ALK. ALK TKIs (ALK TKI), including crizotinib, are effective treatments in preclinical models for patients with ALK-fusion cancers (18).

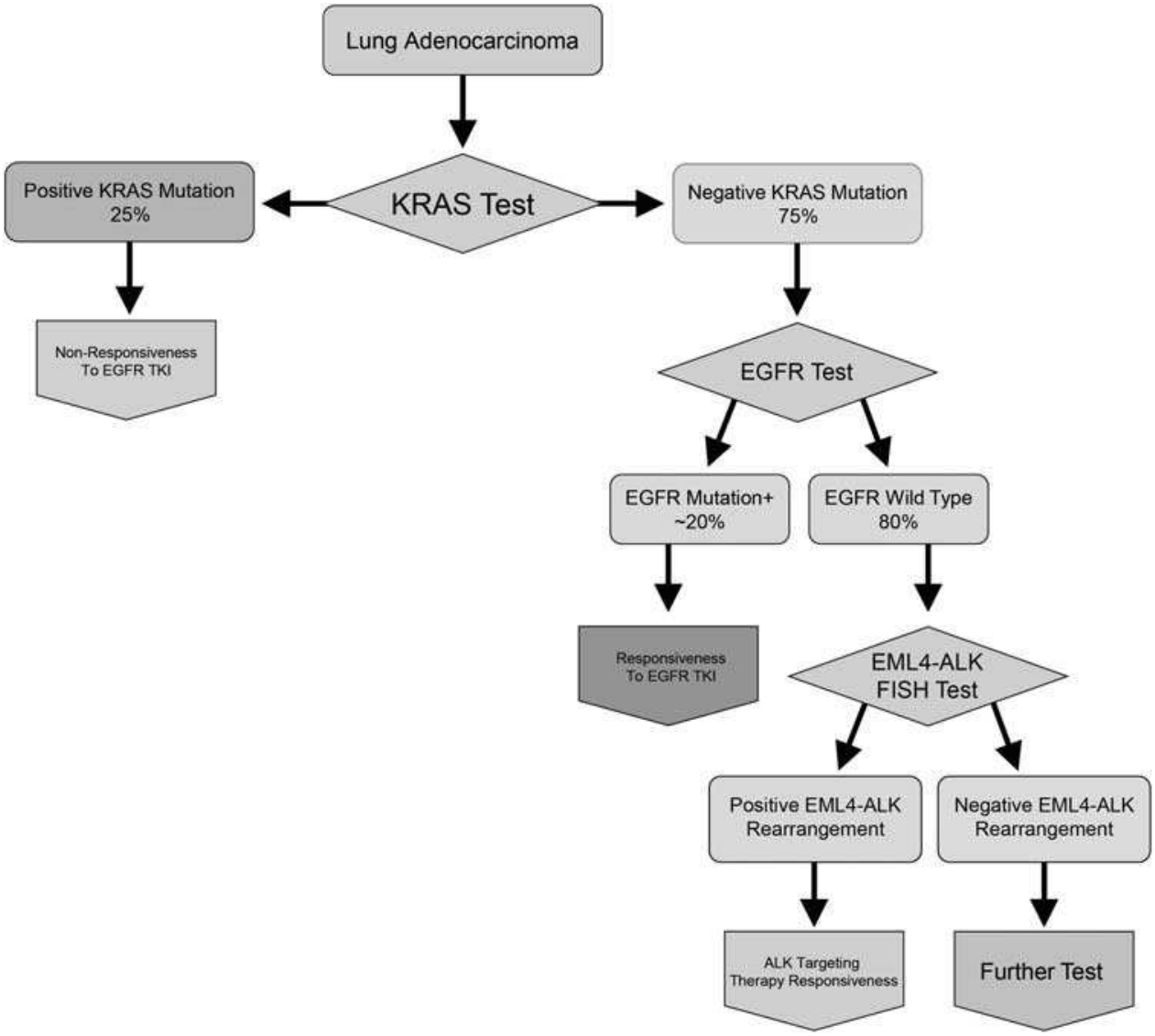
Based on the molecular feature of patients with adenocarcinoma of the lung, an algorithm that defines the rationale in selecting patients who could benefit from EGFR and EML4–ALK targeted therapy have been suggested (Figure 2).

Figure 2. Suggested algorithm for molecular testing for patients with lung adenocarcinoma. The algorithm defines the rationale in selecting patients who could benefit from EGFR and EML4–ALKA targeted therapy. Adenocarcinoma cases are subjected to testing for EGFR mutations. The EGFR mutation-positive cases (15%) are further divided into responsive and resistant groups according to their mutation profiles. A responsive mutation predicts a response rate of 91% and a resistant mutation predicts a response rate of 9%. The presence of wild-type EGFR characterizes about 85% of the adenocarcinomas, and predicts the likelihood of non-responsive to EGFR TKI. Tumors with wild-type EGFR are further tested for EML4–ALK rearrangement. Although EML4–ALK rearrangement is found in only 3% of patients with lung adenocarcinoma, its presence predicts a 53% probability of response to targeted therapy (16).



Another common alteration of NSCLC is the KRAS mutation. Mutations in KRAS are one mechanism of primary resistance to gefitinib and erlotinib. KRAS mutations are almost exclusively detected in codons 12 and 13 of exon 2, resulting in EGFR independent intracellular signal transduction activation. The KRAS mutations were found in 17% (21/121) of African–American patients compared with 26% (125/476) of Caucasian patients (19). KRAS mutations in adenocarcinoma are usually associated with wild-type EGFR and non-responsiveness to EGFR TKI therapy. Considering the mutually exclusive presence of EGFR, EML4-ALK and KRAS mutations, an alternative alghoritm of analysis have been proposed (Figure 3).

Figure 3. Alternative algorithm for molecular testing for patients with lung adenocarcinomas. Approximately 25% of lung adenocarcinomas harbor KRAS mutations, which predict non-response to EGFR TKI therapy. Of the remaining KRAS-negative lung adenocarcinomas, B20% harbor EGFR mutations, which are associated with responsiveness to EGFR TKI therapy. EGFR mutation negative cases may benefit from additional testing for the EML4–ALK rearrangement, which will be helpful in selecting patients potentially eligible for ALK targeted therapy (16).



1.1.6 Conventional chemotherapy

Despite remarkable advances in the targeted treatment of non-small cell lung cancer (NSCLC) over the past several years, chemotherapy remains of paramount importance in the treatment of advanced NSCLC. Even in patients whose tumors contain *EGFR* activating mutations or *ALK* gene rearrangements and are treated with first line tyrosine kinase inhibitors, resistance invariably develops, with chemotherapy remaining the cornerstone of subsequent therapy. In profiling mutations of 1,000 metastatic lung adenocarcinoma patients, although the Lung Cancer Mutation Consortium was able to identify actionable mutations, including molecular aberrations linked to approved drugs and clinical trials in 54% of cases (1), in only a small minority, about 14-18% in Western populations, are there approved targeted drugs (EGFR and ALK TKIs) with which to treat them. As of yet, no drugs targeting oncogenic-driver pathways have been approved in squamous cell lung cancers, though clinical trials are ongoing. With the majority of advanced lung cancer patients not harboring actionable driver mutations with paired targeted agents that effectively improve outcomes, advancing chemotherapy regimens through rational drug combinations and discovery of new potent chemotherapeutics remains critical.

Although recently implemented treatment guidelines recommend that patients with advanced stage NSCLC whose tumors harbor *EGFR* activating mutations or *ALK* gene rearrangements be treated first line with erlotinib or crizotinib, respectively, it is with the realization that there is no overall survival benefit to patients with *EGFR* mutated cancers whether they receive an EGFR TKI first line or second line. This TKI first recommendation is true even in patients with tumor-related poor performance status.

Cytotoxic agents active against NSCLC are platinum analogues (cisplatin-carboplatin), ifosfamide, mytomycin C, vindesine, vinblastine, etoposide, gemcitabine, paclitaxel, docetaxel, vinorelbine, pemetrexed. For ‘fit’ patients who do not have an oncogene-driven cancer, platinum doublet chemotherapy (with consideration of bevacizumab in non-squamous histology patients) remains the cornerstone of treatment. In an attempt to preserve efficacy and minimize toxicity, platinum free combinations of newer agents have been tested against conventional

platinum-based combinations. Although a recent meta-analysis of 16 randomized trials found that the efficacy was comparable between non-platinum doublets of third-generation agents and platinum-based doublets for pooled overall survival (HR =1.03, 95% CI: 0.98-1.08, P=0.290), all evidence based guidelines support platinum-based therapy as standard of care (20).

Utilizing DNA repair enzymes as biomarkers for better selecting front-line chemotherapy is an area of active investigation. Low ERCC1 expression by either IHC or RT-PCR has been shown in preliminary studies to be a potential biomarker of benefit to platinum compounds and low RRM1 a potential biomarker of benefit to gemcitabine. The ERCC1 enzyme removes platinum-induced DNA adducts, and thus low ERCC1 levels are associated with platinum sensitivity (21). RRM1 is a subunit of ribonucleotide reductase which is the main target of gemcitabine; thus, low RRM1 levels are associated with gemcitabine sensitivity (22).

Pemetrexed is a multi-targeted anti-folate employed: with platinum derivates for first-line treatment, as single agent for subsequent lines of treatment, and as maintenance therapy.

In the landmark JMDB trial, Scagliotti *et al*. demonstrated no difference in overall survival between cisplatin/gemcitabine and cisplatin/pemetrexed as first-line treatment of patients with metastatic NSCLC. However, in a preplanned subset analysis the cisplatin-pemetrexed combination was superior in non-squamous histology with a median overall survival of 12.6 months in the cisplatin-pemetrexed arm and 10.9 months in the cisplatin-gemcitabine arm (HR =0.84; 95% CI: 0.71-0.99; P=0.03) (23). By contrast, patients with squamous carcinoma had a worse median overall survival in the cisplatin-pemetrexed arm than in the cisplatin-gemcitabine arm (9.4 *vs.* 10.8 months; HR =1.23; 95% CI: 1.0-1.5; P=0.05).

A consistent survival advantage with pemetrexed was observed especially in non-squamous NSCLC (which represented the majority of the patients). A meta-analysis of five trials (three first-line trials, one second-line trial, one maintenance trial) confirmed that pemetrexed, when compared with alternative treatments or placebo, is consistently associated with a significant overall survival improvement in non-squamous histology (HR =0.82) but not in squamous histology (HR =1.19) (24).

In Table 3 is presented the response of 3rd generation cytotoxic drugs as monotherapy and in combination with platinum analogues.

Table 3. Results of six new agents in advanced NSCLC as monotherapy and in combination with platinum analogues (Pt)

Agent	Complete response + Partial response	Complete response + Partial response combination with (Pt) analogues
Vinorelbine	>15%	30-45% (C)
Gemcitabine	>15%	28-54% (C)
Paclitaxel	>15%	27-44% (C)
Docetaxel	>15%	25-62% (C)
Docetaxel	>15%	26-51% (Cb)
Irinotecan	>15%	50% (C)
Pemetrexed	<15%	30.6% (C)

The diagnosis and management paradigm of metastatic NSCLC has transitioned into an algorithm of presence or absence of oncogene addiction as a key branch point to selecting appropriate treatment. As described above, with the identification of driver mutations such as *EGFR* and *ALK*, EGFR-TKIs and crizotinib are supplanting traditional chemotherapy for upfront treatment of these patients. However, initial TKI responders inevitably relapse due to acquired resistance. More recently, an added layer of complexity related to intrapatient tumor heterogeneity has been observed, particularly relevant to the clonal evolution of somatic mutations from the primary tumor to metastatic lesions and the mixed response to treatment in different tumor sites. Therefore, an area of focus has therefore been on interrogating the combination of novel targeted agents together with chemotherapy to optimize efficacy, survival and overcome acquired resistance.

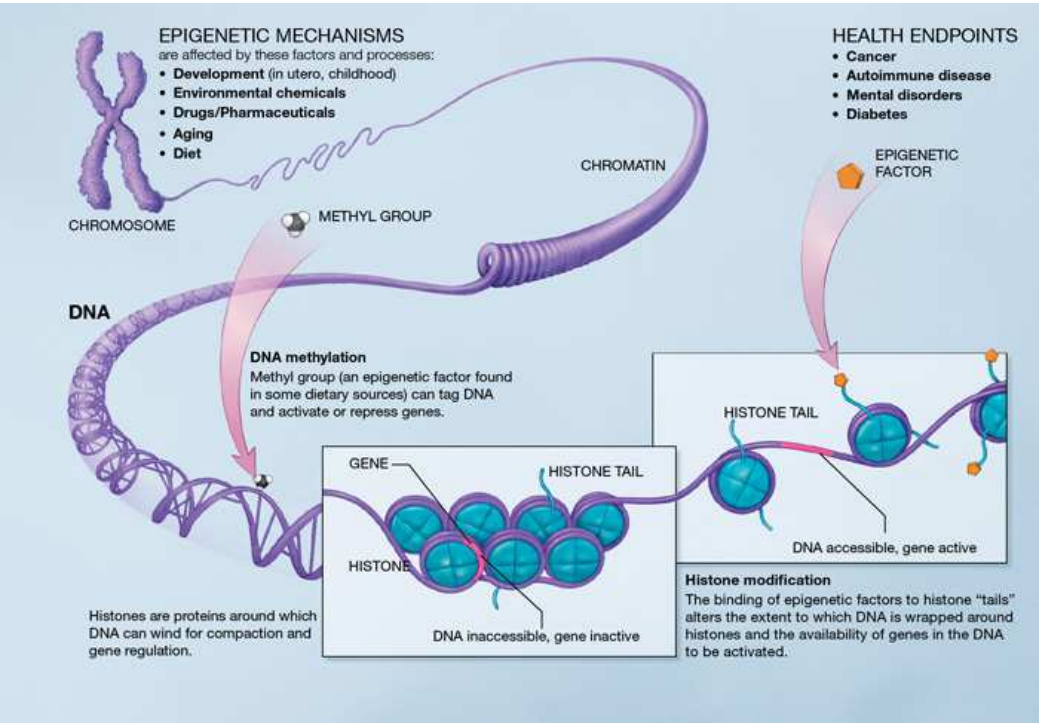
1.2 Epigenetic and cancer

Epigenetics is the study of heritable changes in gene activity that are not caused by changes in the DNA sequence; the term is also used to describe the study of stable, long-term alterations in the transcriptional potential of a cell, not necessarily heritable. Unlike simple genetics based on changes in DNA sequence, the changes in gene expression or cellular phenotype of epigenetics have other causes, thus use of the term *epi- genetics.*

Examples of mechanisms that produce such changes are DNA methylation and histone modification, each of which alters how genes are expressed without altering the underlying DNA sequence.

Figure 4. Epigenetic mechanisms

(http://en.wikipedia.org/wiki/File:Epigenetic_mechanisms.jpg)



1.2.1 Methylation

DNA methylation is probably the most well known epigenetic mark able to discriminate between normal cells and tumor cells in humans. The "normal" CpG methylation profile is often inverted in cells that become tumorigenic (25). In normal cells, CpG Islands localized at gene promoters are generally unmethylated, while other individual CpG dinucleotides throughout the genome tend to be methylated. Conversely, in cancer cells, CpG islands preceding tumor suppressor gene promoters are often hypermethylated, while CpG methylation of oncogene promoter regions is often decreased.

Hypermethylation of gene promoters can result in silencing of those genes. This type of epigenetic mutation is dangerous when genes that regulate the cell cycle are silenced, allowing cells to grow and reproduce uncontrollably, leading to tumorigenesis (26). Genes commonly found to be transcriptionally silenced due to promoter hypermethylation include: Cyclin-dependent kinase inhibitor p16, a cell-cycle inhibitor; p53, a tumor suppressor gene; MGMT, a DNA repair gene; APC, a cell cycle regulator; MLH1, a DNA-repair gene; and BRCA1, another DNA-repair gene (25, 26).

Hypomethylation of CpG dinucleotides in other parts of the genome leads to chromosome instability due to mechanisms such as loss of imprinting and reactivation of transposable elements.(27) In healthy cells, CpG dinucleotides of lower densities are found within coding and non-coding intergenic regions. Parasitic repetitive sequences, centromeres and oncogenes are often repressed through methylation.

The entire genome of a cancerous cell contains significantly less methylcytosine than the genome of a healthy cell. In fact, cancer cell genomes have 20-50% less methylation at individual CpG dinucleotides across the genome (27). In cancer cells “global hypomethylation” due to disruption in DNA methyltransferases (DNMTs) may promote mitotic recombination and chromosome rearrangement, ultimately

resulting in aneuploidy when the chromosomes fail to separate properly during mitosis (27).

CpG island methylation is important in gene expression regulation, yet cytosine methylation can lead directly to destabilizing genetic mutations and a precancerous cellular state. Methylated cytosines make hydrolysis of the amine group and spontaneous conversion to thymine more favorable. They can cause aberrant recruitment of chromatin proteins. Cytosine methylations change the amount of UV light absorption of the nucleotide base, creating pyrimidine dimers. When mutation results in loss of heterozygosity at tumor suppressor gene sites, these genes may become inactive. Single base pair mutations during replication can also have detrimental effects (26).

1.2.2 Histone modifications

Eukaryotic DNA has a complex structure. It is generally wrapped around special proteins called histones to form a structure called a nucleosome. A nucleosome consists of 2 sets of 4 histones: H2A, H2B, H3, and H4. Histone H1 contributes to DNA packaging outside of the nucleosome. Specific histone modifying enzymes can add or remove functional groups to the histones, and these modifications influence the level of transcription of the genes wrapped around those histones and the level of DNA replication. Therefore, the histone modification profiles of healthy and cancerous cells are different.

In comparison to healthy cells, cancerous cells exhibit decreased monoacetylated and trimethylated forms of histone H4 (decreased H4ac and H4me3). Interestingly, loss of histone H4 Lysine 16 acetylation (H4K16ac), which is a mark of aging at the telomeres, specifically loses its acetylation.

Other histone marks associated with tumorigenesis include increased deacetylation (decreased acetylation) of histones H3 and H4, decreased trimethylation of histone H3 Lysine 4 (H3K4me3), and increased monomethylation of histone H3 Lysine 9 (H3K9me) and trimethylation of histone H3 Lysine 27 (H3K27me3). These histone

modifications can silence tumor suppressor genes despite the drop in methylation of the gene's CpG island (an event that normally activates genes) (28).

Modification of histones by acetylation affects gene transcription by changing the chromatin structure that modulates the accessibility of transcription factors to their target DNA. As consequence, it plays an important role in regulation of gene expression. Additionally, acetylation and/or deacetylation of non-histonic proteins modify many important cell functions (29-30).

The acetylation state of histones and other proteins is maintained by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. HATs catalyze the transfer of an acetyl group from acetyl-CoA to lysine residues in proteins, whereas HDAC removes it. Depending on the mechanisms of removing the acetyl group, HDACs can be divided into two distinct families. The "classical family" comprises Zn2+-dependent HDACs, the second family of HDACs depends in catalysis on NAD+ and subsequently, *O*-acetyl-ADP-ribose and nicotinamide are formed as a result of the acetyl transfer (31). Furthermore, based on the homology to their yeast analogues, HDACs are divided into four classes. Class I are located in the nucleus and includes HDACs 1, 2, 3 and 8. HDACs 4, 5, 7 and 9 are members of class IIa, while isoforms 6 and 10 that are located both in the cytoplasm and nucleus are classified as class IIb. Class IV, which exhibits features of class I and II, includes only HDAC11. NAD+-dependent homologues 1-7 of the yeast Sir2 proteins (sirtuins) are designed as class III of HDACs, and have mono-ADP-ribosyltransferase activity. HATs, "functional opponents" of HDACs, are divided into Gcn5/PCAF *N*-acetyltransferases (GNATs) and MYST HATs. Although these two groups of HATs are the major enzymes catalyzing *N*-acetyltransferase activity, other proteins also exhibit this acetylase activity (32).

1.3 Histone deacetylase inhibitors

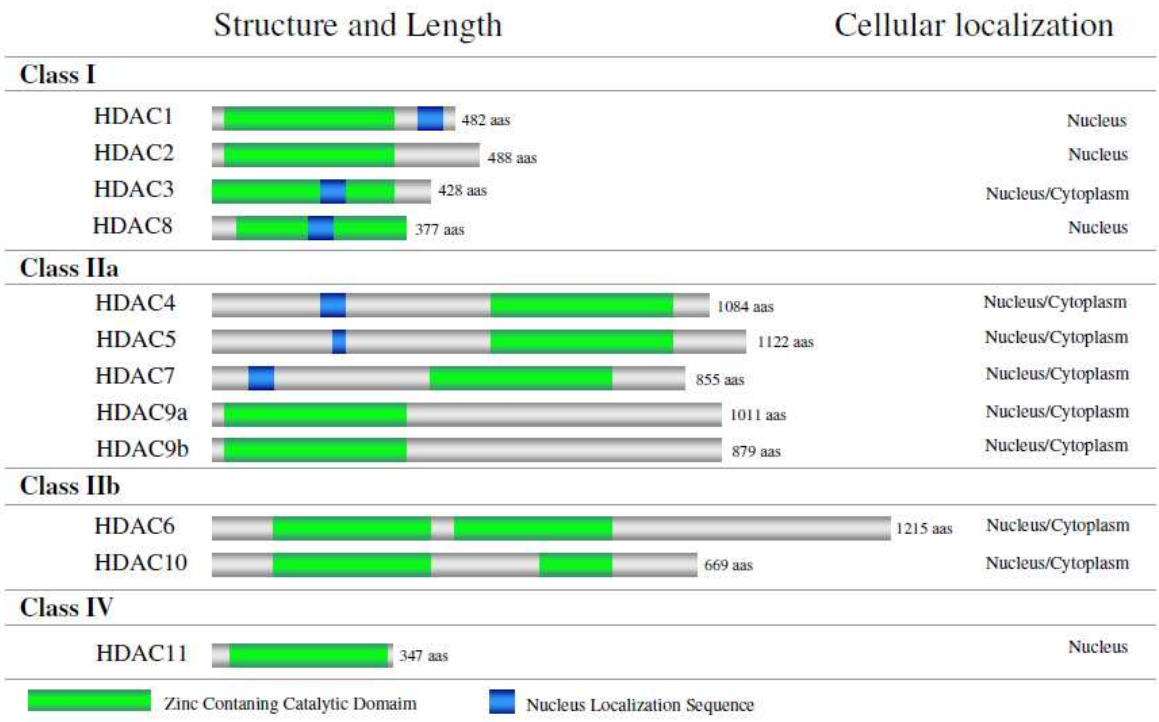
During the last few decades, several approaches have been used in an effort to discover new more effective anticancer drugs. Many promising compounds have been investigated. However, chemoresistance that may arise during chemotherapy is one of the main causes of failure of treatment. Epigenetic changes are emerging as part cause of the chemoresistence. Of these, histone acetylation and deacetylation have been investigated as therapeutic targets because of their importance in regulation of gene expression. Changes in histone acetylation influence chromatin condensation and these alterations influence gene transcription. The balance between histone transacetylases and deacetylases is often damaged in cancer, leading to changed expressions of tumor suppressor genes and/or proto-oncogenes (33, 34).

1.3.1 Histone deacetylases and cancer

HDACs class I and II levels vary in different cancer cells. HDAC1 is overexpressed in prostate and gastric cancers, where it signalizes poor prognosis, as well as in lung, esophageal, colon and breast cancers (35-37). High levels of HDAC2 have been found in colorectal, cervical and gastric cancers (38, 39). In addition, HDAC3 is overexpressed in gastric, prostate and colorectal cancer (40), and high expression of HDAC1 and 2 correlates with reduced patient survival in colorectal carcinomas. HDAC6 is highly expressed in breast cancer, HDAC8 is over-expressed in neuroblastoma cells and its overexpression correlates with metastasis and advanced stage of disease with poor prognosis. Expression of HDAC11 is increased in rhabdomyosarcoma (41).

Class III HDACs play an important role in carcinogenesis. Some act as antioncogenes while others influence tumors by controlling the cell metabolism (42). Decreased activities of HDACs are associated with suppressed tumor cell development and growth (43,44). Moreover mutations of HDAC4 have been identified in breast cancer samples (45) and mutation of HDAC2 that cause protein truncation was found in human epithelial cancer cell lines.

Figure 5. HDAC structure and localization (46)



1.3.2 Type of Histone deacetylase inhibitors

The results from various studies indicate that HDAC inhibitors increase the anticancer efficacy of additional therapy modalities and they therefore would be very efficient in the clinic together with other anticancer treatment modalities including ionizing radiation and/or chemotherapy. For this reason, investigation of the clinical application of HDAC inhibitors has increased with over 490 clinical trials for cancer and a few for other diseases (47). HDAC inhibitors have also be found to be effective for treatment of other diseases. Some HDAC inhibitors have antimalarial properties and are studied as new possible drugs for the treatment of malaria. There is also some evidence that HDAC pan-inhibitors and HDAC III inhibitors possess anti-inflammatory effects in models of asthma (48). They can be classified according to their chemical structure into

four groups: 1) hydroxamic acids; 2) aliphatic acids; 2) benzamides; 4) cyclic tetrapeptides.

1) Hydroxamic acids trichostatin A (TSA), vorinostat (suberoylanilide hydroxamic acid, SAHA) which was approved by the FDA as the first HDAC inhibitor for the treatment of relapsed and refractory cutaneous T-cell lymphoma (CTCL) (49), belinostat (PXD-101) and panobinostat (LBH589) are pan-HDAC inhibitors.

2) The aliphatic acids [valproic acid (VPA), butyric acid and phenylbutyric acid] are weak inhibitors of HDAC I and IIa (50).

3) Benzamides that include entinostat (SNDX-275, MS-275) and mocetinostat (MGCD0103) are isoform selective inhibitors of HDAC I and mocetinostat inhibits also IV HDAC (51).

4) The cyclic tetrapeptides, inhibitors of class I HDACs (romidepsin inhibits also HDAC 4 and 6), are cyclic hydroxamic acids containing peptides: romidepsin (depsipeptide, FK228, FR901228), apicidin and trapoxinand. Of these, romidepsin that was approved by the FDA and the EuMedicines Agency to treat CTCL and peripheral T cell lymphomas, is most effective (52). It is a prodrug which is activated to a metabolite that chelates the zinc ions in the active center of the HDAC of class I.

1.3.3 HDACi as anticancer drugs

The clinical development of HDACi is an active area of study. There are currently at least 15 different HDACi in clinical trials both as monotherapy and in combination for both hematological malignancies and for solid tumors.

Vorinostat HDACi is the most advanced in clinical use. In fact it is the first that have been approved by the FDA for the treatment of cutaneous T-cell lymphoma (53, 54).

LBH-589, which is in phase I and II clinical trials, appears to have greater efficacy of Vorinostat. Preliminary evidences indicate a capacity to induce disease stabilization, partial response, and, in few cases, a transient complete response.

Clinical trials with several HDACi are performed on patients with a wide variety of hematological and solid tumors including chronic lymphoid leukemia, acute myeloid leukemia, multiple myeloma, cancer of the head and neck, melanoma, and cancers of various organs such as brain, lung , breast, ovary, kidney and pancreas. The evidence accumulated to date show that the HDACi may be more useful in combination with other cytotoxic drugs and / or targeted.

Since many chemotherapeutic agents exert their antitumor activity damaging directly or indirectly the DNA, the combined treatment with HDACi can sensitize cancer cells with a combination of DNA damage and altered chromatin remodeling (55).

In fact it is known that the HDACi synergize with γ radiation, increasing the cytotoxic effect in various types of tumors. The mechanisms by which the HDACi sensitize cancer cells to DNA damage are different.

The loss of chromatin compaction as a result of the core histone hyperacetilation can increase the accessibility of genotoxic agents and increase efficiency. So in this model the HDACi potentiate the effect induced by genotoxic agents.

This may be due to the effect induced by the production of ROS, acting genotoxic, following treatment with HDACi. In addition there is also evidence that HDACi can suppress the mechanisms of DNA repair.

In fact, the modulation of the phosphorylation of histone H2AX in the site where it is damaged the DNA is important to allow repair. The phosphorylated form of H2AX can be induced by treatment with HDACi and the sites of H2AX phosphorylated by DNA damage

persist after exposure to HDACi, this results in a decreased ability to repair DNA strand break (56). Many of the components of the path of DNA repair such as ATM, NBS1, PARP1, Ku 70 and 80 are adjusted by acetylation.

Finally, the HDACi can sensitize cells to apoptosis induced by cytotoxic substances by decreasing the activation threshold for apoptosis by regulating the expression of pro-and antiapoptotic proteins (57).

As already said, HDACi may act on mechanisms that regulate protein degradation via the proteasome involvement, in which also operates the Hsp90 chaperone. For this reason, studies have been conducted to evaluate the efficacy of cotreatment with HDACi and Hsp90 inhibitors such as 17-AAG.

The synergistic effect of these two treatments leads to a more efficient degradation of oncoproteins whose half-life is regulated by this chaperone such as BCR-ABL, resulting in a greater mortality of tumor cells.

Another cotreatment with promising results is that with HDACi together with proteasome inhibitors, such as bortezomib.

The inhibition of proteasomal activity leads to an accumulation of ubiquitilated proteins.

This causes the endoplasmic reticulum stress and apoptosis. Cancer cells try to survive this stress "bundling" ubiquitilated proteins into a structure called cytoprotective perinuclear aggresome.

HDAC6 binds ubiquitilated proteins bringing to aggresome. So the inhibition of HDAC does not allow the formation of aggresome leading to the dispersion of microaggregates toxic in the cell. This proteotoxicity promotes the activation of the ER stress and induction of apoptosis (58).

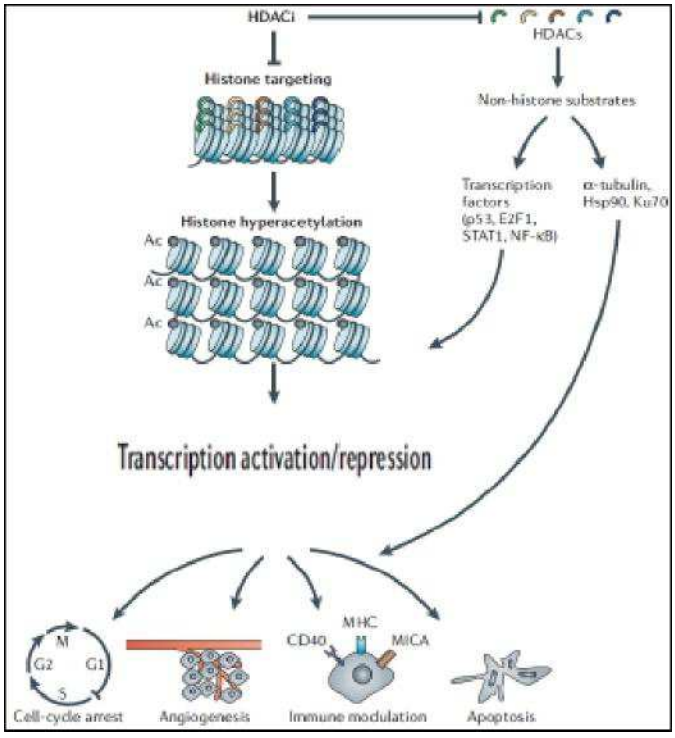
The main side effects associated to HDACi include fatigue, nausea, dehydration, diarrhea, and thrombocytopenia. In general, these effects are transient and cease with the end of treatment.

The development of clinical approaches with HDACi are still a matter of study with different substances. The HDACi constitute a group of promising anti-tumor substances, since they can induce death of malignant cells of a broad category of solid tumors and not. The mechanism by which it performs this action is yet to fully understand. But it is clear that these chemicals can cause growth arrest and death of cancer cells by different

mechanisms such as apoptosis, or autophagy, inhibition of metastasis and angiogenesis and impaired immune response. Normal cells are relatively resistant to cell death induced by HDACi. The basis of this resistance are not known. It has been speculated that the multiple defects of cancer cells do not allow to counteract stress induced by HDACi which fail, however, to carry out normal cells. In fact, the HDACi are well tolerated in clinical trials. While HDACi have shown good efficacy as anticancer agents in preclinical studies, in clinical approaches on a wide variety of tumors, only a portion of patients with certain diagnoses have responded to treatment with HDACi.

From this it is clear that is still important to study the mechanisms of action and the markers that can predict the effectiveness of response (59)

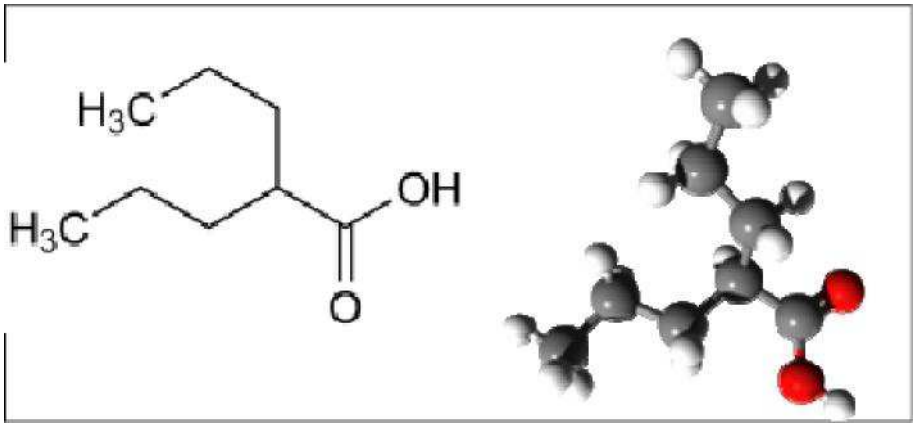
Figure 6. HDACi as anticancer drugs



1.3.4 Valproic Acid

Valproic acid (VPA) has been synthesized for the first time in 1882 but only in 1963 his efficacy as antiepileptic was discovered. Since then, the VPA has been used as a drug for the treatment of epilepsy, and also for other neurological disorders. Only in recent years its function as an inhibitor of histone deacetylase has been discovered (Figure 7) (60). Valproic acid is a small branched fatty acid with 8 carbon atoms, its molecular structure offers many possibilities for chemical modifications (Figure 7).

Figure 7. Chemical Structure of Valproic Acid (60)



The analogues of VPA, including its metabolites, show overlapping but distinct activities that affect seizures, neuronal processes, cell proliferation and differentiation. Studies carried out in recent years have confirmed that VPA and its analogues show antitumor action. Following the discovery of this antiproliferative activity of VPA in cancer cells, several studies have shown that this event is associated with the differentiation of neoplastic cells and is correlated with the inhibition of histone deacetylases.

Since it is already used as a drug, some effects on humans are already known. Unlike other short chain fatty acids such as butyric acid in the serum half-life of this drug is somewhat prolonged, between 7-16 h.

Valproic acid is generally well tolerated, at therapeutic concentration in plasma between 50 and 100 micrograms / ml, from patients treated for neurological problems. The major side effects are detectable in the liver, is also known to have teratogenic effect.

As deacetylase inhibitor, it has been observed that Valproic acid induces the hyperacetilation of the amino-terminal tails of histones H3 and H4 *in vitro* and *in vivo* and has been demonstrated its ability to inhibit histone deacetylase from the standpoint of enzyme (60). It was subsequently shown that valproic acid and similar compounds, inhibit histone deacetylase class I through two different mechanisms: inhibiting the catalytic activity and inducing the proteosomal degradation (61).

The VPA unlike the TSA, does not inhibit the activity of HDAC-6 and 10. The anti-proliferative effect of valproic acid was shown initially by Regan in 1985, who observed a decrease in mitotic index in murine neuroblastoma and glioma. This effect was reversible and not caused by cytotoxicity (62)

Valproic acid shows a potent antitumor effect in a wide range of models both *in vivo* and *in vitro*, by modulating several mechanisms including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation and senescence . These effects appear to be cell type specific and may depend on the level of differentiation and genetic alterations.

The majority of preclinical studies as anticancer drug have been addressed so far in hematologic malignancies, but there are also many studies on solid tumors used as a template. It has been observed that valproic acid induces apoptosis in many human leukemic lines (branch B, T and myeloid) by triggering the release of cytochrome c from mitochondria and activation of caspase-3, -8 and -9.

When the cell line MV4-11 was pre-treated with an inhibitor of caspases, the pro-apoptotic effect of valproic acid was inhibited in the nucleus, but not on the cell surface (63). These results allow to assume that valproic acid can activate two different apoptotic signaling pathways: a caspase-dependent and the other caspase-independent. The antiproliferative effect of VPA was also examined HCC cell lines and primary human hepatocytes.

In spite of the fact that the hepatocellular carcinomas are generally very resistant to chemotherapy, VPA inhibits proliferation in cancer cell lines but not in primary hepatocytes (64). The inhibition of proliferation was associated with increased expression of p21 in tumor cells of glioma, thyroid, melanoma, ovarian cancer, and medullo blastoma. The pro-apoptotic effect was observed in many models and

associated with different components of the apoptotic pathways. In particular, an increase of pro-apoptotic factors such as Bik, in breast cancer cell lines, and Bax in thyroid cells, were detected. Moreover a decrease of anti-apoptotic proteins such as Bcl-2 and Bcl-xL in prostate cancer cells and thyroid were also observed. These studies have been addressed using concentrations ranging from 0.2 mM to 10 mM.

Unfortunately, however, the doses of valproic acid necessary to obtain an in vivo antitumor effect are very high, thus causing the significant side effects and limiting its use in clinical practice. Studies have shown that the weak activity of the drug could be attributed to its inability to access the zinc cation in the pocket of HDAC enzyme activity. Coupling valproic acid with molecules capable of enhancing this activity could boost its pharmacological effects. In particular, the addition of a tiosulphonate group, linked via an ester or an amide group, would lead to the liberation of a-SH group, required for the de acetylation of lysine residues acetylated (65).

The hydrogen sulfide (H2S) is produced endogenously in a constant and is involved in many processes such as neuro-modulation, hypertension, inflammation, hemorrhagic shock and edema. It plays some protective roles against oxidative stress and in the maintenance of vascular tone (66).

1.3.5 Combination of Histone Deacetylase Inhibitors with Other Therapeutic Regimens

The results from *in vitro* and *in vivo* experiments using various cancer cells have demonstrated that combination of HDAC inhibitors with a variety of anticancer drugs have synergistic or additive effects (67). Chemotherapeutic combinations with HDAC inhibitors have also been used in clinical trials (68). Several types of therapies have been investigated in combination with HDAC inhibitors.

1) HDAC inhibitors were combined with other epigenetic modifiers. Inhibitors of DNA methyl transferases 5-azacytidine (azacitidine) and 5-aza-2'-deoxycytidine (decitabine) had increased antitumor effects when used with HDAC inhibitors (69, 70).

2) Promising results have been reported for combinations of HDAC inhibitors and ROS-generating agents. One such agent, adaphostin, increases entinostat and

vorinostat induced apoptosis in leukemia cells (71). In addition depletion of GSH, that is a ROS scavenger, increases the effects of vorinostat on AML cells (72).

3) Other drugs that have been combined with HDAC inhibitors are microtubule stabilizers. VPA increases the toxic effects of paclitaxel in anaplastic thyroid carcinoma cells due to their interaction with the tubulin β subunit. VPA enhances tubulin hyperacetylation that stabilizes microtubule structures (73). Similar enhancement of apoptosis was observed in endometrial carcinoma cells treated with trichostatin A and paclitaxel caused by the activation of the intrinsic mitochondria-dependent pathway. Trichostatin A also stabilizes microtubules via α-tubulin acetylation both *in vitro* and *in vivo* (74).

4) Another effective combination of HDAC inhibitors is that with proteasome inhibitors. Cancer cell death due to a combination of proteasome and HDAC inhibitors is caused by induction of oxidative stress, endoplasmic reticulum (ER) stress and stimulations of JNK. Bortezomib, marizomib (NPI-0052) and carfilzomib are proteasome inhibitors which have been combined with HDAC inhibitors. Treatment of multiple myeloma cells with bortezomib

made the cells more sensitive to vorinostat and sodium butyrate induced apoptosis (75). Mechanisms of the anticancer effects of a combination of proteasome and HDAC inhibitors are mitochondrial damage, disruption of aggresome formation, stimulations of JNK and caspases and enhancement of oxidative and ER stress (75, 76).

5) Numerous studies show synergisms or additive effects combining the HDAC inhibitors and DNA damaging agents such as topoisomerase inhibitors, DNA intercalators, inhibitors of DNA synthesis and agents covalently modifying DNA (i.e. doxorubicin, epirubicin, etoposid, cisplatin, 5-fluorouracil, melphalan, and temozolomide and ionizing radiation in many cancer cell lines) (77).

1.3.6 Clinical Studies and Registered Drugs

Several HDAC inhibitors of different structural classes are under clinical development (see Table 4). These include the short-chain fatty acids (phenyl butyrate and valproic acid); the hydroxamic acids [vorinostat (ZolinzaR, SAHA); panobinostat (LBH589); PCI-24781 and belinostat (PXD101)]; the cyclic tetrapeptides [romidepsin (IstodaxR, FK228); and the benzamides entinostat (MS-

275)]. Two HDAC inhibitors, vorinostat and romidespin, have been approved by the US FDA for treating patients with progressive, persistent or recurrent cutaneous T-cell lymphoma (CTCL) after one or more lines of chemotherapy and romidepsin for patients suffering from peripheral T cell lymphoma who received at least one prior Therapy (78, 79). Vorinostat had modest activity as a single agent. Its response rate is 10-20% in AML and MDS patients. However this HDAC inhibitor, in combination with 5-azacitidine, increased response rate by 30%. The combination of vorinostat with idarubicin and cytarabine had synergistic activity that was maximal when vorinostat preceded cytarabine. In a phase II trial, the response rate of 85% of the combination was superior to that of idarubicin and cytarabine alone; notably, there were responses in all patients with FLT3-ITD mutations (80, 81)

Table 4. HDAC inhibitors under clinical development

Chemical structure	Name	HDAC specifity	Study phase
Hydroxamates	SAHA (vorinostat)	Pan-inhibitor	Approved for CTCL, phase III alone or in combination
	PXD101 (belinostat)	Pan-inhibitor	Phase II alone or in combination
	LBH589 (panobinostat)	Classes I and II	Phase III alone or in combination
	ITF2357 (givinostat)	Pan-inhibitor	Phase II alone or in combination
	4SC-201 (resminostat)	Pan-inhibitor	Phase II alone or in combination
	PCI 24781 (abexinostat)	Classes I and II	Phase II alone or in combination
Cyclic peptides	Depsipeptide/FK228 (romidepsin)	Class I	Approved for CTCL and PCTL, phase III alone or in combination
Benzamides	MS-275 (entinostat)	Class I	Phase II alone or in combination
	MGCD0103 (mocetinostat)	Class I	Phase II alone or in combination
Aliphatic fatty acids	Valproic acid	Classes I and IIa	Phase II alone or in combination (approved for epilepsy and some other nonmalignant disseases)
	Butyrate	Classes I and IIa	Phase II alone or in combination

HDAC inhibitors also appear to be active in AML, lymphomas and myelodysplastic syndromes (MDS). Inhibition of HDACs mediates the epigenetic gene silencing in common translocations associated with certain hematological malignancies such as AML/ETO fusion protein (82). MGCD0103 (Mocetinostat) was evaluated in a

clinical phase II trial for the treatment of patients with refractory chronic lymphocytic leukemia (CLL). This HDAC inhibitor alone showed only limited efficacy. For this reason, mocetinostat in combination with other agents such as conventional chemotherapeutic drugs was recommended (83). LBH-589 (Panobinostat) underwent phase I and II clinical studies for the treatment of solid and hematologic maligancies and phase III clinical trials against CTCL and CML. Two phases I clinical trials showed promising results using LBH-589 in an oral and intravenous form against CTCL (84) and leukemias, respectively (85). Both studies found increased acetylation of histones in tumor cells that was associated with apoptosis. LBH-589 also underwent several phase III clinical trials against CTCL too and leukemia in its oral form and showed positive effect for the treatment of those diseases. Despite promising results in the treatment of CTCL, vorinostat and romidepsin have not been effective in studies that involved solid tumors. Clinical trials have assessed their efficacy against different solid tumors, e.g. neuroendocrine tumors, glioblastoma multiforme, mesothelioma, refractory breast, colorectal, NSCL, prostate, head and neck, renal cell, ovarian, cervical and thyroid cancers. None of the patients included in these trials showed at least partial response to treatment and they suffered from side effects (86). Study that assessed whether VPA modulates the efficacy of radiochemotherapy with temozolomide in glioblastoma patients showed that combined therapy with VPA was more effective over patients treated without HDAC inhibitors. The authors of this study reasoned that the improvement in the arm with VPA was due to the inhibition of HDAC (87). VPA with doxorubicin appeared to be an effective chemotherapy regimen (16% response rate) in patients with refractory or recurrent mesothelioma (88). Vorinostat enhanced the efficacy of carboplatin and paclitaxel in patients with advanced non-small-cell lung cancer (89). One clinical study showed that the combination of vorinostat and tamoxifen exhibited encouraging activity in reversing hormone resistance of breast cancer (90). The most common side effects of HDAC inhibitors are thrombocytopenia, neutropenia, diarrhea, nausea, vomiting and fatigue. Most toxicities are not class-specific and have been observed in all HDAC inhibitors (91, 92).

Recently, new sulforilated compounds, derivated from valproic acid have been syntesized by Sulfidris (Milan, Italy). These compounds seem to have a marked

histon-deacetilase activity, with respect to the parental compound valproic acid. The release of H_2S from this molecole is able to reduce collateral effects of the drugs, reducing their cardiotoxicity. Moreover H2S is able to increase the pharmacological effect of the drug, increasing the ability to inhibit HDAC (65).

2. Aim of the project

The aim of this project was to analyse the antitumoral activity of two compounds (ACS 2 and ACS 33), derived from Valproic Acid and conjugated with H2S, in human cancer cell lines derived from non-small-cell lung cancer tissues.

The antitumoral activity of the compounds was analysed together with molecular mechanisms of their activity, with the aim to design rationale for combination treatment strategy. Different schedules of treatment with conventional chemotherapic drugs were tested to identify new potential strategy for the therapy of NSCLC patients.

3. Materials and Methods

Cell lines

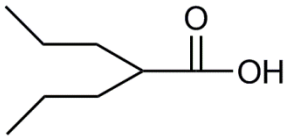
Three human stabilized cell lines derived from lung cancer were used for the different studies. In particular: CAEP cell line, isolated and characterized in our laboratory, starting from a fresh sample of epidermoidal lung cancer; the commercial Chago K1 cell line, derived from a bronchogenic carcinoma; and the commercial NCIH1915 cell line, derived from a cerebral metastasis of scarcely differentiated lung carcinoma. Cells were maintained in culture in DMEM/HAM F12 (1:1) with the adding of fetal bovine serum (10%), glutamine(2 mM), non essential aminoacids (1%) (Mascia Brunelli S.p.A. Milano), and insulin (10 μM/ml) (Sigma Aldrich). Cells were used in the exponential phase of their growth.

ACS 2, ACS 33 and Valproic Acid

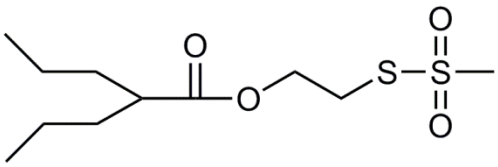
ACS 2 compound was solubilized in PEG 400 or DMSO, due to its scarcely solubility in aqueous solution. Valproic Acid and ACS 33 compounds were dissolved in DMSO, all at concentration of 100 mmol/L. Final concentrations of PEG 400 and DMSO never were over 0.5% and 1%, respectively.

Valproic Acid, ACS 2 e ACS 33 (chemical structures are represented in Figure 1) were analysed at concentrations of 1, 35, 70, 140, 210, 280 μM for 72h. Antiproliferative activity of the 3 compounds was analysed using sulforodamine B assay (SRB), according to Skehan et al. (93). The ability of the compounds to induce apoptosis was analysed by TUNEL assay using flow cytometry method.

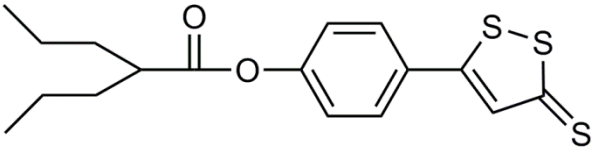
Figure 1. Chemical structures of Valproic Acid, ACS 33 and ACS 2.



VPA M.W.=144.2



ACS 33 M.W.=282.4



ACS 2 M.W.=352.5

Drugs combination experiments

The activity of the ACS 2 compound was evaluated in combination with cisplatin and doxorubicin. Different schedules of combination were used. Regarding cisplatin: a) simultaneous exposure to ACS 2 compound and cisplatin for 6 hours, followed by ACS 2 for 66 hours; b) exposure to the sequence ACS 2 for 72 hours, cisplatin for 6 hours, 18 hours wash out and 72 hours with ACS 2 again; c) inverse sequence, cisplatin for 6 hours, 18 hours wash out, ACS 2 for 72 hours.

In these experiments ACS 2 was used at the concentrations of 70, 140, 210, and 280 µM, whereas cisplatin at the concentrations of 0.01, 0.1, 1 and 10 µM. Each different

experiment was carried out using the different controls (cell line not treated with drugs, maintained in culture for the same time expected for samples treated with drugs).

For combination experiments with doxorubicin: a) doxorubicin exposure for 1 hour, followed by 71 hours wash out, and exposure to ACS 2 for 72 hours: b) exposure to ACS 2 for 72 hours, followed by Doxorubicin for 1 hour, and wash out for 71 hours; c) simultaneous exposition to ACS 2 and doxorubicin for 1 hour, followed by exposition to ACS 2 for 71 hours.

In these experiments ACS 2 was used at the concentrations of 70, 140, 210, and 280 µM, whereas doxorubicin was used at the concentrations of 0.005, 0.05, 0.5 and 5 µM.

To analyse the obtained results different methods are present in literature, but some of these aren't applicable to drug with low cytotoxic effect or without a dose-effect curve, as in the case of cisplatin. So we choose to use the method of Romanelli et al. (94).

In brief, the expected cell survival (Sexp, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) and the observed cell survival (Sobs) for the combination of A and B were used to construct an R index (RI): RI¼Sexp/Sobs. RI>1.5 indicated a synergistic interaction, RI<0.5, antagonism and RI>=0.5 and <=1.5, additivity.

Matrigel invasivity test

Invasion assays were performed in triplicate in 24-well multiwall plates containing BD Falcon Cell Culture inserts with 8-mm filters coated with Matrigel basement membrane matrix (BD Biosciences, Milan, Italy). NIH 3T3 cells were used as control cells. After drug exposure, each well was loaded with 10 x 10^4 cells and incubated for 22 h at 37°C in 5% CO2 atmosphere. Non-invasive cells were removed from the upper surface of the membrane with a cotton swab. The invasive cells on the underside of the membrane were fixed in 100% methanol and stained with 1% toluidine blue. The air dried membrane was placed on a slide and cells were counted under light microscope at 40 X magnification. The percentage of invasion was calculated according to the manufacturer's instructions.

PCR Real Time

PCR Real Time was performed for MMP1 and HPRT1 and GAPDH housekeeping genes using TaqMan Gene Expression Assay, and using TAqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Monza, Italy). PCR reactions were carried out

in triplicate on 7500 PCR Real Time System (Applied Biosystems) under the following conditions: 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The obtained data were analyzed by AbiPrism 7000 software. Reproducibility of RT-PCR was verified in triplicate reactions and the coefficient of variation (CV), calculated from three Ct values, was always <1.5%.

Western-blot

After ACS 2 treatment, cells were lysed in 50 mM di Tris-HCl (pH 8.0), 150 mM di NaCl, 1% Triton X-100, e 0.1% SDS, with the adding of 1 mM phenylmethylsulfonyl Fluoride (PMSF) and a proteases inhibitor cocktail (Sigma-Aldrich). An equal amount of proteins were underwent electrophoresis in 12% SDS-poliacrilamide gel. Proteins were transferred on a Immobilon-P Transfer Membrane (Millipore). After blockage of aspecific sites, membrane was incubated with the specific antibodies: anti-acH3 and anti-acH4 (Upstate Millipore, Milan, Italy), and anti-b-actin (Santa Cruz Biotechnology, Inc-DBA., Segrate, Italy) as loading control. Densitometric analysis was made using Image Lab software (BioRad).

DNA denaturation assay

DNA denaturability was probed using acridine orange (AO), the metachromatic fluorochrome which differentially stains double-stranded (ds) versus single stranded (ss) DNA sections (95). AO, when bound to ds DNA, yields green fluorescence, whereas its interaction with ss DNA results in red fluorescence (96). The fluorescence color of DNA-bound AO shows the status of DNA denaturation, which in turn, correlates with chromatin condensation. Briefly, cells were fixed with 1% formaldehyde in PBS on ice, then treated with RNAse A (MP Biomedicals, 5 Kunitz units/ml) and exposed to 0.1M HCL for 30 sec at room temperature. Cells were subsequently stained with AO (10 mg/ml) (Sigma) dissolved in 0.1M citric acid-phosphate buffer at pH 2.6. Data were analyzed by flow cytometry using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA) and experiments were repeated twice.

TUNEL assay

At the end of drug exposure, the percentage of apoptotic cells was evaluated by flow cytometric analysis according to the previously described TUNEL assay procedure (97). Briefly, after treatment cells were trypsinized, fixed, exposed to the TUNEL reaction mixture, counterstained with propidium iodide, and then analyzed by FACS.

Mitochondrial membrane potential (DC) depolarization assay

After a 72-h exposure to ACS 2 140 mM, mitochondrial membrane potential was evaluated by flow cytometric analysis according to the previously described JC-1 method (97). Data acquisition and analysis were performed using CELLQuest software. 15,000 events were recorded for each sample.

Cytochrome c release assay

Cells were treated according to the manufacturer's instructions (Inno CyteTM Flow Cytometric Cytochrome c Release Kit, Calbiochem1, EMD Chemicals, Inc., Darmstadt, Germany). Briefly, cells were washed once in PBS and then immediately incubated in permeabilization buffer for 10 min on ice, fixed in 8% paraformaldehyde and washed twice with 1 X wash buffer.

Cells were then incubated with anti-cytochrome c antibody diluted 1:1,000 in blocking buffer for 1 h, washed and incubated with anti-IgG FITC diluted 1:300 in blocking buffer, according to the manufacturer's instructions. Cells were then resuspended in 1_ wash buffer and analyzed using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA).

Statistical analysis

Differences between treatments in terms of dose-response, apoptosis and gene expression modulation were determined using the Student's t-test for unpaired observations. $P<0.05$ was considered significant.

4. Results

Valproic acid didn't show antiproliferative activity in the examined cell lines. At the contrary, the two compounds ACS 2 and ACS 33 showed a cytotoxic activity in all 3 cell lines. In particular, ACS 2 was the most active compound, with a growth reduction of over 50% at concentrations ranging from 64.5 µM and 72 µM (Figure 2) (Table 1).

Figure 2. Cytotoxic activity of valproic acid and of its derivatives ACS 33 and ACS 2 in human lung cancer cell lines ChaGo-K1, CAEP and NCIH1915 after a 72-h exposure. Each point indicates the mean of at least three experiments. Standard deviation (SD) never exceeded 5%.

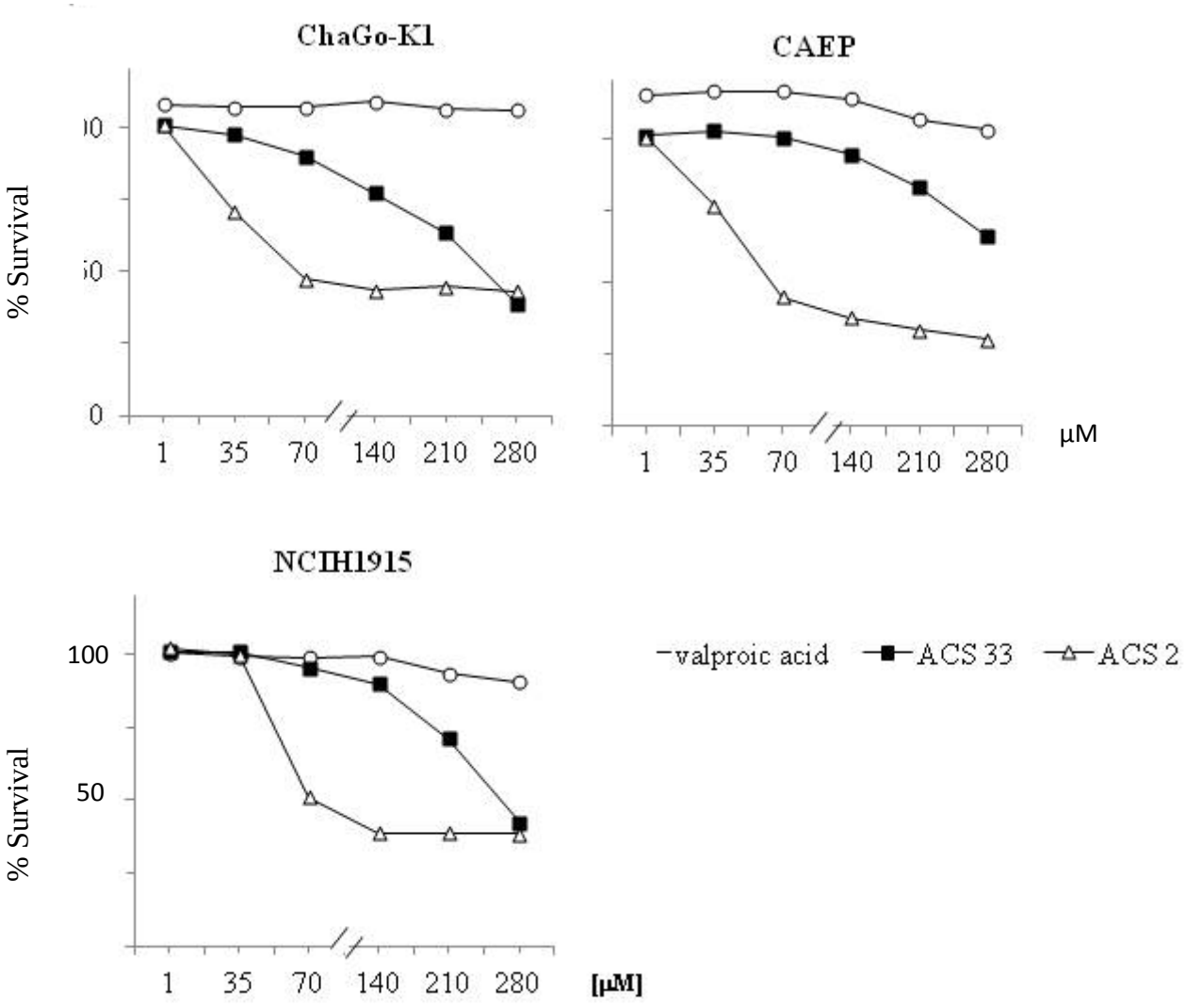


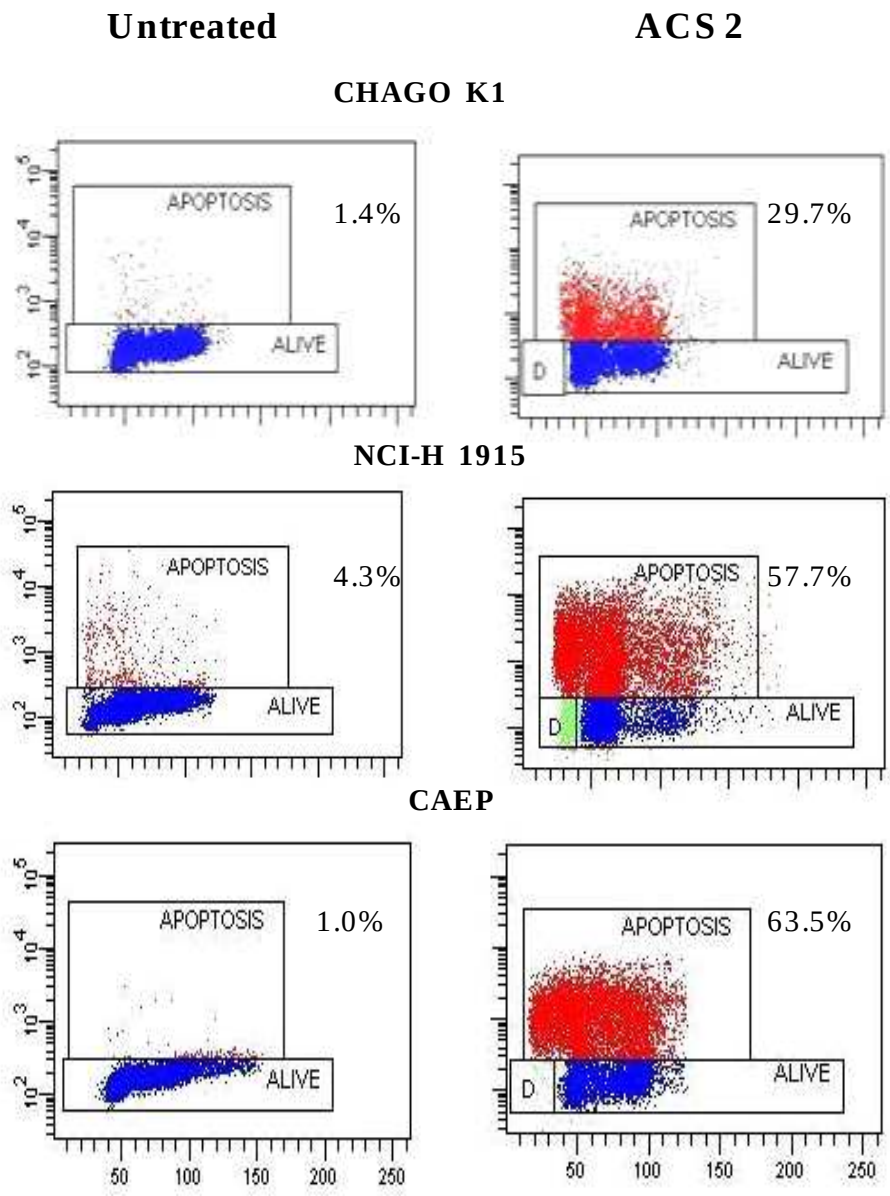
Table 1. Valproic acid, ACS 2 e ACS 33 concentrations able to induce a cell growth reduction of 50% in the 3 analysed cell lines.

Drugs	IC_{50} values [μM]		
	ChaGo-K1	**CAEP**	**NCIH1915**
Valproic acid	n.r.*	n.r.	n.r.
ACS33	248	n.r.	261
ACS2	66,5	64,5	72

***not reached**

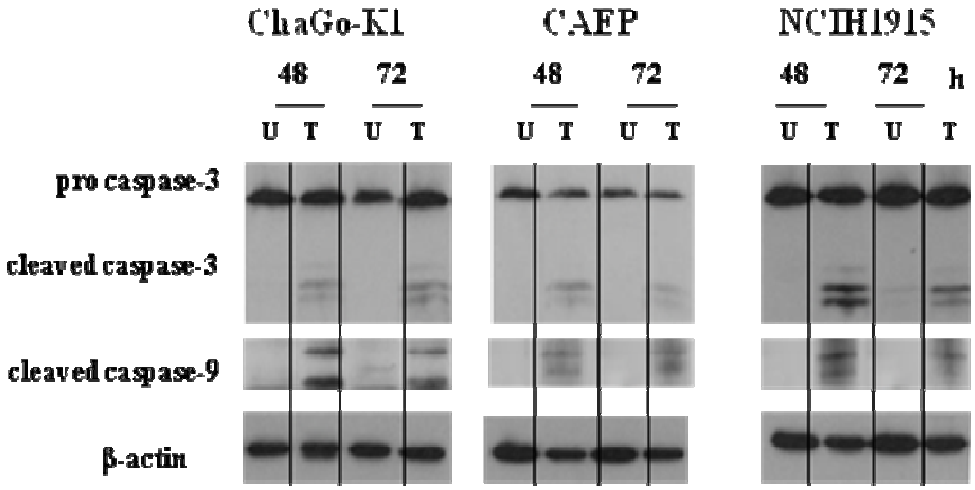
TUNEL analysis was performed in all 3 cell lines using ACS 2, which showed the higher antiproliferative activity. The compound was able to induce apoptosis in all 3 cell lines. In particular, 30% of apoptosis was observed in Chago K1 cell line, whereas 60% of apoptosis was observed in NCIH1915 and CAEP cells, after treatment with the compound at the concentration of 140 μM for 72h (Figure 3).

Figure 3. TUNEL analysis



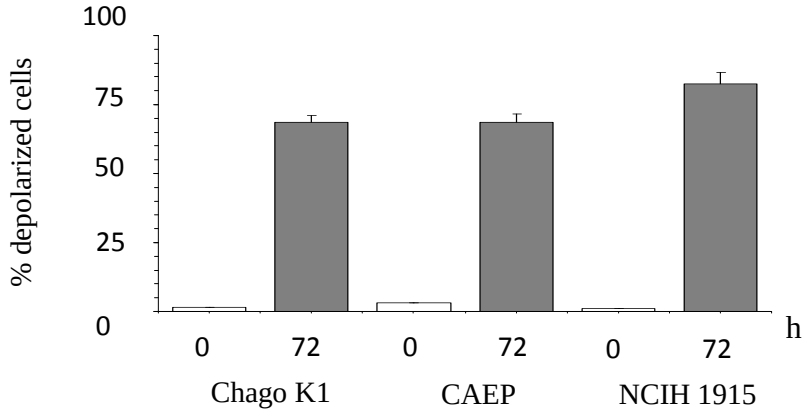
At the same conditions (ACS 2 140 µM for 72h) caspase 3 and 9 activation was observed (Figure 4), confirming the induction of apoptosis and suggesting a mitochondrial activation of the apoptotic process.

Figure 4. Western-blot analysis of caspase 3 and 9.



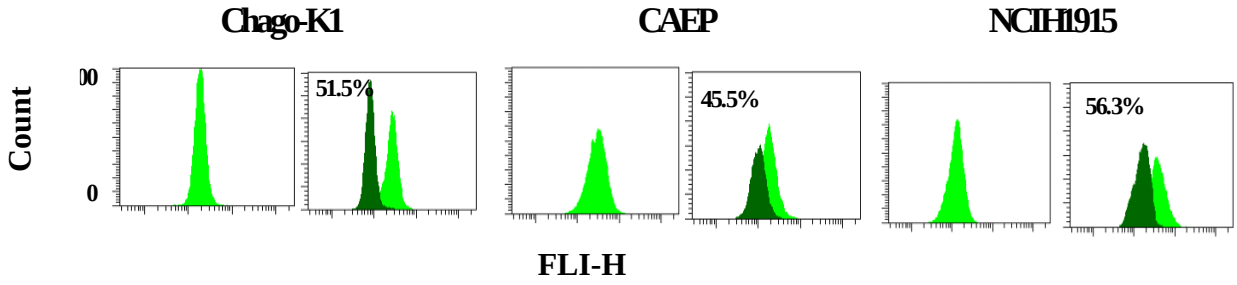
The mitochondrial activation of apoptosis was confirmed also by a marked depolarization of mitochondrial membrane potential, observed after ACS 2 treatment in all 3 cell lines (Figure 5).

Figure 5. Percentage of mitochondrial membrane potential depolarization (DC) after a 72-h exposure to ACS 2 140mM. Samples were run in triplicate, and data are the average of three experiments. SD never exceeded 5%.



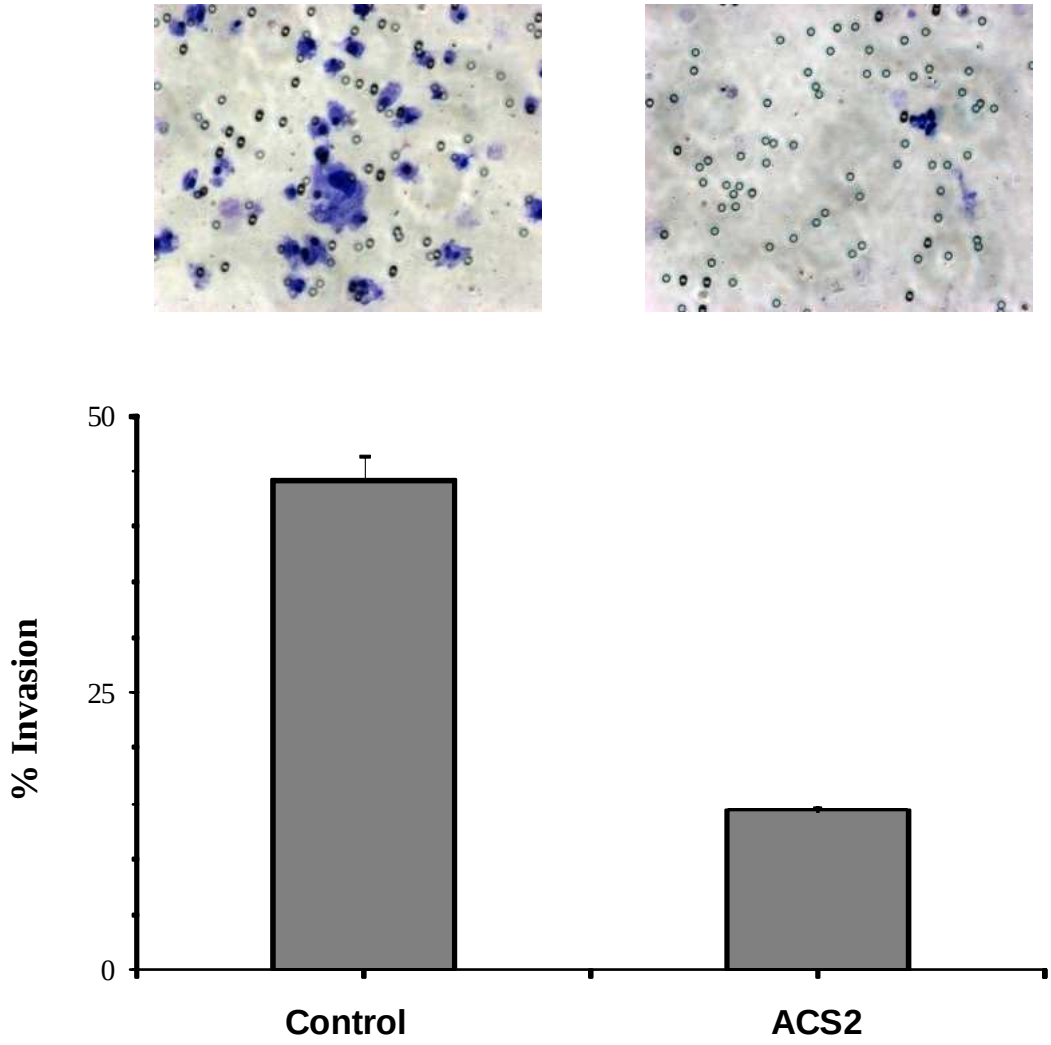
In all 3 cell lines an increase of cytocrome c release was observed after treatment with ACS 2 (Figure 6).

Figure 6. Cytocrome c release.



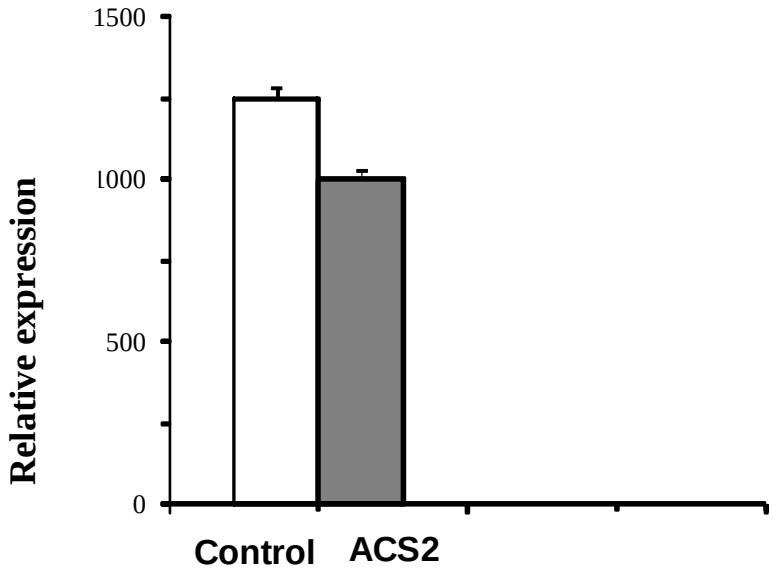
To test the anti-invasivity capacity of ACS 2, Matrigel analysis was performed on NCIH1915 cell line. After treatment with the compound for 72h at the concentration of 140 µM, the invasion capacity of cells decreased of about 30% (Figure 7).

Figure 7. Invasivity test on NCIH1915 cells after exposure to ACS 2.



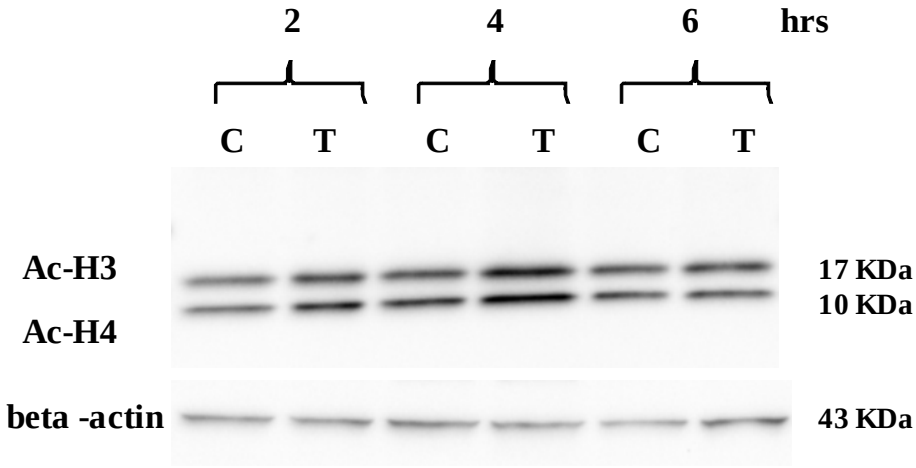
The reduction of invasive capacity of cells was accompanied by a reduction of expression levels of MMP 1, protein involved in the process of invasion and metastatization (Figure 8).

Figure 8. Expression levels of MMP1 analysed through Real Time PCR, and using as housekeeping genes HPRT1 and GAPDH.



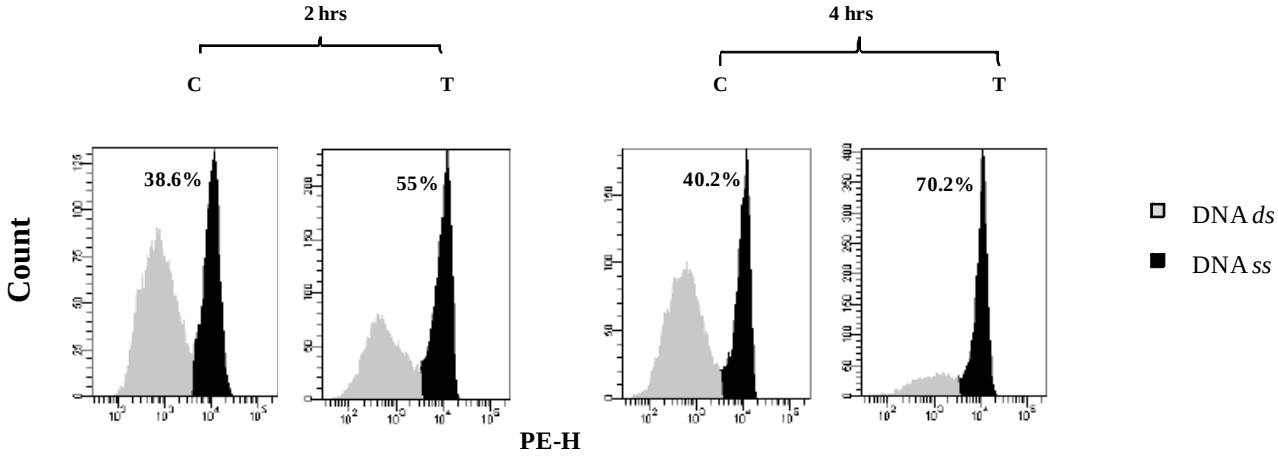
The ability of ACS 2 to interfere with the denaturation process of DNA, and as consequence with chromatin condensation grade, was also analysed. This search was finalized to found a rationale to combine ACS 2 compound with chemotherapic drugs with the ability to induce DNA damage, with the aim to design specific drug combination schedule of treatment. First of all the expression levels of the acetylated form of two histonic proteins, H3 e H4, was analysed in CAEP cell line. An increase of 1.61 and 1.58 fold in the expression level of ac-H3 e ac-H4, respectively, was found after 4 h exposure to ACS 2 at the concentration of 140 µM, with respect to control (Figure 9).

Figure 9. Expression levels of acetilated form of H3 e H4 histones, evaluated by Western-blot.



To confirm these findings, we used the acridine orange (AO) assay to analyze the different grades of susceptibility to denaturation of CAEP cells after exposure to ACS 2 140mM. Such an approach takes advantage of the fact that DNA in extended rather than condensed chromatin differs in susceptibility to denaturation, which in turn, correlates with chromatin condensation grades. Once again, we observed an increment of up to 30% in AO bound to ssDNA with respect to ds DNA, which is symptomatic of chromatin decondensation (Figure 10). Similar results were obtained in ChaGo-K1 and NCIH1915 lines (data not shown).

Figure 10. Orange acridine assay.



ACS 2 was then analysed in combination with cisplatin. The activity was different in relation to the different schedules of treatment. In particular, the sequence cisplatin for 6h, wash out 18h, ACS 2 72h showed an additive effect in NCIH1915 and CAEP cell lines, whereas showed an antagonistic effect in ChaGo-K1 cells. The inverse combination, ACS 2 72h followed by cisplatin for 6h and 18h of wash out resulted in an additive effect in all cell lines.

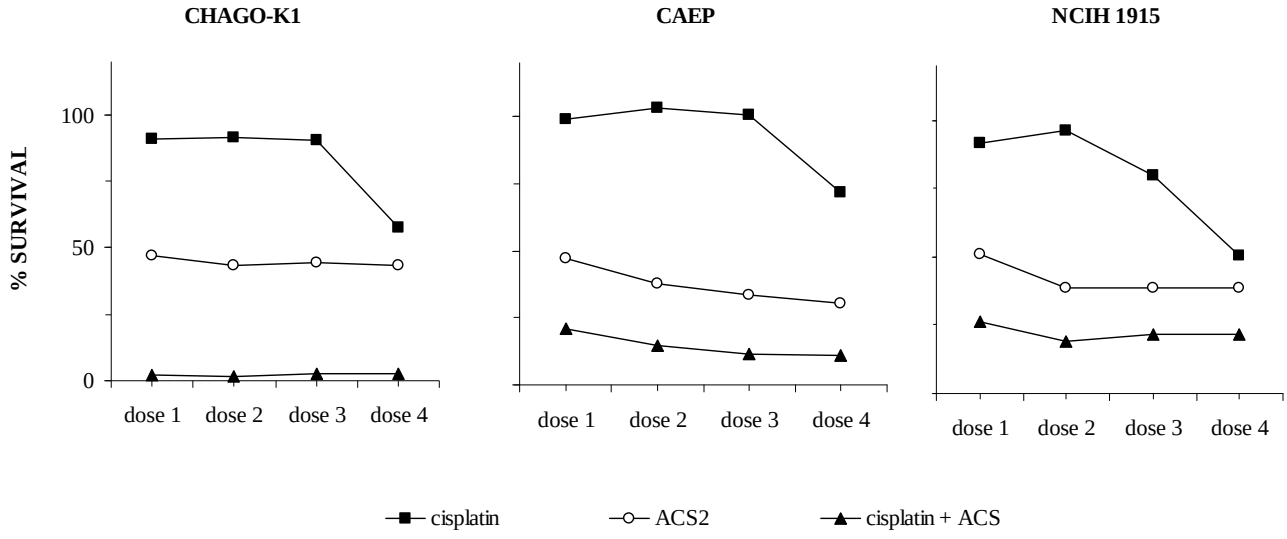
Conversely, a strong synergistic interaction was observed when ACS 2 and cisplatin were administered together. (Table 2 and Figure 11).

Table 2. Interaction between ACS 2 and cisplatin.

	Interaction type		
Drug schedule	NCIH1915	CAEP	ChaGo-K1
Pt (6 h) → 18-h w.o. → ACS2 (72 h)	Additive	Additive	Antagonistic
ACS2 (72 h) → Pt (6 h) → 18-h w.o.	Additive	Additive	Additive
ACS2 + Pt (6 h) → ACS2 (66 h)	Synergistic (RI* = 1.9)	Synergistic (RI_m = 2.9)	Synergistic (RI_m = 5.6)

Pt, cisplatin; w.o., washout; *RI, R index >1.5 = synergism; RI < 0.5 = antagonism; RI >0.5 and < 1.5 = additivity.

Figure 11. Synergistic interaction between ACS 2 and Cisplatin used simultaneously (ACS 2 + cisplatin for 6h followed with ACS 2 for further 66h).



DRUG SCHEDULE	dose 1 (µM)	dose 2(µM)	dose 3(µM)	dose 4(µM)
Pt (6h)→ 66h w.o.	0.01	0.1	1	10
ACS 2 (72h)	70	140	210	280
ACS 2 + Pt (6h) → ACS 2 (66h)	0.01 (*Pt*); 70 (*ACS*)	0.1 (*Pt*); 140 (*ACS*)	1 (*Pt*); 210 (*ACS*)	10 (*Pt*); 280 (*ACS*)

Moreover, the combination of ACS 2 with doxorubicin was also analysed, and all treatment schedule showed an additive results (Table 3).

Table 3. Interaction between ACS 2 and doxorubicin

DRUG SCHEDULE	INTERACTION TYPE		
	NCIH 1915	CAEP	CHAGO-K1
DOXO (1h)→ 72h w.o. → ACS (72h)	additive	additive	additive
ACS (72h) → DOXO (1h)→ 72h w.o.	additive	additive	additive
ACS + DOXO (1h) → ACS (72h)	additive (RI_m^* = 1.5)	additive	additive

5. Discussion

Histone deacetylases (HDACs) are a family of enzymes that regulate chromatin remodeling and gene transcription, and there is growing interest in HDAC inhibitors as promising anticancer agents. Short chain fatty acids such as butyric and valproic acid were the first HDAC inhibitors to be identified as tumor growth inhibitors and inducers of apoptosis both in vitro and in vivo. However, they were found to have low potency, with IC50 in the millimolar range. Despite such weak in vitro activity, VPA's anticancer mechanism of action has been investigated in preclinical models of skin, breast, colon, prostate and small cell lung cancer, and the drug is currently used in phase I–III clinical trials (98, 99). Unfortunately, therapeutic doses of VPA are necessarily very high and cause limiting side-effects. An attempt to overcome the weak potency of VPA, due mainly to its inability to access the zinc cation in the HDAC active-site pocket, has been made by inserting sulfurated groups, selected amongst those known to be endowed with cancer chemopreventive activity and described as potent HDAC inhibitors, in the valproate moiety (100- 102).

In this study we evaluated the antitumor activity of valproic acid and its derivatives ACS 33 and ACS 2 bearing a thiosulfonate and dithiolthione moiety, respectively, in a panel of NSCLC cell lines. Both derivatives exhibited a much higher cytotoxic activity than that of the parent compound, with ACS2 proving to be the most effective drug, capable of reducing cell survival by 50% in all cell lines tested and at low concentrations.

Such data are in agreement with those reported by Moody et al. (103), although it must be pointed out that these authors used different cell lines and cell survival assays. The possibility of using ACS 2 at low concentrations has therapeutic implications as it would eliminate the problem of side-effects of the valproate component, making ACS 2 a potential ‘‘lead’’ compound for clinical development. On the basis of these data, we investigated the mechanism of action underlying the improved antitumor effect of ACS 2 with respect to the parent compound, and discovered that the strong cytotoxic activity of ACS 2 is largely due to its pro-apoptotic action observed in all cell lines. Our findings also showed that ACS 2 triggers apoptotic machinery via the mitochondrial pathway, as highlighted by the strong mitochondrial membrane depolarization, cytoplasmatic cytochrome c release and caspase-9 and -3 cleavage. These data differ from the results

obtained by Moody and coworkers who did not register apoptosis after exposure of NCI-H1299 cells to ACS 2. Such a difference can probably be attributed to the specific experimental conditions used by the authors, i.e., the short exposure time (24 h) and the lower drug concentration (28mM) (103).

Matrix metalloproteinase-1 (MMP-1), an interstitial collagenase, plays an important role in the breakdown of extracellular matrix and mediates pathways of apoptosis, angiogenesis, and immunity. It has also been demonstrated that the overexpression of this enzyme is associated with tumor initiation, invasion, and metastasis of many types of human cancer including lung cancer (104-106). Furthermore, a specific polymorphism of MMP-1 (MMP-1-1607 1G-to-2G) was recently found to be associated with susceptibility to both growth and progression of lung cancer (107). In our study, we showed that ACS 2 significantly reduces cell survival, diminishes the invasive capacity of NCIH1915, an established cell line obtained from a metastatic brain lesion, and downregulates MMP-1 expression. These data, together with the anti-angiogenic activity described by Isenberg et al. (108), highlight the potential therapeutic effectiveness of ACS 2 in advanced NSCLC where patient survival is very poor due to the high incidence of metastases. Conventionally, NSCLC is treated with surgery in its early stages, while combination chemotherapy consisting of platinum-based regimens is standard practice in advanced disease. In our study we explored the potential for using ACS 2 in combination with cisplatin and aimed to design an effective schedule based on the plasmatic half-life of both drugs. Our findings show that the simultaneous use of ACS 2 and cisplatin for 6 h followed by a further 66-h exposure to ACS 2 produced a highly tumoricidal effect in all cell lines tested starting from the lowest doses of both drugs. Furthermore, renal protection against the effect of cisplatin afforded by another dithiolethione (109), in addition to the possibility of using low doses of both drugs, highlights a potentially good safety profile of the drug schedule. Finally, it can be hypothesized that the synergism between the two drugs is ascribable to the effect of ACS2 on chromatin remodeling through HDAC inhibition, which makes DNA less compact and more sensitive to cisplatin induced damage. In fact, lower chromatin condensation was confirmed when an increase in acetylation of the two core histonic proteins (H3 and H4) was detected, a hallmark of chromatin relaxation, after a short exposure to ACS 2. We also observed an increase in DNA susceptibility to denaturation after the same exposure time, which in turn correlates with chromatin decondensation (95).

Doxorubicin, a widely used antiblastic agent, is considered ineffective against a number of tumor types, including NSCLC, because of its narrow therapeutic range, outside of which

side effects such as severe cardiotoxicity have been observed (110, 111). A recent study has shown that deficiencies in H2S synthesis may contribute to the pathogenesis of doxorubicin-induced cardiomyopathy and that administration of NaHS, an H2S donor, ameliorates doxorubicin-related cardiac dysfunction by inhibiting oxidative stress injury (112). The use of H2S donors could therefore prove to be a promising therapeutic strategy to prevent doxorubicin-induced cardiotoxicity. We thus attempted to define an effective drug schedule for ACS 2 and doxorubicin in our preclinical models, observing an additive interaction independently of the combination scheme used. These preliminary data are nevertheless interesting because of the potential cardiovascular protection provided by the H2S component of ACS 2. Further research is warranted into this specific area.

In conclusion, our findings, in addition to demonstrating the increased cytotoxic activity of ACS 33 and ACS 2 with respect to valproic acid, highlight the strong pro-apoptotic activity of ACS 2, a promising lead compound of this new class of HDAC inhibitors. Furthermore, we identified a highly effective combination schedule of ACS 2 and cisplatin capable of inducing a synergistic interaction even when the two drugs are used at low concentrations. This increased efficacy is probably due to the modification induced by the valproate derivative on chromatin condensation, facilitating the establishment of cisplatin-DNA abducts. For these reasons, the VPA derivative ACS 2 in combination with platinum compounds could represent a promising alternative to traditional chemotherapeutic regimens used for advanced lung cancer.

Further in vivo studies are needed to confirm these results.

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