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DEPARTMENT OF PHARMACY

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TRANSCRIPTION FACTORS AS TARGETS OF NEW MOLECULES WITH POTENTIAL ANTI-CANCER ACTIVITY.

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INTRODUCTION

1. Melanoma

1.1 Etiology and Epidemiology

Melanoma is the least common but the most aggressive form of skin cancer occurring when melanocytes, melanin-producing cells of the skin, eye, mucosal epithelia, and meninges undergo changes and become malignant. So, melanoma may occur in all parts of the body where are normally present melanocytes, as the skin of course, but also the mucous membranes, meninges and uvea. Has greater impact on the left side of the body\(^1\). Melanocytes are derived from neural crest progenitors, and their development is modulated by the receptor tyrosine kinase (RTK) c-KIT and microphthalmia-associated transcription factor (MITF). Melanocytes produce two main types of pigment: brown/black eumelanin, that protects from ultraviolet radiation (UVR) and red pheomelanin that contributes to melanomagenesis through a mechanism involving ROS\(^2\)\(^-\)\(^3\).

Genetic predisposition or an environmental stressor contributes to the genesis of melanoma. The strongest melanoma risk factors are a family history of melanoma, atypical moles or dysplastic nevi. Sun sensitivity, immunosuppression and exposure to ultraviolet radiation are additional risk factors\(^4\). In particular the exposure to
Ultraviolet light causes variations in particular genes (polymorphisms) that affect both the defensive response of the skin to ultraviolet light and the risk of melanoma. Ultraviolet radiation has multiple effects on the skin: causes genetic changes, impairs cutaneous immune function, increases the local production of growth factors, and induces the formation of DNA-damaging reactive oxygen species that affect keratinocytes and melanocytes. Clinically, variations in pigmentation and the protective response to ultraviolet light are associated with variations in susceptibility to melanoma. Also, specific phenotypic characteristics have been shown to correlate with increased risk of melanoma such as green/blue eye color, red/blond hair color, fair skin, the presence of freckles and inability to tan. Caucasians have a 20-fold increased risk of developing skin melanoma when compared to dark skin populations.

According to the World Health Organization, in the last decades the incidence of cutaneous malignant melanoma is increasing faster than any other cancer in the world, about of 2-7% annually. This fact has led to the use of the term melanoma “epidemic”. In 2014 the American Cancer Society and the National Cancer Institute estimated 76,100 new cases of patient with invasive melanoma and 9,710 deaths only in USA. Although melanoma represent less of 5% of cases of skin cancer it counts more of 75% of skin-related
deaths. Melanoma is very common in young adults aged 15-34 years. Melanoma incidence is also dependent on gender, in fact in the U.S. women have a higher risk of melanoma than men before age 40, but they have a better prognosis in terms of survival. In men, the predominant anatomical location for melanoma lesions is the trunk and in women lesions are mainly found in the lower limbs. This impressive increase in incidence may be due to several factors, including behavioural changes, better early detection by screening instruments, changes in diagnostic criteria in histopathology, and perhaps also the change in the medico-legal climate.

A simple procedure to diagnose melanoma is founded on its pigmentation. The American Cancer Society has devised a simple A-B-C-D-E acronym to guide in the identification of pigmented lesions that should be evaluated with biopsy (Figure 1).

**A** = **asymmetry**, when one half the lesion is looking different from the other half; the common nevi are round and symmetrical whereas the most melanomas is asymmetric.

**B** = **border irregularity**, the common nevi have smooth edges whereas margins of melanomas in the initial phase are often irregular and jagged.

**C** = **color variegation**, the common nevi have the same shade of brown whereas the lesion is not one uniform color...
but rather a combination of black, brown, blue, or even white.

**D = diameter**, lesions whit a diameter greater than 5-6 mm is exposed to a higher risk of turning into melanoma.

**E = evolution** or **enlargement**, any change or evolution of a previously present pigmented lesion should raise suspicion for the lesion being melanoma and trigger a biopsy.

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**Figure 1.** The ABCs of Melanoma Diagnosis
1.2 Stadiation

The Clark model describes the histopathological changes that occur in the progression of melanoma, from normal melanocytes to malignant. In the Clark model, the first phenotypic change in melanocytes is the development of benign nevi, which are composed of neval melanocytes (Figure 2). Normal melanocytes progressively develop a malignant phenotype through the acquisition of various phenotypic features. The particular histologic features characterizing each step of progression are the visible manifestations of underlying genetic changes. When the growth of a nevus is controlled, probably by the oncogene-induced cell senescence, they rarely progresses to cancer. Instead, abnormal activation of the mitogen-activated protein kinase (MAPK) signaling pathway due to somatic mutations of N-RAS or BRAF stimulates growth in melanoma cells. These mutations occur at a similar frequency in benign nevi and in primary and metastatic melanomas. So most nevi stop proliferation and remain static for decades, other may acquire additional molecular lesions and become malignant.

The second step in the progression of melanoma is the transformation of some benign nevi in dysplastic nevi, which are not really invasive malignancies, but rather just moles that have some architectural disorder. The third step is the radial growth phase melanoma in which there is
growth through the epidermis but not deeply into the
dermis. In the fourth step, namely vertical-growth phase,
appear the invasive characteristics of the melanoma cells.
They acquire a potential to spread, grow intradermally and
gains the capacity to invade the vascular and lymphatic
systems.

Finally, a metastatic melanoma develops when tumor
cells dissociate from the primary lesion, migrate through the
surrounding stroma, and invade blood vessels and
lymphatics to form a tumor at a distant site, whether it be in
an organ, or a skin, or lymph node through vascular or
lymphatic invasion11.

![Biologic Progression of Melanoma](image)

**Figure 2.** Biologic Progression of Melanoma
*(Miller et al., N Engl J Med 2006)*
The **Breslow model** measures the melanoma thickness (in millimetres) as the distance between the upper layer of the epidermis and the deepest point of tumor penetration. Tumor depth is most accurately measured by evaluating the entire tumor via an excisional biopsy. Clinically, the Breslow index is considered one of the most significant factors in predicting the progression of the disease and primary criterion in melanoma staging\(^\text{12}\) (Fig. 3).

**Figure 3.** Clark and Breslow’s model  
*(Miller et al., N Engl J Med 2006)*

The current melanoma staging refers at the seventh edition (2209) of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual that was made on the basis of a multivariate analysis of 30,946 patients with stages I, II, and III melanoma and 7,972 patients with stage IV
melanoma. In this system histological features as tumor thickness, mitotic rate, and ulceration, are important hallmarks of melanoma prognosis and staging.

According to AJCC the melanoma stages are classified as reported:

**STAGE 0**: In situ (non-invasive) melanoma remains confined to the epidermis.

**STAGE I and II**: Localized Melanoma. These stages are divided each other in: Stage IA-IB and IIA-IIB-IIC on the base of tumor thickness “T” (thresholds of 1.0, 2.0, and 4.0 mm) and the presence of microscopic ulceration. The presence of at least one mitosis (cancer cell division) per millimeter squared (mm²) can upgrade a thin melanoma to a later stage at higher risk for metastasis.

**STAGE III**: Regional Metastatic Melanoma. These stages are divided each other in: Stage IIIA-IIIB-IIIC on the base of number of nodal metastases “N” (thresholds of 1, 2-3 and 4+ nodes, depends on factors such as whether the metastases are in-transit or have reached the nodes, the number of metastatic nodes, the number of cancer cells found in them) and micrometastases. This can be determined by examining a biopsy of the node nearest the tumor, known as the sentinel node.

**STAGE IV**: Distant Metastatic Melanoma, defined on the evaluated of the site(s) of the distant metastases “M”
(nonvisceral, lung, or any other visceral metastatic sites) and elevated serum lactate dehydrogenase (LDH) level (Table 1 e 2).

<p>| Table 1. TNM Staging Categories for Cutaneous Melanoma (Balch et al., J Clin Oncol. 2009) |</p>
<table>
<thead>
<tr>
<th>Classification</th>
<th>Thickness (mm)</th>
<th>Ulceration Status/Mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>≤ 1.00</td>
<td>a: Without ulceration and mitoses &lt; 1mm²  b: With ulceration or mitoses ≥ 1mm²</td>
</tr>
<tr>
<td>T2</td>
<td>1.01-2.00</td>
<td>a: Without ulceration      b: With ulceration</td>
</tr>
<tr>
<td>T3</td>
<td>2.01-4.00</td>
<td>a: Without ulceration      b: With ulceration</td>
</tr>
<tr>
<td>T4</td>
<td>&gt; 4.00</td>
<td>a: Without ulceration      b: With ulceration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>No. of Metastatic Nodes</th>
<th>Nodal Metastatic Burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>N1</td>
<td>1</td>
<td>a: Micrometastasis² b: Macrometastasis²</td>
</tr>
<tr>
<td>N2</td>
<td>2-3</td>
<td>a: Micrometastasis² b: Macrometastasis²</td>
</tr>
<tr>
<td>N3</td>
<td>4+ or metastatic nodes, or matted nodes, or in transit metastases/septiles with metastatic nodes</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>Site</th>
<th>Serum LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastases</td>
<td>NA</td>
</tr>
<tr>
<td>M1a</td>
<td>Distant skin, subcutaneous, or nodal metastases</td>
<td></td>
</tr>
<tr>
<td>M1b</td>
<td>Lung metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>M1c</td>
<td>All other visceral metastases</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Any distant metastases</td>
<td>Elevated</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase. *Micrometastases are diagnosed after sentinel lymph node biopsy. †Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.
As with nearly all malignancies, the outcome of melanoma initially depends on the stage at presentation. An estimated 82% to 85% of patients present with localized disease, 10% to 13% with regional disease, and 2% to 5% with distant metastatic disease. In general, the prognosis is excellent for patients who present with localized disease and primary tumours 1.0 mm or less in thickness, with 5-years survival achieved in more than 90% of patients. For patients

Table 2. Anatomic Stage Groupings for Cutaneous Melanoma (Balch et al., J Clin Oncol. 2009)
with localized melanomas more than 1.0 mm in thickness, survival rates range from 50% to 90%. The likelihood of regional nodal involvement increases with increasing tumour thickness. When regional nodes are involved, survival rates are roughly halved. However, within stage III, 5-year survival rates range from 20% to 70%, depending primarily on the nodal tumour burden. Long-term survival in patients with distant metastatic melanoma, taken as a whole, is less than 10%. However, even within stage IV, some patients have a more indolent clinical course that is biologically distinct from that of most patients with advanced disease\textsuperscript{13}.

### 1.3 Molecular pathways activated in melanoma

Cell signaling pathways regulate cell growth and death, cell metabolism migration and angiogenesis. In cancer progression there is a loss of control of these signalling that result hyperactivated and/or silenced irreversibly allowing to cancer cells to acquire specific phenotypes, such as the ability to resist to apoptosis, abnormal proliferation, angiogenesis, and invasion.

**RAS/RAF/MEK/ERK pathway**

The mitogen-activated protein kinase (MAPK) pathway plays an important role in cellular proliferation and
differentiation. Under normal physiological conditions, growth factors bound their tyrosine kinases receptors and activate a small GTP binding protein RAS (existing in three isoform: ARAF, BRAF and CRAF in humans) on cell membranes, which triggers intracellular signaling. This is followed by the sequential stimulation of several cytoplasmic protein kinases. The first of the cascade is RAF, a serine/threonine kinase protein that leads to phosphorylation of MEK (mitogen-activated protein/ERK kinase) proteins that activate in highly specific manner the downstream components: the extracellular signal-related kinase-1 and 2 (ERK-1 or p44MAPK and ERK-2 or p42MAPK). These isoforms can be activated in response to a wide variety of growth factors and mitogens such as stem cell factor (SCF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and glial-cell-derived neurotrophic factor (GDNF) as a result of phosphorylation of threonine and tyrosine residues in a -TXY- motif and then translocate into the nucleus and stimulate pro-growth signals. In particular, the main substrates of ERKs are the transcription factors and other nuclear proteins like c-Fos ATF-2, c-Myc, c-Myb, Ets2, NF-1L6, TAL-1 and p53. In addition to transcription factors, another set of substrates for the ERKs are PHAS, tyrosine hydroxylase, phospholipase A2, phospholipase Cy, RNA polymerase II, CDC2 and also apoptosis regulator protein.
as Bim, Bax, Bcl-2, Bad. Therefore, ERK activation promote cell-cycle progression and proliferation\textsuperscript{14}.

Unregulated activation of the MAPK pathway can lead to malignancy. The MAPK pathway is one of the most frequently deregulated signaling pathways in the great majority of melanomas. ERK is hyperactivated in 90\% of human melanomas by growth factors and by genetic alterations of upstream factors, RAS and RAF proteins as well as mutations at upstream membrane receptors (e.g., KIT) \textsuperscript{15}. Mutations in BRAF appear to be the most common genetic alteration and major driver of this pathway, which occur in 50–70\% of melanomas. Although BRAF is thought to exert its oncogenic effects almost entirely through MEK, CRAF can lead to MEK-independent pro-survival effects, in part through its interaction with nuclear factor-kappa B (NF-\(\kappa\)B), as well as through inhibitory effects of critical regulators of apoptosis, such as ASK-1 and MST-2\textsuperscript{16,17}. These mutations can be identified in benign melanocytic proliferation and all stages of invasive and metastatic melanoma promoting proliferation, survival, invasion, and angiogenesis of melanoma. Specifically, the presence of a BRAF mutation has been associated with a more aggressive disease course and poorer prognosis compared with patients without BRAF mutations\textsuperscript{18}. 
In melanoma, NRAS gene also is mutated in 15-30% of tumor samples leading to its permanent activation and serial activation both the downstream components of the RAS effector pathways and the non-MAPK pathways, such as the phosphatidylinositol-3 kinases (PI3K) pathway resulting in increased v-akt murine thymoma viral oncogene homolog (AKT) activity\textsuperscript{19-20}.

**PI3K/AKT/mTOR pathway**

The PI3K/AKT/mTOR is an intracellular signaling pathway promoting cell survival and proliferation, and is hyperactivated in most malignancies, including melanoma\textsuperscript{21-22}. Stimulation of the PI3K pathway arises via GTP binding of RAS proteins and stimulation of receptor tyrosine kinases (RTKs). PI3Ks, constitute a lipid kinase family characterized by their ability to phosphorylate inositol ring 3′-OH group in membrane phospholipids generating phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) which is a key propagator of intracellular signaling.

AKT is a family of serine/threonine protein kinase that comprise three highly homologous members known as AKT1, AKT2 and AKT3. All three AKT isoforms consist of a conserved domain structure including a specific PH domain, a central kinase domain and a carboxyl-terminal regulatory
domain that mediates the interaction between signaling molecules. AKT normally exist in the cytoplasm in inactive conformation. After the interaction with PIP3 by its PH domain it traslocates to the inner membrane where it is phosphorylated in two critical residues, Thr308 and Ser473. Thr308 is in the activation loop within the kinase domain of AKT and it is phosphorylated by PDK1 (phosphoinositide-dependent kinase 1). Ser473 is in the C-terminal regulatory domain of AKT and it is phosphorylated by mTORC2 complex. Phosphorylation of Thr308 partially activates AKT, while phosphorylation of both sites is required for full activation. The numerous substrates of AKT include cellular regulators of insulin signaling, proliferation, and survival. AKT is inactivated by phosphatases (PP2A, PHLPP1, and PHLPP2) that remove the phospho-group from these residues. The activity of the PI3K/AKT pathway overall is regulated by the tumour suppressor phosphatase and tensin homolog (PTEN).

PTEN dephosphorylates the 3’ position of PIP2 and PIP3 antagonizing PI3K activity. Activated AKT phosphorylates a broad range of proteins involved in the control of apoptosis (the FOXO family of transcription factors, BAD or NF-κB), cell cycle regulation (GSK3β, p27kip1), and growth (TSC2), thereby facilitating the proliferation and survival of cells.
mTOR (mammalian target of rapamycin), a serine/threonine protein kinase, is a downstream effector of PI3K and AKT. mTOR carries out several function via two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 includes mTOR regulatory-associated protein of mTOR (Raptor), mLST8, and proline-rich AKT substrate 40 (PRAS40)\textsuperscript{26} and is allosterically inhibited by the macrolide antibiotic rapamycin\textsuperscript{27}. It is an important effector of cell growth and proliferation and regulates protein synthesis acting on several components of the translation machinery as eukaryotic translation initiation factor (eIF4E) and p70S6 kinase\textsuperscript{28}. mTORC2 includes rapamycin-insensitive companion of mTOR (Rictor), mLST8, and stress-activated MAPK-interacting protein 1 (Sin1)\textsuperscript{29}. It plays important roles in signal transduction as well as activates directly AKT\textsuperscript{30}. The major mechanisms of PI3K pathway activation in melanoma are loss of PTEN and NRAS mutations. Lack of PTEN antagonism (observed in 12% of melanomas through mutation or methylation) causes increased levels of PIP3 and active (phosphorylated) AKT\textsuperscript{31}. Mutated AKT may also independently activate the PI3K/AKT/mTOR signalling pathway. Elevated AKT phosphorylation and/or activated mTOR functioning arises in 70% of malignant melanomas\textsuperscript{32-33}. In particular, increased levels of the active form of AKT
were found in the radial-growth phase of melanoma and was found correlate with poor prognosis in melanoma patients\textsuperscript{34-35}. Activation of AKT in cancer cells induces cell survival, proliferation, metastasis, invasion, and angiogenesis. AKT, in fact, inhibits apoptosis through the inactivation of BCL-2 antagonist of cell death (BAD) protein and of caspase-9, and promotes cell proliferation by increasing CCND1 expression. Moreover, affects many other cell-survival and cell cycle genes through the activation of transcription factor as NF-κB\textsuperscript{36-37}.

**NF-κB pathway**

Nuclear Factor-kappa B (NFκB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. The NF-κB proteins constitute a family of proteins with homology to the chicken oncogene, *rel*. There are five known mammalian NF-κB subunits, each characterized by ankyrin repeat elements: p65 (RelA), RelB, Rel (c-Rel), p50/p105, and p52/p100 which form homo- and hetero-dimers. These proteins share an approximately 300 amino acid N-terminal domain called the Rel homology (RH) domain containing important sequences for binding DNA or inhibitor of NF-κB (IκB) as well as sites of dimerization. However, they differ in their C-terminal domain in that RelA, RelB and c-Rel exhibit
transactivating functions, while p100 and p105 contain inhibitory domains. In fact, NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of the p50 and p52 proteins, respectively. The most common Rel/NF-κB dimer in mammals contains p50-p65. In unstimulated cells, NF-κB is retained in the cytoplasm through interaction with inhibitory proteins called IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α). NF-κB activation is rapidly induced by primarily inflammatory stimuli, as well as proinflammatory cytokines (IL-1 and TNF-α), endotoxins, viruses and UV, that lead to activation of the IκB kinase (IKK) complex. IKK activated phosphorylates IκB proteins that are recognized by the ubiquitin ligase machinery, leading to their polyubiquitination and subsequent degradation by the proteasome 26S. During this process, also p65 is phosphorylated and p100 and p105 are cleaved into active forms p52 and p50 respectively. These events generate active NF-κB complexes that is free to traslocate into the nucleus where they bind to specific sequence in the promoters of target genes, inducing transcription. These genes include: immunoregulatory and inflammatory genes (cytokines and chemokines, acute phase proteins, cell adhesion proteins, inducible nitric oxide synthase (iNOS), immunoglobulins, and viral genes; anti-apoptotic genes as tumor necrosis factor receptor-associated
factor 1 and 2 (TRAF1 and TRAF2) inhibitor-of-apoptosis protein 1 and 2 (c-IAP1 and c-IAP2), caspase8/FADD, c-FLIP, members of BCL2 family; genes that positively regulate cell cycle progression (cyclin D1, c-myc, MMP9); and genes that encode negative regulators of NF-κB.

The earliest indication for its role in tumorigenesis was emerged following RelA/p65 cloning and sequencing, which revealed its homology to c-Rel and its viral oncogenic derivative v-Rel that leads to carcinogenesis in avian lymphoid cells. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. It was demonstrated that some components of NF-κB family, such as p50 and p65 are overexpressed in the nuclei of dysplastic nevi and lesions of human melanoma. The activation of NF-κB promotes the multiple steps in melanoma progression such as transformation, initiation, promotion, angiogenesis, invasion and enhanced metastatic potential of malignant melanoma cells. Moreover, NF-κB activated would be responsible of resistance of cancer cells to chemotherapeutic agents. NF-κB activation in melanoma may be the result of either exposure to proinflammatory stimuli in the tumor microenvironment or mutational activation of IKK resulting in enhanced transactivating capacity of the NF-κB complex and an increased survival of melanoma cells.
Data indicate that the enhanced activation of NF-κB may be due to deregulations in upstream signaling pathways such as Ras/Raf and PI3K/AKT\textsuperscript{46}. It has recently been suggested that AKT phosphorylates IKKα at the consensus sequence RXXRS/T, leading to the p65 phosphorylation and also may facilitate directly the phosphorylation of p65 on serines 529 and 536\textsuperscript{47}. Mutational activation of BRAF, common in human melanomas, has been also associated with an enhanced IKK activity and the activation of NF-κB transcriptional activity resulting in increased survival of melanoma cells\textsuperscript{48}. Infact, constitutively active ERK may indirectly activate NF-κB through the upregulation of inflammatory cytokines such as TNF-α and IL-1α/β, as well as chemokines, that activate NF-κB\textsuperscript{49-50}.

Therefore, NF-κB is a downstream target of both RAF and AKT and the PI3K/AKT pathway can activate by oncogenic RAS\textsuperscript{51-52}. The extensive interaction of these signaling pathways in malignant melanomas provides a possible mechanism for compensatory signaling to make resistance to molecular-targeted therapy (Figure 4).
1.4 Common genetic alteration in melanoma

The development and progression of malignant melanoma is related to gene mutations. In recent years, next-generation sequencing of melanoma has revealed the enormous genetic complexity of melanoma, with thousands...
of mutations, deletions, amplifications, translocations, and DNA methylation changes being present in the genomes of individual tumors. The mutation rate of melanoma is higher than those reported for other aggressive tumors (Table 3). This may due to the mutagenic effect of ultraviolet (UV) radiation involved in the melanoma pathogenesis\textsuperscript{53}. In fact, a recent analysis of whole-exome sequences revealed that 46% of melanoma driver mutation can be attributed to the cytidine to thymidine (C-to-T) transition, characteristic alteration due to UVR; a 9% of melanoma driver mutations, instead, showed the guanine to thymidine (G-to-T) transversion, characteristic of UVA-associated oxidative damage. Most of these occur in tumour suppressor genes, including CDKN2A, PTEN, and TP53. However, although UVR cause many mutations, other mutagenic mechanisms are likely important in melanoma development\textsuperscript{54}. Melanoma mutations it must be differentiated which mutations is causative in the disease “driver mutations” (confer a fitness advantage to the tumor cell) and which is only a bystander mutation “passenger mutations” (that never conferred a fitness advantage)\textsuperscript{55}.

One of the best-studied oncogenic events in melanoma is mutation of **BRAF** (v-Raf murine sarcoma viral oncogene homolog), a key protein kinase acting in the RAS–RAF–MEK–ERK mitogen-activated protein kinase (MAPK)
signal transduction pathway which regulates cell growth and proliferation. The RAF isoforms include ARAF, BRAF, and CRAF/RAF-1. BRAF mutations are found in about 60% of all melanomas and in most of the benign nevi, which implies that the mutation by itself is not responsible for malignancy in melanocytic proliferations. BRAF mutations are more frequent in melanomas that develop in intermittently sun-exposed skin and less so in acral and mucosal melanomas. BRAF mutations are not found in uveal melanomas. The most common mutation of BRAF are found in exon 15, at codon 600. In about 75% of all mutations in that area a valine is substituted by glutamic acid at codon (V600E). Less common mutations include valine by lysine (V600K) (about 20%), valine by aspartic acid (V600D) and valine by arginine (V600R) contribute another 5–6%. All this BRAF mutation cause the permanent activation of the protein and consequently induce MEK activation. BRAF mutation, and in part also the special type of substitution, correlates with age, localization of the primary tumor, sun damage, and, in part, geographic region. A small number of BRAF mutants other than V600 were identified in human melanoma, as G469E, D594G, G466A, and N581S. These mutants have low kinase activity but shown to still activate ERK via a mechanism involving their ability to strongly activate CRAF. Melanomas with wild type BRAF usually carry
oncogenic mutations in upstream components of the MAPK pathway.

**NRAS** (neuroblastoma RAS viral oncogene homolog), a GTPase is mutated in approximately 20% of melanomas\(^6^0\).

**KIT** (v-Kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog) is a receptor tyrosine kinase activated by binding of the cytokine stem cell factor (SCF); in melanoma KIT mutation and amplification are quite rare, they are found in particular subsets of melanoma, acral melanoma, mucosal melanoma, and lentigo maligna melanoma\(^6^1\). Approximately 70% of KIT mutations identified in melanoma are found in exon 11, most commonly L576P, and in the kinase domain in exon 13, most often K642E\(^6^2\). KIT mutations activate signal-transduction pathways (MAPK and PI3K) and increased melanocyte proliferation and melanoma survival\(^6^3\).

**GNAQ** (guanine nucleotide-binding protein, q polypeptide) and **GNA11** (guanine nucleotide-binding protein, a11), encoding members of the \(G\alpha (q)\) family of \(G\) protein \(\alpha\) subunits, are driver oncogenes in uveal melanoma. Mutations in GNAQ and GNA11 activate the MAPK pathway\(^6^4\)-\(^6^5\).

Genetic alterations in components of the p16\(^{INK4A}\)–cyclin D/CDK4-RB cell cycle checkpoint are found in virtually all melanoma cell lines in a mutually exclusive
fashion, underlining the importance of this checkpoint in preventing melanoma development\textsuperscript{66-67}. \textbf{CDKN2A} is a well-known melanoma tumor suppressor gene that encodes for two tumor suppressor proteins through alternative splicing: p16\textsuperscript{INK4A}, a key negative regulator of the cell cycle that activates retinoblastoma protein (RB) through negative regulation of CDK4, and p14\textsuperscript{ARF} which activates p53 through inhibition of its major negative regulator, MDM2\textsuperscript{68}. CDKN2A locus is mutated in about 25\% of melanoma families and inactivated by mutation, deletion, or promoter hypermethylation in 50–80\% of sporadic melanoma cases\textsuperscript{69}. Loss of CDKN2A and CCND1 correlate with poor responses to dabrafenib\textsuperscript{70}. Moreover, expression of oncogenic \textit{BRAFV600E} in melanocytes induces expression of p16\textsuperscript{INK4A}\textsuperscript{71}. p53 mutations was found in 19\% of melanoma tumors\textsuperscript{72}, but several alterations in other genes affecting p53 activity inhibiting its function. Other known genetic alterations are: mutation of p14\textsuperscript{ARF}\textsuperscript{73}; overexpression of MDM2 or amplification of MDM4, the major negative regulators of p53 that promote its degradation and inactivation\textsuperscript{74-75}; overexpression of p63, an antiapoptotic protein related to p53 that in melanoma cell lines prevents translocation of p53 to the nucleus\textsuperscript{76}; increased expression of iASPP, a conserved ankyrin repeat protein that shuttles between nuclear and cytoplasmic compartments and
inhibits the pro-apoptotic function of p53. **CDK4**, a cell cycle G1/S kinase, and cyclin D1 (CCND1), were shown to be amplified in melanoma. CDK4 mutations are associated with familial melanoma. Significantly, CDK4 pathway is deregulated in most melanomas as a consequence of increased activity of ERK or deletion of CDK4 inhibitor p16INK4A.

A cooperating event in BRAF-initiated melanomagenesis is constitutive activation of the PI3K/AKT/mTOR signaling pathway. BRAFV600E mutation are often associated with the PTEN aberrations inducing hyperactive–mTOR signaling in melanomas.

**PTEN** is a negative regulator of the PI3K/AKT pathway and it is mutated in 25% to 30% of melanoma, most commonly via allelic loss and focal deletions. PTEN (or rather lack of PTEN) was shown to play a key role in the predisposition to melanoma in individuals carrying certain variants of melanocortin-1 receptor (MC1R). The mechanisms through which MC1R variants associated with red hair and fair skin predispose to melanoma were not understood until recently, when it was shown that wild-type but not variant MC1R protein associates with PTEN and protects it from degradation after UVB exposure. Disrupted MC1R-PTEN association was shown to be responsible for the
oncogenic transformation promoted by MC1R variants in presence of BRAFV600E\textsuperscript{81}.

By contrast, activating mutations in PIK3CA, which encodes the catalytic subunit of PI3K, or in AKT1, AKT2, and AKT3, are rare in melanoma\textsuperscript{82}. Only AKT3 is deregulated in 25\% of melanoma tumours promoting development of malignant melanoma\textsuperscript{83}. Mutations in other PI3K pathways genes mTOR, IRS4, PIK3R1, PIK3R4, and PIK3R5 were detected in 17\% of BRAFV600 and in 9\% of NRAS-mutant tumors\textsuperscript{84}.

Phosphoinositide-dependent kinase-1 (PDK1) is a serine/threonine protein kinase that phosphorylates and activates kinases of the AGC family, including AKT. Increased expression of PDK1 was observed in a large cohort of melanoma samples compared to nevi, and deletion of PDK1 in a GEMM of melanoma BRAFV600E/PTEN-/- significantly delayed development of tumours and metastases\textsuperscript{85}.

MAP2K1 and MAP2K2 (MEK1 and MEK2, respectively) was found mutated in several melanoma cell lines with a frequency of 8\%, resulting in constitutive ERK phosphorylation and higher resistance to MEK inhibitors\textsuperscript{86}.

Microphthalmia-associated transcription factor (MITF) is a survival oncogene, amplified in 20\% of melanoma cases. MITF acts both as a transcription activator to promote expression of genes involved in cell cycle, but
also as a transcriptional repressor of genes involved in invasion\textsuperscript{87}; MITF amplification/alteration resulting in dysregulation of anti-apoptotic proteins. MITF is a melanoma-predisposition gene because is mutated in some familial melanomas\textsuperscript{88}. The most significant mutation in MITF that occurs with higher frequency in patients affected with melanoma is a missense substitution (Mi-E318K). Codon 318 is located in a small-ubiquitin-like modifier (SUMO) consensus site (ΨKXE) and Mi-E318K severely abolishes a SUMOylation of MITF inhibiting its transcriptional activity\textsuperscript{89}. 
<table>
<thead>
<tr>
<th>PRIMARY SUBTYPES</th>
<th>PATHWAY</th>
<th>ABDERRATION</th>
<th>FOUND IN TUMORS WITH...</th>
<th>FREQUENCY</th>
<th>POSSIBLE THERAPIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF*</td>
<td>MAPK</td>
<td>Point mutation</td>
<td>NRAS wild type</td>
<td>50-60%</td>
<td>BRAF1 + MEKI, BRAF1 + EGFRi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS</td>
<td>MAPK, PK, RALGDS</td>
<td>Gene fusions</td>
<td>BRAF wild type</td>
<td>20-23%</td>
<td>MEKI + CDK4i</td>
</tr>
<tr>
<td>KIT</td>
<td>MAPK, PTK</td>
<td>Point mutation</td>
<td>NRAS, BRAF wild type</td>
<td>1% overall, 10% in unaccultured, 10% in acral</td>
<td>SU-mitansib, imatinib, nilotinib</td>
</tr>
<tr>
<td>GNAQ/GNA11</td>
<td>Go(g) family of G protein α subunits; MAPK activators</td>
<td>Point mutation</td>
<td>NRAS, BRAF wild type</td>
<td>1%, 40-50% each in uveal</td>
<td>MEKI + PI3Ki, enzastaurin</td>
</tr>
<tr>
<td>MTT*</td>
<td>Transcription, translation, cell cycle</td>
<td>Amplification</td>
<td>ALL</td>
<td>20%</td>
<td>HDACi</td>
</tr>
<tr>
<td>NF1*</td>
<td>MAPK, PTK</td>
<td>Negative regulator of Raf</td>
<td>Mutations, loss of expression</td>
<td>BRAF, NRAS wild type and less often in mutated</td>
<td>4% overall, 25% of BRAF, NRAS wild type</td>
</tr>
<tr>
<td>TERT*</td>
<td>Tumor suppressor</td>
<td>Mutations in the promoter of catalytic subunit</td>
<td>ND</td>
<td>70-80% overall, 33% primary, 60% metastatic</td>
<td>TERT inhibitor in preclinical</td>
</tr>
<tr>
<td>ERBB4</td>
<td>PTK, MAPK</td>
<td>Point mutation</td>
<td>All types</td>
<td>15-20%</td>
<td>Lapatinib (PI3Ki) + PI3Ki</td>
</tr>
<tr>
<td>MET</td>
<td>PTK, MAPK</td>
<td>Activation by growth factor</td>
<td>All types</td>
<td>ND</td>
<td>Cobimetinib?</td>
</tr>
<tr>
<td>AKT3</td>
<td>PTK</td>
<td>Amplification</td>
<td>All types</td>
<td>25%</td>
<td>AKT1, PI3Ki, mTORi</td>
</tr>
<tr>
<td>PTEN</td>
<td>PTK</td>
<td>Point mutation or deletions</td>
<td>BRAF mutated; NRAS and NRAS wild type</td>
<td>40-60%</td>
<td>PI3Ki</td>
</tr>
<tr>
<td>MAGI</td>
<td>PTK, stabilizes PTEN</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TACC</td>
<td>Possibly stimulates PTK/AURKA signaling</td>
<td>--</td>
<td>BRAF and NRAS mutated</td>
<td>5%</td>
<td>PI3Ki, AURKAi</td>
</tr>
<tr>
<td>PRED2</td>
<td>RHO-ROCK/PP2</td>
<td>Rac exchange factor</td>
<td>Point mutations</td>
<td>BRAF or NRAS mutated</td>
<td>14%</td>
</tr>
<tr>
<td>RAC1</td>
<td>RHO-ROCK/PP2</td>
<td>Regulator of cell adhesion, invasion, migration</td>
<td>Point mutations</td>
<td>BRAF or NRAS mutated</td>
<td>6% of unexposed</td>
</tr>
<tr>
<td>MAPK1, MAPK2, MAPK3</td>
<td>MAPK</td>
<td>Mutations</td>
<td>BRAF mutated, NRAS wild type</td>
<td>9%</td>
<td>ERKi</td>
</tr>
<tr>
<td>MAPK3, MAPK9</td>
<td>RHO-ROCK/PP2</td>
<td>Rac exchange factor</td>
<td>Mutations, loss of heterozygosity</td>
<td>All types</td>
<td>85% and 67%</td>
</tr>
<tr>
<td>MYC</td>
<td>Transcription</td>
<td>Amplification</td>
<td>All types</td>
<td>20-40%</td>
<td>mTORi</td>
</tr>
<tr>
<td>ETV1</td>
<td>Transcription</td>
<td>Amplification</td>
<td>All types</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>Cell cycle, apoptosis</td>
<td>Point mutation</td>
<td>All types</td>
<td>15-20%</td>
<td></td>
</tr>
<tr>
<td>MDM4</td>
<td>Negative regulator of p53</td>
<td>Overexpression</td>
<td>All types</td>
<td>65%</td>
<td>p53-MDM4i</td>
</tr>
<tr>
<td>CDKN2A (PI3K/NF1, m14ARF)+</td>
<td>Negative regulator of TP53 and RB</td>
<td>Point mutation, deletion</td>
<td>BRAF and NRAS mutated, KIT amplified</td>
<td>30-40%</td>
<td>CDK4i</td>
</tr>
<tr>
<td>BCL2, BCL2A1</td>
<td>Suppression of apoptosis</td>
<td>Elevated expression, amplification (BCL2A1)</td>
<td>All types</td>
<td>ND 30% (BCL2A1)</td>
<td>BH3 mimetics</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cell cycle, G1/S cycle</td>
<td>Amplifications</td>
<td>More frequent in BRAF, NRAS wild type</td>
<td>11%</td>
<td>CDK4i</td>
</tr>
<tr>
<td>CDK4*</td>
<td>Cell cycle, G1/S cycle-dependent kinase</td>
<td>Amplifications</td>
<td>More frequent in BRAF, NRAS wild type</td>
<td>3%</td>
<td>selective CDK4i</td>
</tr>
</tbody>
</table>

Table 3. Common genetic alteration in melanoma
1.5 Therapy

SURGERY and ADIUVANT Therapy

Currently there isn’t a “gold therapy” for melanoma but surgical excision is considered the primary treatment for patient with cutaneous melanoma because renders “disease-free” with relatively limited associated morbidity. The management of early-stage disease, melanoma in situ (margin of 0.5 cm) and low-risk primary melanomas (margins greater than 0.5 cm), being treated with wide excision and then follow-up skin exams. For patients with intermediate- and high-risk melanomas measuring 1.01 to 2.0 mm in thickness wide excision is recommended. Sentinel lymph node mapping and biopsy is routinely offered for patients having 1 mm or greater deep melanomas. Also the patients with cutaneous melanoma metastases (Stage IIIb or IVa) are frequently treated with local excision followed by skin exams, imaging for metastases or enrollment in clinical trials. Cutaneous melanoma metastases can be subdivided in 3 types: satellite close the primary tumor (within 2 cm), in transit which are > 2 cm from the primary tumor but within the same region as the primary, and distant metastases far from the primary site. In this patients the surgical approach increases their survival. It was reported a 5-year survival rate of 20% in 144 patients with non-regional melanoma metastases; and overall 3-4-year survival rates
of 36% and 31%, respectively, in stage IV melanoma patients\textsuperscript{92}. However surgery alone cannot identify and address the microscopic in-transit malignant cells present which often give rise to future tumors. For this purpose, in 1996 was approved high-dose interferon-alfa as adjuvant therapy for the stage III or IV disease. Treatment with IFN-\(\alpha\) is associated with a statistically significant improvement in disease-free survival but not in overall survival. Moreover, IFN-\(\alpha\) could cause significant toxic effects as: severe fatigue, depression, and hepatotoxicity\textsuperscript{93}.

Radiation therapy (RT) is used in 1–6\% of patients with melanoma in the US in the setting of inoperable disease, particularly for patients in who tumor occurred predominately in the head and neck region. It is commonly used as adjuvant therapy or as palliative therapy to improve local control or in patient with desmoplastic neurotropic melanoma (DNM) \textsuperscript{94}.

In the last 30 years many drugs and combination of drugs have introduced in melanoma therapy to improve patient survival for longer time. (Figure 5).

\textbf{Figure 5.} Timeline of FDA regulatory approval for metastatic melanoma. \textit{(Lo and Fisher. Science 2014)}
CHEMIOThERAPy

Cytotoxic chemotherapy was the main treatment strategy for metastatic melanoma. Common antineoplastic agents currently being used include dacarbazine, temozolomide, high-dose interleukin-2 and paclitaxel with or without cisplatin or carboplatin. These have shown modest response rates of less than 20% in first- and second-line settings.

**Dacarbazine** [5(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide] was the first chemotherapeutic agent approved by the U.S. Food and Drug Administration (FDA) in 1970 for treatment of metastatic melanoma and it was used for many years. Dacarbazine is a prodrug, one of the triazene derivatives, that is converted in the active metabolite, diazomethane, by cytochrome P450. Dacarbazine acts through DNA alkylation, forming crosslinks within and between helices that lead to local denaturation of the DNA strand, interfering with its form and function and killing the cancer cell. However, dacarbazine monotherapy is characterized with low overall response rates (approximately 10%-15%), and the drug doesn’t offer survival benefit. To improve response rates several chemotherapeutic agents as Temozolomide (a DTIC analog), cisplatin, carmustine, fotemustine, vinblastine, and tamoxifen or cytokine as interleukin-2 (IL-2) and interferon-
alpha were added to DTIC in a variety of regimens but without a statistically significant survival benefit. Toxicities include nausea and vomiting, in over 90% of patients, myelosuppression (both leucopenia and thrombocytopenia) and flu-like illness, consisting of chills, fever, malaise, and myalgia.95-97.

**IMMUNOTHERAPY**

Immunological strategies in therapy of localized and metastatic melanoma have a long tradition. The importance of immune response in melanoma derived from observation of the correlation between higher melanoma incidence and immunosuppressed patient.98 Several therapies was been proposed for disseminated disease modulating the immunologic response like the nonspecific immunostimulant *Bacillus Calmette-Guérin*, interferons, interleukins and others but they have failed to delivery major clinical benefits99-102. Also, melanoma vaccines has not shown significant clinical responses103.

**IL-2**

The first immune-based therapy FDA-approved for advanced melanoma in 1998 was IL-2, an immune cytokine that activates citotoxic T cell activation and promotes their proliferation. It stimulates, also, the development of lymphokine-activated killer (LAK) cells, which have the
ability to lyse tumor cells\textsuperscript{104-105}. The use of high-dose IL-2 showing long-term, durable complete responses in previously treated patients with metastatic melanoma\textsuperscript{106}. However, its use is limited by high toxicity due to anaphylactic shock and development of infectious granulomas at sites of injection and low response rates. To reduce systemic toxicity and increase local therapeutic effects, it was introduced intralesional interleukin-2 for treatment of cutaneous melanoma metastases, which showed a complete response in 78\% of treated lesions\textsuperscript{107}. Early studies showed that “tumor infiltrating lymphocytes” (TIL) present in tumors had potent anti-tumor activity\textsuperscript{108}. So, adoptive cell therapies utilizing TILs in combination with IL-2 have been promising\textsuperscript{109}. Lymphodepletion prior to TIL infusion improve effect on adoptive cell transfer therapy because reduce competition for growth factors and cytokines in vivo microenvironment. Although immunotherapy with adoptive transfer of TILs is a promising to treat patients with refractory metastatic melanomas there is a technically challenging to generate sufficient amount of tumor-specific lymphocytes that can maintain their tumor-killing activity in vivo. So, it will be difficult to implement these therapies into routine clinical practice\textsuperscript{110}. 
The most successful immunotherapy approach has been “immune checkpoints” inhibition. This therapy is based on the fact that T lymphocytes are critical to antitumor immunity. Two of the best-studied checkpoints involve cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1), two coinhibitory T cell receptors that mediate immune tolerance\textsuperscript{111}.

**ANTI-CTLA4**

CTLA-4 is a member of the immunoglobulin superfamily and it is a transmembrane inhibitory receptor that downregulates the immune system. CTLA4 is expressed on the surface of T cells CD4\textsuperscript{+} and CD8\textsuperscript{+}. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CD28 transmits a stimulatory signal whereas CTLA4, as a control mechanism around 48 hours after the initial T-cell activation, transmits an inhibitory signal to T cells. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function\textsuperscript{112}. CTLA-4 blockade with anti-CTLA-4 monoclonal antibodies allows appropriate T-cell activation through re-establishment of the costimulatory binding of CD28 to B7. This interaction increases T-cell proliferation and amplifies T-cell–mediated immunity, thus, promoting the antitumor immune response in patients\textsuperscript{113}. The first anti-CTLA-4 agent
to be investigated in patients with metastatic melanoma was **Tremelimumab**, a human monoclonal IgG2 anti-CTLA-4 antibody. Although in phase I/II clinical trials, it demonstrated antitumor activity in patients with stage III/IV melanoma, a phase III study failed because tremelimumab shows any benefit over chemotherapy\textsuperscript{114-115}. More successes has had **Ipilimumab**, a human monoclonal IgG1 anti-CTLA-4 antibody that was approved by the FDA in 2011 for the treatment of patients with metastatic melanoma. In multiple phase III clinical trials in patients with stage III and IV melanoma ipilimumab monotherapy at a dose of 3 mg/kg bodyweight significantly improved overall survival (10.1 months) compared to gp100 peptide vaccine monotherapy (6.1 months) \textsuperscript{116}. Also the treatment with ipilimumab at a dose of 10 mg/kg bodyweight plus dacarbazine compares with dacarbazine alone revealed a higher overall survival rate (11.2 months versus 9.1 months, respectively) with a 28% reduction in the risk of death\textsuperscript{117}. The response to Ipilimumab as an immunologic reaction appears slowly but may be long lasting. Significant immune toxicities were reported in this trials. Common side effects that may result from autoimmune reactions associated with the use of ipilimumab include gastrointestinal immune-related events (diarrhea; colitis) and other autoimmune inflammations (hypophysitis; thyroiditis; hepatitis) \textsuperscript{118}. 
ANTI PD-1/PDL-1

PD-1, like CTLA-4, is an inhibitory receptor expressed on T and B cells and some myeloid cells. The PD-1 ligands PD-L1 and PD-L2 have different expression patterns. PD-L1 acts as a negative regulator of T cells. It is located on multiple normal and tumor cells like melanoma, where, once bound by PD-1, attenuates immune responses inducing peripheral tolerance to “self” antigens and, in cancer, promotes downregulation/termination of the immune response against the tumor. PD-L2 is expressed on antigen-presenting cells (APC), providing tolerance to orally administered antigens. Humanized antibodies against both PDL1 and PD-1 have been developed to inhibit their interaction increasing antitumor activity. In clinical trials for many cancers, including melanoma, they have shown more powerful, with higher response rates and fewer autoimmune toxicities. The human antibody nivolumab (BMS-936558) is directed against PD-1, which in a phase I trial showed a responses in 28% of the melanoma patients, with long-term responses longer than 1 year in 50% of responding patients. The best response was observed with a dosage of 3 mg/kg bodyweight with a low rate of grade 3 or 4 toxicity observed in only 6% of patients. Nivolumab was well tolerated in fact were observed common side effects as diarrhea, rash, and pruritus. The better safety profile of
PD-1 antibody versus CTLA-4 antibody is most likely due to the fact that it acts at tumor sites, preventing interaction with PD-L1 on tumor cells. A second anti-PD-1 agent, pembrolizumab (MK-3475, formerly known as lambrolizumab) is a humanized IgG4 antibody approved by FDA on September 2014 following phase I trial with 135 advanced melanoma patients. It showed a response rate of 38% in patients receiving a larger dose (10 mg/kg of bodyweight intravenously every 2 or 3 weeks). Most of the responses became evident after 12 weeks and were long lasting (in 81%, lasting more than 11 months). Adverse drug reactions, reported in 79% of patients, included fever, chills, fatigue, rash and pruritus. An anti-PD-L1 human IgG4 antibody (BMS-936559) is being tested in phase I trial in 55 patients with melanoma. Preliminary results showed significant tumor long-term response of 29% with the dose 3 mg/kg bodyweight and a comparable rate of grade 3 or 4 adverse effects. Other two anti-PD-L1 antibodies, MPDL3280A (Genentech) and MEDI4736 (MedImmune), are being tested in solid tumors under early phase clinical trials. Recent preliminary results of MDPL3280A trial showed an overall response rate of 29% in 44 patients with melanomas of different origins. Combination therapies utilizing multiple immune modulating agents that block of both immune checkpoints (PD-1 and CTLA4) are showing great
promise. Anti-PD-1/nivolumab/MK-3475 and anti-CTLA-4/Yervoy antibodies are being combined in clinical trials (NCT01024231). This treatment produced an objective response rate of 40\% in 52 patients at a variety of dose levels and in several patients tumors disappeared completely\(^\text{127}\).

**TARGET THERAPY**

Concurrent advances in targeted molecular therapy have also improved the treatment and prognosis of a subset of advanced melanoma patients. Approval of single substances directed against mutated proteins has dramatically changed the options available in melanoma therapy.

**BRAF inhibitors**

BRAF-targeted therapies represent the first major breakthrough in systemic therapy for metastatic melanoma. The earlier BRAF inhibitor is nonselective multikinase inhibitor **sorafenib** (BAY 43-9006). It acts as a pan-inhibitor of RAF, which targets not only BRAF but also CRAF as well as the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinases (RTKs)\(^\text{128}\). However, sorafenib did not show great benefit, in clinical trials neither as monotherapy or in combination with
other anticancer compounds, such as dacarbazine, carboplatin and paclitaxel in patients with metastatic melanoma\textsuperscript{129}. The latest generation of highly specific and potent BRAF inhibitors offers a significant improvement over sorafenib against mutant BRAF. These drugs show a greater selectivity for mutant BRAF and have fewer off-target effects. The first selective BRAF inhibitor targeting the mutant V600E form is PLX4720 (vemurafenib).

Vemurafenib is a potent ATP-competitive RAF inhibitor that co-crystallizes with a protein construct containing the kinase domain of BRAF V600E to enable preferential binding to the ATP-binding domain of mutant BRAF\textsuperscript{130}. Pre-clinical studies showed that vemurafenib inhibits the kinase activity of BRAF harboring the V600E mutation with an half maximal inhibitory concentration (IC\textsubscript{50}) of 13 nM, resulting in selectively blocked the RAF/MEK/ERK pathway and consequent cell-cycle arrest and the induction of apoptosis in melanoma cells\textsuperscript{131}. In 2011 vemurafenib/Zelboraf (960 mg twice daily) received FDA approval for treatment of metastatic or unresectable melanoma harbouring the BRAF mutation V600E following the results of a phase III trial, BRIM-3. The 675 enrolled patients, previously untreated, with BRAF V600E mutation-positive metastatic melanoma were randomized to receive vemurafenib at 960 mg orally twice daily, or dacarbazine
chemotherapy of 1,000 mg/m² intravenously every three weeks. The six-month overall survival was 84% in the vemurafenib arm and 64% in the dacarbazine arm. Many patients treated with vemurafenib had a rapid tumor response with resultant decreased tumor weight as indicated by the high overall response rate that was 48% for vemurafenib compared to 5.5% for dacarbazine.\textsuperscript{132} Vemurafenib is not truly selective for BRAF mutant melanoma cells and can paradoxically activate the MAPK pathway in wild-type BRAF cells\textsuperscript{133} which resulting in several toxicities, concerning frequency and intensity during treatment, as the development of a rash, in some associated with a severe photosensitivity (UVA), arthralgia, keratinocytic neoplasia and squamous cell carcinoma.\textsuperscript{134}

The second BRAF inhibitor that has been approved for the treatment of BRAF V600 mutated metastatic melanoma by the FDA on May 30th 2013 is \textbf{dabrafenib}. Dabrafenib/Tafinlar is a specific inhibitors of BRAF V600E/K. In a phase III clinical trial (BREAK-3), dabrafenib (150 mg twice daily) was compared with dacarbazine in previously untreated patients with advanced melanoma with mutated BRAF. As vemurafenib dabrafenib showed significant high response rates and reduced of 70% in risk for disease progression versus standard therapy.\textsuperscript{135} Toxic side-effects included skin lesions, pyrexia, frequent fatigue,
nausea and pain. The development of photosensitivity and epithelial tumors were less frequent than vemurafenib.

Other BRAF inhibitors that are currently being evaluated in clinical trials are LGX818, BMS-908662, XL281 ARQ736, and RAF265 (clinicaltrials.gov). In particular, LGX818 is in a phase I trial and has shown the similar response rates (58%) but lower toxicity than vemurafenib and dabrafenib\textsuperscript{136}. New inhibitors are in development to eliminate the side effects associated with the paradoxical activation by vemurafenib of the ERK1/2 pathway in wild-type BRAF cells. PLX7904, a new “paradox-breaking” BRAF inhibitor, that inhibits MAPK signaling in BRAF-mutant cells without activating BRAF in BRAF wild-type cells\textsuperscript{137}.

A major problem with the use of these inhibitors and the further advancement of melanoma therapies is de novo or acquired drug resistance. In fact, unfortunately, some patients with BRAF-mutated melanoma do not respond to therapy or show tumours progress or grow again with a median time to progression of approximately 6-8 months\textsuperscript{138}. Primary or de novo resistance may be due to pre-existing mutations (intrinsic) or interactions between tumor cells and microenvironments (extrinsic). Further additional mutations in the MAPK pathway (eg. MEK2) and in the PIK3 pathway (eg. PIK3 and AKT1), or loss of NF1 function (which regulates RAS) have been shown to escape the effects of
BRAF (and MEK) inhibitors activating bypass survival pathways, in particular, PI3K/AKT pathway\textsuperscript{139-141}. Secondary or acquired resistance to BRAF inhibitors, when melanomas begin to grow again following an initial successful treatment, occurs inevitably in most of the patients treated. The secondary resistance to BRAF inhibitor was mainly associated to several MAPK pathway-dependent or-independent mechanisms. Acquisition of new mutations in NRAS or the downstream kinase MEK1, BRAF amplification or splicing, overexpression of cancer osaka thyroid (COT), and activation of RTKs cause rapid regrowth of existing metastases or the emergence of new\textsuperscript{142-145}. Although the resistance mechanisms identified so far are diverse, most appear correlate to reactivation of MEK/ERK signaling and increased of the PI3K/AKT/mTOR pathway. Moreover, cell growth and proliferation are controlled by a network of pathways consisting of various key proteins which might be mutated, so the block of a single checkpoint will not be successful in the long-term. Correspondingly, to overcome the impact of resistance to BRAF inhibitors, the designed drug combination may provide a more durable response rate and may prolong progression-free survival (PFS) in patients with BRAF mutant melanoma, compared with either agent alone. The most promising combination strategy is the simultaneous inhibition of BRAF, MEK and PI3K pathway\textsuperscript{146-}.
Another interesting strategy being investigated in clinical trials that may help overcome BRAF inhibition resistance is the use of intermittent dosing of vemurafenib after the maximum response to therapy to prolong the response duration. A better understanding of the molecular biology of melanoma has helped the development of new therapies.

### MEK inhibitors

There are two major classes of MEK inhibitors, ATP non-competitive and ATP competitive inhibitors. Currently, most MEK inhibitors are non-competitive, indicating that they do not compete for the ATP binding site and instead bind to an adjacent allosteric site, which explains their high specificity. MEK is a downstream kinase of BRAF so its inhibition leads to decreased cell signalling and proliferation in cancer cells inducing apoptosis. Trametinib (GSK1120212) is a non-competitive MEK1/2 inhibitor and it was FDA-approved in 2013 for the treatment of adult patients with the BRAF V600E/K mutation and unresectable or metastatic melanoma at the dose of 2 mg orally once daily. Inhibition of MEK has been explored in clinical trials for melanoma patients with mutant BRAF. In a phase III randomized trial trametinib compared to chemotherapy (dacarbazine or paclitaxel) showed a marked improvement in both progression free and overall survival (respectively 4.8
months and 81% vs 1.5 months and 67%)\textsuperscript{152}. The adverse effects is mild, including rash, cardiac dysfunction, ocular toxicities, diarrhea, peripheral edema, and hypertension which could be managed with dose reduction or interruption. Currently, several MEK inhibitors have showed efficacy in the treatment of melanoma as selumetinib and MEK162, while other are in clinical development as PD-0325901, refametinib, RO-4987655, TAK-733, and XL518. Recently, MEK162 monotherapy revealed a response rate of 21\% in NRAS-mutated melanoma\textsuperscript{153-154}. Patients who did not respond to vemurafenib or who have develop resistance to BRAF inhibitor usually will not benefit from MEK inhibitor monotherapy because the mechanisms involved in development of resistance to mutant BRAF confer resistance to MEK inhibition.

**MEK and BRAF combined therapy**

Numerous preclinical and clinical studies have shown that the combination of BRAF and MEK inhibitors is a successful strategy for the treatment of metastatic melanoma. Preclinical studies have demonstrate that this co-inhibition reduces tumor growth, increases apoptosis and delays the onset of resistance when compared with monotherapy\textsuperscript{155}. The first combination to be tested in clinical trials was the BRAF inhibitor dabrafenib with the MEK inhibitor trametinib. In a phase II trial, in 54 MAPK
inhibitor-naive patients with BRAFV600E/K mutation, the CombiDT (dabrafenib 150 mg twice daily and trametinib 2 mg daily) showed promising activity in patients with stages III or IV melanoma and fewer side effects compared with vemurafenib. They induce a significantly higher response of 76% vs 54% for dabrafenib alone and an evident improvement in PFS (9.4 months in the combination group vs 5.8 months in the monotherapy group). No patient recorded progressive disease as their best response. The CombiDT showed, also, less toxicities than dabrafenib alone. In fact, the addition of trametinib reduced the appearance of cutaneous squamous cell carcinomas. The most common toxicity was pyrexia, which occurred in 71% of patients (5% had grade 3 or 4 reactions). On the basis of these results FDA has granted accelerated approval of the combination of dabrafenib and trametinib for the treatment of patients with metastatic melanomas that carry the BRAFV600E or BRAFV600K mutation in January 2014. A phase III clinical trial comparing the combination of dabrafenib and trametinib with dabrafenib alone (NCT01584648) and dabrafenib and trametinib with vemurafenib monotherapy (NCT01597908) is also in progress. Other BRAF and MEK inhibitor combinations have also shown promise and are in phase III trials as vemurafenib and cobimetinib (CombiVC).
and LGX818 and MEK162 (NCT01909453). Unfortunately, it has been shown that the response to Combi therapy after disease progression with BRAF inhibitors has little efficacy. Moreover, a dual BRAF/MEK inhibitor, RO5126766, has recently shown activity in patients with metastatic melanoma, suggesting that combined inhibition of BRAF and MEK will be of the greatest benefit for metastatic melanoma patients. However, about 50% of individual metastases have a complete response but most patients still relapse within a year although rarely resistance emerges in the lesions that had a complete response\textsuperscript{146}. Other early-phase combination trials with approved BRAF inhibitors explore addition of inhibitors of PI3K, AKT, CDK, HSP90, RTKs and other targets, as well as immunotherapy, to improve durability of responses.

**ERK inhibitors**

ERK1/2 are the kinases below MEK in the MAPK cascade, they are the primary effector of this pathway, and regulates the activity and expression of transcription factors and proteins that result in cell growth, proliferation, and cell cycle progression. Most BRAF inhibitor resistance (and combination BRAF and MEK inhibitor resistance so far) results in reactivation of ERK signalling. Thus, inhibition of ERK might represent an emerging therapeutic strategy for
the treatment of BRAF inhibitor-resistant melanoma. Preclinical evidence suggests that ERK inhibition is effective in BRAF inhibitor-resistant melanoma with common MAPK-reactivating resistance drivers (RAS, MEK1 mutations, BRAF splice variants, and BRAF amplification). Several candidate drugs are in phase I trials, as MK-8353 (NCT01358331)\textsuperscript{160-161}.

**KIT inhibitors**

C-KIT is a receptor tyrosine kinase that when binding to its ligand activates the MAPK, PI3K/AKT and Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathways, resulting in proliferative and survival effects\textsuperscript{162}. Selective inhibitors of KIT (**imatinib; sunitinib; nilotinib; dasatinib**) which were developed and used for the treatment of other types of cancer (such as gastrointestinal stroma tumor [GIST] or chronic myeloid leukemia) became interesting also for melanoma treatment\textsuperscript{163}. In a phase II trial using imatinib (400 mg orally once-daily), in selected melanomas (acral; mucosal; sun-damaged skin), the best overall response rate was 77\% in cases with KIT mutation compared to only 18\% in cases with KIT amplification\textsuperscript{164}. Imatinib was well tolerated and didn’t show very serious adverse effects at utilized dose. On the basis of these data, imatinib has shown promising results as a therapeutic agent in patients with metastatic melanoma.
and mutations in the KIT gene. Other tyrosine kinase inhibitors (sunitinib, dasatinib, and nilotinib) are underway in a phase III trials. The major limitation of this study has been the rarity of mutations in this gene, which occur in less than 5% of patients with melanoma.\textsuperscript{165}

**NRAS inhibitors**

Neuroblastoma RAS viral oncogene homolog (NRAS) is mutated in approximately 15% to 20% of melanoma and seems to correlate with a worse prognosis compared to NRAS/BRAF wild-type patients.\textsuperscript{166} Developing drugs targeting mutated NRAS is challenging because multiply pathways are unregulated due to this mutation. Infact, current drug development focuses on targeting the downstream effectors of NRAS, the MAPK and PI3K pathways. MEK162 is the most promising MEK inhibitor, showing response in 20% of the patients with NRAS-mutated melanoma.\textsuperscript{167} It is most likely that such patients will benefit much more from the combination therapies and a trial (NCT01781572) that combining MEK162 and LEE011, a cyclin-dependent kinase (CDK) 4/6 inhibitor, in patients with NRAS-mutated melanoma is ongoing.
PI3K/AKT inhibitors

The PI3K/AKT pathway regulates several cellular processes including proliferation, survival, motility, metabolism, and angiogenesis. This pathway is activated by receptor tyrosine kinases and RAS proteins and is inhibited by PTEN. Alterations in the PI3K pathway have also been reported in 60% of the melanomas\textsuperscript{168-169}. Several classes of PI3K pathway inhibitors exist including PI3K inhibitors, AKT inhibitors, mTOR inhibitor, dual PI3K\textbackslash mTOR inhibitor and dual mTOR\textbackslash1\textbackslash2 inhibitor that down-regulate the activation of this survival pathway. The first generation of inhibitors of PI3K signaling in melanoma was rapamycin and the second-generation agents were everolimus (RAD001) and temsirolimus (CCI-779), they allosterically inhibit the mTORC1 complex\textsuperscript{170-171}. These agents have long been used as immunosuppressor in patients with organ transplants and have demonstrated anti-proliferative effects against many human cancers\textsuperscript{172}. However, clinical trials in melanoma have shown a lack of objective responses to mTORC1 inhibitors as single agents or in combination with BRAF inhibitors\textsuperscript{173-174}. The reason for this seems to be interruption of negative feedback loops mediated by mTORC1, causing activation of PI3K, AKT and ERK\textsuperscript{175}.

mTOR complex-1 inhibitors are now in use in a multitude of cancer types. Dermatologic side effects are
common and include stomatitis, eruptions, and nail changes, including paronychia. Few data are available about the pharmacodynamics effects of PI3K and AKT inhibitors in patient. Most of them are now under clinical trials in melanoma (both wild-type and BRAF mutant), often in combination with MAPK inhibitors (clinicaltrials.gov; NCT01820364; NCT01616199; NCT01337765; NCT01941927; NCT01166126; NCT01014351; NCT00022464).

**CDK4 and CDK6 inhibitors**

The CDK4-CDK6 pathway sits below the MAPK pathway and involves p16, cyclin D, CDK4 and CDK6, and the RB1 protein. This pathway is aberrantly activated in most melanomas resulting in increased cell cycle progression, reduced induction of apoptosis, and cell senescence. Inhibitors of CDK4, such as palbociclib (PD0332991; NCT01037790), SCH 727965 (dinaciclib), LY2835219, BAY1000394, LEE011), and the combination of LGX818 with LEE011 (NCT01777776), are currently in clinical trials for various advanced cancers including melanoma.

**Other targets**

Several other targets exist in BRAF-mutant melanoma, ranging from growth factors in the tumour microenvironment (eg, HGF and VEGF), receptor tyrosine
kinases at the cell surface (eg, MET, FGFR, and VEGFR), epigenetic regulators (eg, histone deacetylase), and apoptosis regulators (eg, MDM2). Lapatinib, an Epidermal Growth Factor Receptor/Human Epidermal growth factor Receptor 2 (EGFR/HER2) inhibitor, showed activity in cell lines harboring ERBB4 mutations and not in ERBB4 wild-type cells. Mutations in ERBB4 observed in 19% of patients with melanoma can cause an overactivation of the ERK and AKT pathways. Currently, a phase II trial testing lapatinib in patients with advanced melanoma with ERBB4 mutations is ongoing (clinicaltrials.gov; NCT012 64081)\textsuperscript{178}.

Figure 6. Cell signalling pathways and drug targets
(\textit{Menzies and Long, Lancet Oncol.} 2014)
2. **Hydrogen Sulphide**

2.1 **Physical and Chemical Properties of H\textsubscript{2}S**

Over millions of years hydrogen sulphide (H\textsubscript{2}S) caused life destructions and extinctions on the earth. It is believed that in Permian period H\textsubscript{2}S provoked a mass extinctions on earth, than the emissions from massive volcanic eruptions in Siberia caused a chain reaction of environmental events that resulted in oxygen depletion in the world’s oceans and, therefore, the died of many species that lived there. Otherwise, some non-oxygen (anaerobic) breathing organisms called green sulfur bacteria (Chlorobium) continued to grow and reproduce at high rates. These green sulfur bacteria used sulfate dissolved in water for respiration instead of oxygen, and subsequently produced H\textsubscript{2}S. According to this theoretical perspective the large amounts of H\textsubscript{2}S produced by these organisms created so much lethal gas in the ocean that it then diffused into the air and land destroyed the plant and animal life as well. By the end of the Permian period, 95% of marine species and 70% of terrestrial ones had vanished\textsuperscript{179}. Actually a great diversity of microorganisms, bacteria, fungi as well as plants, are be able to produce H\textsubscript{2}S\textsuperscript{180-181}. Interestingly, plants are the primary producers of organic sulfur compounds and are able to couple photosynthesis to the reduction of sulfate,
assimilation into cysteine, and further metabolism into methionine, glutathione, and many other compounds. Animals, instead, have a dietary requirement for sulfur amino acids. Cysteine is the central intermediate from which most sulfur compounds are synthesized.  

H$_2$S, along with nitric oxide (NO) and carbon monoxide (CO), forms part of a group of biologically active gases that are termed gasotransmitters or gasomediators. H$_2$S is a colorless, water-soluble and flammable gas with an unpleasant smell of rotten eggs. H$_2$S is the predominant sulphur contaminant of natural gas and ranges in concentration from <1 to >90%.

H$_2$S has a molecular weight of 34.08 and its chemical structure is analogue to that of water molecule with an atom of sulfur being less electronegative than the first and much less polar (Figure 7). Infact, H$_2$S has a vapor density (d) of 1.19, heavier than air (d=1.0), boiling point (-60.7 °C) and melting point (-82.3°C) is much lower than they are in water and freezing point is -86°C.

![Figure 7. Similarity in molecular structure between water (H$_2$O) and hydrogen sulfide (H$_2$S).](image-url)
H$_2$S is a weakly acidic, dissociating in aqueous solution into hydrogen cation (H$^+$) and hydrosulfide anion (HS$^-$) (K$_{a1}$= 1.3*10$^{-7}$ M), which subsequently may decompose to H$^+$ and sulfide anion (S$^{2-}$) (K$_{a2}$= 1*10$^{-19}$ M) in the following reaction$^{183-185}$:

\[
\begin{align*}
\text{H}_2\text{S} & \rightleftharpoons \text{H}^+ + \text{HS}^- \\
\text{HS}^- & \rightleftharpoons \text{H}^+ + \text{S}^{2-}
\end{align*}
\]

**Hydrogen Sulfide Dissociation**

Under physiological conditions, at 37°C and pH 7.4, equal amounts of H$_2$S and HS$^-$ are within the cell, and approximately a 20% H$_2$S/80% HS$^-$ ratio in extracellular fluid and plasma. Conversely, the chemical form S$^{2-}$ is not present in appreciable amounts, since the dissociation of HS$^-$ occurs only at high pH values.

H$_2$S present in the air comes from organic and inorganic sources. Organic sources include bacteria and decomposition of matters such as released from septic tanks, sewers, or water treatment plants, while inorganic sources are natural gas, petroleum refinery, rayon manufacturing, paper and pulp mill industry, sulfur deposits, volcanic gases, and sulphur springs. The half-life of H$_2$S in air varies from 12 to 37 h. The temperature would change the half-life and the solubility of H$_2$S. H$_2$S is a highly lipophilic molecule, such as it can easily penetrates lipid bilayer of cell membranes.
The toxic effects of this small molecule were known long before its physiological properties. Back to World War One, H₂S was used by the British as a chemical agent. It has been postulated that H₂S exerts dose-dependent toxicity with millimolar concentrations. Accumulation of H₂S gas in confined or closed spaces, including septic tanks and cesspools, animal processing plants, pump mills, and sludge plants has been the cause of numerous cases of human deaths. Sudden release of H₂S gas in a huge amount from oil wells or refineries leads to infamous “knock-down” phenomenon of oilers and other petrochemical workers, an instant loss of consciousness often associated with respiratory failure. Most organ system are susceptible to the effects of H₂S and each system exhibits a different response according as a concentration, time or rate of exposure. The lethal concentration for 50% of humans for 5 min exposure (LC50) is 800 ppm, and concentrations over 1,000 ppm cause immediate collapse with loss of breathing, with only a single inhalation needed. Tissues most susceptible to H₂S toxicity are those with exposed mucous membranes and those with high oxygen demands. This gas is considered a broad-spectrum poison to humans in the nervous system, respiratory system, and cardiovascular system; infact, at concentrations <1000ppm, can have toxic effects that occur with eye irritation, sore throat, dizziness, nausea, difficulty breathing and chest stiffness; at concentrations >1000ppm, it can cause serious adverse effects.
resulting in respiratory paralysis, pulmonary edema, cardiac arrhythmias, stroke and death.

The molecular mechanisms underlying the toxicological effects of H₂S are mostly attributed to its ability to induce K (+)-channel-mediated hyperpolarization of neurons and potentiation of other inhibitory mechanisms, or to inhibit cytochrome c oxidase in mitochondria, at micromolar concentrations, reducing ATP production\(^{190}\). However, over the last decade it has become clear that it is not only detectable in significant amounts in mammals, including humans, where it appeared in blood from nanomolar to micromolar concentration range, but that it is also produced endogenously and may play a significant role in both normal physiology and the pathophysiology of disease.

### 2.2 Endogenous Production of H₂S in mammalian cells

**Enzymatic Production of H₂S**

In mammals, H₂S is endogenously produced by enzymatic reactions and by nonenzymatic pathways. The key precursors for endogenous H₂S synthesis are sulfur-containing amino acids as L-cysteine, that derives from alimentary sources, or synthesizes from L-methionine, through the so-called “trans-sulfuration pathway” with homocysteine (Hcy) as an intermediate, or liberates from endogenous protein. H₂S is generated by four separate
enzymes that are cystathionine gamma-lyase (CSE, EC 4.4.1.1), cystathionine beta-synthase (CBS, EC 4.2.1.22), cysteine aminotransferase (CAT, EC 2.6.1.3) and 3-mercaptopyruvate sulfurtransferase (3-MST EC 2.8.1.2)\textsuperscript{191} (Figure 8).

Interestingly, these enzymes are evolutionarily conserved and occur in many lower species as well as in mammals and their expression is tissue-specific\textsuperscript{192}. CBS is the predominant enzyme responsible for H\textsubscript{2}S generation in the brain and the nervous system, and is also highly expressed in the liver and kidney\textsuperscript{193}. CSE is expressed abundantly in mammalian cardiovascular system and respiratory system. It also appears to be the main H\textsubscript{2}S-forming enzyme in the liver, kidney, uterus, placenta, smooth muscle as well as pancreatic islets\textsuperscript{194}-\textsuperscript{195}. 3-MST can be found in proximal tubular epithelium in the kidney, pericentral hepatocytes in the liver, cardiac cells in the heart and neuroglial cells in the brain\textsuperscript{196}. Both CSE and CBS are expressed in the human colon in the myenteric plexus neurons, and evidence indicates that human colonic mucosa metabolizes L-cysteine to H\textsubscript{2}S\textsuperscript{197}. Importantly, while CBS and CSE are hemeproteins primarily located in the cytosol, 3-MST is a zinc-dependent protein found in both the mitochondria and cytosol. CBS and CSE are pyridoxal 5'-phosphate (PLP) dependent enzymes and they
are responsible for the majority of the endogenous production of $\text{H}_2\text{S}$ in mammalian tissues.

CBS is a cystathionine-forming enzyme and it catalyzes $\beta$-replacement reactions between L-serine, L-cysteine, cysteine thioethers, or some other $\beta$-substituted $\alpha$-L-amino acids, and a variety of mercaptans. Homocysteine is the physiological substrate for CBS and in presence of cysteine their condensation generate, as final products, cystathionine and $\text{H}_2\text{S}$\textsuperscript{198}. In addition to $\text{H}_2\text{S}$ production, CBS has a prominent role in maintaining homocysteine levels \textit{in vivo}. Patients with homocysteinaemia exhibit impaired CBS activity, due to mutations of the regulatory domain of CBS, and display markedly increased levels of homocysteine and low $\text{H}_2\text{S}$ levels that contribute to the cardiovascular disease\textsuperscript{199-200}. Human CBS is a homotetramer consisting of 63 kDa subunits which bind two cofactors, PLP and heme. Each CBS subunit of 551 amino acid residues binds two substrates (homocysteine and serine). The NH\textsubscript{2} terminal of CBS contains the binding sites for both PLP and heme (protoporphyrin IX). Although, there is no clear a role of heme in the catalytic activity of CBS, seems to be redox sensor because its deletion renders CBS insensitive to oxidative stress\textsuperscript{201}. The PLP binding domain is the catalytic domain, and it is deep in the heme domain, linked by a Schiff base. The COOH terminal of CBS contains a regulatory
domain of 140 residues, playing an autoinhibitory role for the activity of full-length CBS. Binding of the allosteric activator, S-adenosyl-L-methionine (AdoMet or SAM), to this domain will cause a conformational change so that CBS is instantly activated. Deletion of the regulatory domain constitutively activates CBS\textsuperscript{202}. The activity of CBS is, also, regulated at the transcriptional level by glucocorticoids and cyclic AMP. The activity of CBS can be directly inhibited by nitric oxide (NO) and carbon monoxide (CO)\textsuperscript{203}.

CSE has been described as an exclusively beta-replacing lyase with a strict specificity for the primary substrate L-cysteine and for several sulfur-containing cosubstrates. It catalyses a β-disulphide elimination reaction that results in the production of thiocysteine, pyruvate, and NH\textsubscript{4}\textsuperscript{+} from cystathionine. Thiocysteine may react with cysteine or other thiols to form H\textsubscript{2}S. CSE is able to convert, also, directly L-cysteine in pyruvate, NH\textsubscript{3} and H\textsubscript{2}S and L-homoserine in H\textsubscript{2}O, NH\textsubscript{3}, and 2-oxobutanoate (o α-ketobutyrate)\textsuperscript{204}. Rat CSE (but not human CSE) may also use cystine, the disulfide form of Cys, as a substrate to generate H\textsubscript{2}S in the presence of a reductant\textsuperscript{205}. The concentration of cystine in the reducing environment of cells is extremely low, so it is unlikely that cystine contributes to H\textsubscript{2}S biogenesis under normal conditions. CSE seems to be the dominant enzyme for the formation of H\textsubscript{2}S in mammalian tissues as demonstrates by
CSE-KO mice that exhibit a profound depletion of H$_2$S in peripheral tissues$^{206}$. Human deficiency of CSE may lead to cystathioninuria, a metabolic disorder characterized by an excess of cystathionine in the urine that is inherited in an autosomal recessive manner. Other diseases related to CSE mutation include hypercystathioninemia and increase the risk of developing atherosclerosis and bladder cancer$^{207}$. CSE is a protein of 405aa and is a tetramer formed by two homodimers, both contributing to the active site pocket. There are two isoforms of human CSE as the consequence of alternative splicing$^{208}$. Activity of CSE is regulated at transcriptional level by myeloid zinc finger 1 (MZF1) and specificity protein 1 (SP1; also known as Sp1 transcription factor), and the enzyme can be upregulated by bacterial endotoxin$^{209}$. A recent study has reported the crystal structures of human CSE (hCSE), in apo form (apo-hCSE), complexed with PLP (hCSE·PLP) and with the inhibitor DL-propargylglycine (PAG) (hCSE·PLP·PAG). The structure of hCSE·PLP·PAG complex highlights the particular importance of Tyr114 in hCSE and the mechanism of PAG-dependent inhibition of hCSE$^{210}$.

In mitochondria 3-MST produces H$_2$S through a reaction involving the generation of pyruvate from 3-mercaptopyruvate (3-MP). It also catalyse the transsulfuration of a thiol to a persulfide, which can
subsequently join a second thiol to form a disulfide and release H2S. 3-MP is provided through the reaction of cysteine and α-chetoacid (e.g. α-ketoglutarato) by CAT. In the cytosol, the thiocysteine formed by cystathionase can act as an acceptor of the sulfur transferred from 3-mercaptopyruvate by MST211.

**Non-enzymatic Production of H2S**

Non-enzymatic production of H2S occurs through glucose, glutathione, inorganic and organic polysulfides and elemental sulfur. H2S can be generated from glucose via glycolysis or from phosphogluconate via NADPH oxidase. Glucose reacts with methionine, homocysteine or cysteine to produce gaseous sulfur compounds. H2S is also produced through direct reduction of glutathione and elemental sulphur that is mediated by NADH or NADPH. Increased oxidative stress and hyperglycemia will promote H2S production from this path212. The enterobacterial flora is an important source of H2S. The intestinal epithelium expresses specialized enzyme namely sulfate-reducing bacteria (SRB) that efficiently degrade sulphide to thiosulphate and sulphate to protect itself against high local concentrations of sulphide and to prevent an excessive entry of H2S into the systemic circulation213. H2S can also be released from thiosulfate and persulfides. Garlic and garlic-derived organic polysulfides induce H2S production in a thiol-dependent
manner. H$_2$S is excreted in the urine primarily as sulfate (either free sulphate or thiosulfate) and in feces and flatus as free sulfide.

**Figure 8.** The biosynthesis of hydrogen sulfide in mammalian cells. *(Kolluru et al., Nitric Oxide, 2013)*

### Catabolism of H$_2$S

The main catabolic pathways of H$_2$S are represented by oxidation and methylation (Figure 9). Much of the inactivation of H$_2$S occurs via mitochondrial oxidation. Two membrane-bound sulphide, quinone oxidoreductases (SQR), oxidize sulfide to elemental sulfur and reduce their cysteine disulfides. Then, sulfur dioxygenase (SDO) oxidizes one of
the persulfides to sulfite (H\textsubscript{2}SO\textsubscript{3}). Sulfur from the second persulfide is transferred from the SQR to sulfite by sulfur transferase (ST) producing thiosulfate (H\textsubscript{2}S\textsubscript{2}O\textsubscript{3}). Thiosulfate is, subsequently, converted in sulfite (SO\textsubscript{3}\textsuperscript{2-}) and sulphate (SO\textsubscript{4}\textsuperscript{2-}), by thiosulfate reductase and sulfite oxidase or is conjugated with cyanide ions (CN-) to generate thiocyanic acid by rhodanase enzyme (RHOD; also known as thiosulfate cyanide sulfurtransferase, TST). Also, sulfite originating through this reaction is quickly oxidized to sulfate, the major end-product of H\textsubscript{2}S metabolism under physiological conditions\textsuperscript{214}. For every mole of H\textsubscript{2}S oxidized 1.5 mole of oxygen is consumed along the electron transport chain.

Metabolism of H\textsubscript{2}S through SQR appears ubiquitous in all tissues except the brain\textsuperscript{215}. This process maintains very low intracellular H\textsubscript{2}S concentrations. In cytosol and in the gastrointestinal tract, H\textsubscript{2}S is catalyse mainly by thiol-S-methyltransferase (TSMT) that converts sulfide to methanethiol (CH\textsubscript{3}SH), which is less toxic to colonocytes than sulphides, by methylation. These enzymes are presumed to have a protective function, preventing excessively high local concentrations of sulfide and H\textsubscript{2}S entry into the systemic circulation\textsuperscript{216-217}.

Methemoglobin, considered a “common sink” for endogenous gases including CO and NO, also binds H\textsubscript{2}S to form sulfhemoglobin. Therefore, the half-life of free H\textsubscript{2}S in
blood may also be short\textsuperscript{218}. It was demonstrated that pretreating wild-type HEK-293 cells with methemoglobin for 1 h prior to adding 100\textmu M H\textsubscript{2}S significantly abolished the antiproliferative effect of H\textsubscript{2}S\textsuperscript{219}. H\textsubscript{2}S can also be scavenged by metallo- or disulfide-containing molecules such as horseradish peroxidise, catalase, and oxidized glutathione\textsuperscript{220}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{catabolism_of_hs.png}
\caption{Catabolism of H\textsubscript{2}S (Kolluru et al., Nitric Oxide, 2013)}
\end{figure}

\subsection*{2.3 Molecular targets of H\textsubscript{2}S}

Once produced in mammalian cells, H\textsubscript{2}S can directly exert its biological effects via interaction with different signalling molecules or can store as sulfur and released later in response to a physiological signal (Figure 10). Acid-labile sulfur and sulfane sulphur are the two main forms of sulfur stores in mammalian cells\textsuperscript{221}. 

\addcontentsline{toc}{section}{References}
In the cells H$_2$S exists in equilibrium with its anionic form HS$^-$, (ratio 1:4). As a gasotransmitter, H$_2$S does not use a specific transporter, and its lipophilic nature allows it to rapidly travel through cell membranes$^{222}$, whereas HS$^-$ anions cannot cross cell membranes and therefore might only target intracellular proteins. Recently was identified a channel permeable to HS$^-$ anions in the bacterium *Clostridium difficile*$^{223}$. The best mode of H$_2$S signalling is through protein sulfhydration, which occurs at reactive Cys residues of target proteins. In this process, the sulfhydryl group of a reactive Cys is modified to a -SSH group, resulting in increased reactivity of the Cys residue. The regulation machineries for S-sulfhydration depend by ATP level, pH value, ionic strength, and oxygen partial pressure$^{224}$. The first reported molecular target for H$_2$S was the ATP-sensitive potassium channels (K$_{ATP}$) which activation accounts in numerous distinct pharmacological effect of H$_2$S$^{194}$. The stimulatory effect of H$_2$S on K$_{ATP}$ channel complex relied on a direct interaction between H$_2$S molecule and the disulphide bond on the extracellular loop of the SUR1 subunit of K$_{ATP}$ channels$^{225}$. A direct confirmation of the effect of this gas on the K$_{ATP}$ channel has come from electrophysiological studies in which NaHS increased the K$_{ATP}$ current in rat aortic and mesenteric smooth muscle cells. H$_2$S is also known to act on a number of other ion channels. H$_2$S inhibits BKCa (big conductance Ca$^{2+}$-sensitive K$^+$) channels in stably
transfected HEK-293 cells\textsuperscript{226}, whereas the opposite effect was noted in rat pituitary tumor cells\textsuperscript{227}; inhibits L-type Ca\textsuperscript{2+} channels in cardiomyocytes\textsuperscript{228}, T-type Ca\textsuperscript{2+} channels mediating visceral pain in the mouse\textsuperscript{229} and intracellular chloride Cl\textsuperscript{-} channels in rat heart lysosomal vesicles\textsuperscript{230}; it activates transient receptor potential vanilloid (TRPV) channels in both urinary tract\textsuperscript{231} and airway smooth muscle\textsuperscript{232}. Important targets of H\textsubscript{2}S are the intracellular transcription factors. The first evidence was done by Oh and colleagues that demonstrated NaHS reduce LPS-induced NF-κB activation in cultured RAW 264.7 macrophages inhibiting IκB-α degradation and NF-κB nuclear translocation\textsuperscript{233}. As NaHS, many H\textsubscript{2}S donors, like GYY4137, S-diclofenac, garlic compounds such as diallyl sulfide (DAS), can also downregulate NF-κB activation\textsuperscript{234-236}. The functional consequence of reduced NF-κB activation in inflammatory cells is downregulation of a number of proinflammatory genes, including those that encode for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as downregulation of inflammatory cytokines/chemokines and adhesion molecules.

However, under some experimental circumstances, NaHS can be proinflammatory and can augment (not inhibit) IκB-α degradation and thence increase (not reduce) NF-κB activation\textsuperscript{237}. Moreover, recently, was established that H\textsubscript{2}S
synthesized by CSE, than TNF-α stimulation, is able to sulphydrylate the p65 subunit of NF-κB at cysteine-38, enhancing its binding to the co-activator RPS3, thereby augmenting the transcription of several anti-apoptotic genes\textsuperscript{238}. Clearly, there is considerable variation in the way that the NF-κB system responds to H\textsubscript{2}S that depends on the cell type and/or culture conditions used during the studies and, also, on the precise stage of the inflammatory response. In fact NF-κB has a proinflammatory role in the early stages of inflammation and anti-inflammatory role in the resolution phase. Additional transcription factors are also targets for H\textsubscript{2}S. The signal transducer and activator of transcription 3 (STAT3), that regulate the expression of many genes that mediate cell survival (e.g., survivin), proliferation (e.g., c-fos), and angiogenesis (e.g., vascular endothelial growth factor), results activated by H\textsubscript{2}S\textsuperscript{239}. NaHS also induces the nuclear localization of the transcription factor NF-E2-related factor 2 (Nrf-2) that regulates gene expression of a number of cellular cardioprotective enzymes, including heme oxygenase-1 (HO-1) and thioredoxin-1 (Trx-1)\textsuperscript{240}. Finally, H\textsubscript{2}S increases hypoxia-inducible factor-1 (Hif-1) activity in the nematode, Caenorhabditis elegans whereas in mammalian has not seen yet\textsuperscript{241}.

The effect of H\textsubscript{2}S on MAPK signalling is not clear due to the cell type and concentrations used. The MAPK
superfamily is composed of three main members, stress-activated protein kinase/c-JunNH₂-terminal kinase (SAPK/JNK), p38-MAPK, and ERK. MAPK regulate cell proliferation, apoptosis, differentiation, inflammation, and cycle progression. The activation of different MAPK members by H₂S in different types of cells results in inducing apoptosis. In fact, H₂S induces apoptosis of human aortic SMCs via activating ERK²⁴², of insulin-secreting beta cells (INS-1E) by the activation of p38 MAPK²⁴³ and of human derived dopaminergic neuroblastoma cell line (SHSY5Y) by downregulation of JNK²⁴⁴. The activation of the same MAPK member by H₂S in different cells may be responsible for the opposite functional consequences. For instance, H₂S increase endothelial cell proliferation (but also rat vascular smooth muscle cells and human colon cancer cells²⁴⁵-²⁴⁶) via stimulating a sustained phosphorylation\activation of ERK²⁴⁷ and increase the proliferation of nontransformed intestinal epithelial cells (IEC-18) cells by upregulation of JNK expression²⁴⁸. Instead, the activation of p38 by H₂S is responsible of survival of human polymorphonuclear cells, anti-inflammation of microglial cells and SH-SY5Y cells²⁴⁴-²⁴⁹-²⁵⁰.

Additional kinases are also targets of H₂S. In isolated hearts subjected to ischemic preconditioning, a brief infusion of NaHS stimulates both cardiac AKT and protein
kinase C (PKC) activity; this effect has been associated with improved cardiac mechanical performance and reduced injury\textsuperscript{251}.

Cell cycle is realized by the progression from G1 phase to S phase, G2 phase, and M phase (mitosis). Once this progression is stopped at any checking point, the cells enter G0 phase or the so-called quiescence status. The fate of cells going through this cycle is determined at three checkpoints. H$_2$S can affect cell proliferation or death by altering the fate of the cell going through cell cycle. On epithelial-like cells H$_2$S induced cell cycle arrest downregulating cyclin D1 and upregulating p21Cip/WAF-1, involved in the control of cyclin-cdk activity\textsuperscript{252-253}. In nontransformed intestinal IEC-18 cell NaHS (1 mM) promotes the proliferation facilitating the cell cycle entry\textsuperscript{254}.

It has been reported that H$_2$S increase [Ca$^{2+}$], by the activation of cAMP pathway in primary cultures of brain cells, neuronal and glial cell lines, and Xenopus oocytes selectively enhance N-methyl-d-aspartate (NMDA)-receptor mediated responses and facilitate the induction of hippocampal long-term potentiation in the CNS\textsuperscript{255}. Another signaling pathway activated by cAMP/PKA in the CNS is the PI3K/AKT/p70 ribosomal S6 kinase (p70S6K). NaHS has been shown to increase cAMP concentration and expression of PI3K, AKT, and p70S6K in isolated rat hippocampal
neurons. In the kidney NaHS reduce renovascular hypertension in hypertensive rats lowing level of cAMP, and, consequently, of angiotensin II. Differential effects of H$_2$S on cAMP/PKA pathway have been related to the expression of different isoforms of AC and/or PDE in different types of cells. As regard the effect of H$_2$S on cGMP/PKG pathway it was demonstrated that NaHS increased the level of cGMP in cultured rat aortic smooth muscle cells inhibiting phosphodiesterase (PDE), isoenzymes responsible for the hydrolysis of both cGMP and cAMP, without to modify the activity of guanylyl cyclase.

H$_2$S is a strong reducing agent and may scavenges of reactive oxygen species and nitrogen species. H$_2$S significantly inhibites ONOO$^-$ with a potency like to that of glutathione (GSH), a major intracellular antioxidant. The antioxidant property of H$_2$S may also be indirectly realized due to its capability in increasing the level of GSH. This could partially result from the stimulatory effect of H$_2$S on $\gamma$-glutamylcysteine synthetase and cysteine transport in neurons.

The antioxidant effects of H$_2$S can affect the remodelling or proliferative process of the blood vessels but doesn’t affect the vascular contractility. H$_2$S reduces, also, H$_2$O$_2$, ONOO$^-$, and O$_2$, homocysteine-induced, in cultured vascular smooth muscle cells and hydrogen peroxide and
oxLDL-induced cytotoxicity of cultured HUVECs. H₂S also can bind to haem proteins as an axial ligand for the prosthetic group enhancing the activities of haem-containing enzymes in vitro, such as haemoglobin, neuroglobin and cytochrome c oxidase, which contributes to some of its (patho) physiological effects. H₂S reacts with heme proteins in distinct ways: (i) by incorporation of H₂S into one of the pyrrole rings of the heme, (ii) generating the sulfheme derivative, (iii) binding to alternate sites of heme such as cysteine, copper, and zinc ions; or to the ferric iron with subsequent reduction of the heme, and (iv) through coordination with the ferric heme iron without inducing reduction or sulfheme production.

**Figure 10.** Molecular targets of H₂S (Li et al., 2011. *Annu. Rev. Pharmacol. Toxicol.*)
2.4 Physio-pathological functions of \( H_2S \) in mammalians

\( H_2S \) is involved in numerous physiological and pathophysiological functions. \( H_2S \) exerts its biological effects at low concentrations and toxic effects at high concentrations acting on various biological targets without a specific receptors for intracellular signaling\(^{264}\). At micromolar (physiological) concentrations, multiple studies have demonstrated the cytoprotective (antinecrotic or antiapoptotic) effects of \( H_2S \) which may be related to its antioxidant activities. \( H_2S \) is able to neutralize a variety of reactive species including oxyradicals, peroxynitrite, hypochlorus acid, and homocysteine\(^{261-266}\). Moreover, low levels of \( H_2S \) exerts cytoprotective effect increasing activity of endogenous antioxidant cellular systems as glutathione, by the activation/expression of \( \gamma \)-glutamylcysteine synthetase, N-acetylcysteine and superoxide dismutase as demonstrated in primary cortical neurons exposed to the excitotoxin NMDA\(^{255}\). Higher (millimolar) \( H_2S \) concentrations are cytotoxic to the cells; this is due to free radical and oxidant generation, calcium mobilization, glutathione depletion, intracellular iron release, as well as induction of mitochondrial cell death pathways.
**H₂S and the Cardiovascular System**

In the cardiovascular system H₂S induces dilation of blood vessels by stimulating K<sub>ATP</sub> channels in vascular smooth muscle cells (SMCs)<sup>194</sup>. H₂S shows, also, cardioprotection effect during ischemia/reperfusion injury in isolated hearts inducing negative inotropic effect and reducing central venous pressure by the opened of K<sub>ATP</sub> channels in the myocardium<sup>267</sup>. All these effects can be prevented by pretreatment with glibenclamide a classical K<sub>ATP</sub>-channel inhibitors, to demonstrate the specificity of H₂S cardiac effects<sup>268</sup>. H₂S possesses many common features of an endothelium-derived hyperpolarizing factor (EDHF). The absence of endothelium shifted the H₂S concentration-response curve to the right with the IC₅₀ changed from 136 to 273 µM. The endothelium-dependent vasorelaxing effect of H₂S becomes even more significant in small resistance arteries. The vasorelaxant effect of H₂S is due to the activation of both K<sub>ATP</sub> channels on vascular SMCs and charybdotoxin/apamin-sensitive K<sub>Ca</sub> channels in vascular endothelial cells<sup>194; 269</sup>.

H₂S reduces atherogenesis by interfering with vascular inflammation<sup>250</sup>, by inhibiting leukocyte infiltration and adhesion<sup>270</sup> and by reducing calcification<sup>271</sup>. Deficiency of CSE significantly enhances atherosclerosis development in ApoE knockout mice involving disruption of vascular redox
status, increased intimal proliferation and inflammatory adhesion molecule expression\textsuperscript{272}, and whereas treatment with NaHS of the same mice resulted in reduced atherosclerotic plaque thanks to reduction of ICAM-1 level in circulation and its expression in aortic endothelial cells\textsuperscript{273}. H\textsubscript{2}S can also inhibit calcification and osteoblast differentiation of vascular smooth muscle cells that can contribute to atherogenesis\textsuperscript{274}.

H\textsubscript{2}S play an important role in regulating platelet function and thrombosis. It inhibits cyclooxygenase activity and collagene-induced platelet aggregation reducing, moreover, thromboxane formation\textsuperscript{275-276}. It was demonstrate that chronic intake of garlic powder and garlic oil also inhibits platelet aggregation in humans\textsuperscript{277}. Several reports indicate a potential synergistic effect between NO and H\textsubscript{2}S in controlling various biological responses of vascular function. Several study reveale that inhibition of NO production by blocking eNOS attenuates H\textsubscript{2}S mediated angiogenic activity and reduces H\textsubscript{2}S-dependent vasorelaxation highlighting the importance of NO in vascular H\textsubscript{2}S signalling, and viceversa\textsuperscript{278}.

**H\textsubscript{2}S and the Nervous System**

H\textsubscript{2}S exerts important effects in central nervous system (CNS) through regulation of neurotransmission and
neuromodulation. The potential physiological functions of H$_2$S in the brain may include calcium homeostasis, potentiation of hippocampal long-term potentiation (LTP), suppression of oxidative stress and regulates survival/death of neurons. Most of these effects are mediate by activation of N-methyl-D-aspartate (NMDA) receptors, normal receptor for glutamate, express in both central and peripheral nervous systems. H$_2$S selectively enhance NMDA-receptor-mediated currents by stimulating the production of cAMP in primary cultures of brain cells, neuronal and glial cell lines, and *Xenopus* oocytes, which then activates protein kinase A (PKA). PKA activated, consequently, phosphorylates the NMDA receptor and induces also LTP$^{79}$.

Moreover, H$_2$S is able to increase directly glutamate secretion$^{80}$. The interaction of H$_2$S and NMDA receptors affects other neuronal activities, such as epilepsy, neuropathic pain, stroke, and Alzheimer's and Parkinson's diseases. Infact, prolonged activation of NMDA receptors by H$_2$S causes calcium overload in cells and leads to their death$^{281-284}$. Thus, inhibition of its production has been suggested to be a potential treatment approach in stroke therapy. Neuromodulator effects of H$_2$S is, also, due increasing release of GABA, a major inhibitory neurotransmitter in CNS, that induces upregulation of GABA$_B$ receptors located at pre- and postsynaptic sites$^{285}$.
The increased GABAergic inhibition by H\textsubscript{2}S may find its application in several situations where the excitation/inhibition balance in CNS is disturbed, such as seizures and epilepsy, stimuli leading to pain, and cerebral ischemia\textsuperscript{282, 286}. In addition, H\textsubscript{2}S regulates synaptic activity inducing Ca\textsuperscript{2+} wave from neurons\textsuperscript{287} and increases norepinephrine and epinephrine levels in the hippocampus, striatum, and brain stem by inhibiting monoamine oxidase\textsuperscript{288}.

\section*{H\textsubscript{2}S and Inflammation}

Another area attracting considerable attention is the role of H\textsubscript{2}S in inflammation. H\textsubscript{2}S has been reported to exert both pro-inflammatory and anti-inflammatory effects.

Among anti-inflammatory effects of H\textsubscript{2}S there are inhibition of proliferation of T lymphocytes\textsuperscript{289}, induction of apoptosis in polymorphonuclear cells\textsuperscript{290}, increases phagocytosis of bacteria by macrophages and promotes a shift of phenotype of macrophages to the “M2”, pro-resolution state\textsuperscript{291}. Several studies in recent years showed that, in some inflammation model as carrageenan-induced paw edema and air pouch, injection of rats with NaHS inhibited leukocyte infiltration and their adherence to vascular endothelium and edema formation. The mechanism underlying anti-inflammatory role seems due to activation of
KATP channels on endothelial cells and leukocytes because they are attenuated by pretreatment with glibenclamide\textsuperscript{270}. H\textsubscript{2}S donors can also down-regulates inflammatory process by reducing the expression of a number of proinflammatory cytokines, e.g. tumour necrosis factor (TNF)-\(\alpha\), interleukin (IL)-1\(\beta\), IL-8, IL-1\(\alpha\), interferon (IFN)-\(\gamma\) or proteins, such as heme oxygenase (HO) and increasing the anti-inflammatory cytokine IL-10. These effects are most likely due to suppression of NFkB activity by H\textsubscript{2}S\textsuperscript{234; 292-294}. In model of neuroinflammation H\textsubscript{2}S also attenuates LPS-induced production and release of NO and TNF-\(\alpha\)\textsuperscript{250}. Moreover, H\textsubscript{2}S reduces the gastrointestinal mucosal damage caused by nonsteroidal anti-inflammatory drugs. There are a number of mechanisms of action underlay this beneficial effects. For example, H\textsubscript{2}S stimulates secretion of bicarbonate in the stomach and duodenum, inhibits gastric acid secretion and reduces gastric mucosal blood flow and prevents the adherence of leukocytes to the vascular endothelium in the early stages of gastric injury. Therefore, it has been synthetized a new class of NSAID derivatives that release H\textsubscript{2}S. These drugs results the same efficacy in terms of retaining anti-inflammatory activity with reduced GI toxicity\textsuperscript{295-297}.

Experimental evidence has showed pro-inflammatory role for H\textsubscript{2}S in various animal models, as hindpaw edema\textsuperscript{298},
acute pancreatitis, LPS-induced endotoxemia and cecal ligation and puncture-induced sepsis. Abnormal synthesis or activity of H$_2$S may play a part in septic or endotoxic shock because its capacity to open K$_{ATP}$ channels. Intriguingly, plasma H$_2$S concentrations were also markedly increased in patients with septic shock. Therefore, inhibiting the activity of H$_2$S generating enzymes would be beneficiary. In fact, in cecal ligation and puncture induced sepsis, PPG treatment attenuated inflammatory response by reducing neutrophil infiltration and reducing animal mortality, whereas NaHS treatment significantly aggravated septic inflammatory damages. H$_2$S has also been reported to stimulate the generation of pro-inflammatory cytokines from human monocytes. The mechanisms for these pro-inflammatory effects of H$_2$S would be the releasing endogenous tachykinins such as substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A, and activation of TRPV-1 that mediate neurogenic inflammation. A number of factors are involved in determining whether H$_2$S is anti-inflammatory or pro-inflammatory. The first is the dose–response relationships. At low, physiological concentrations H$_2$S is predominantly anti-inflammatory, whereas at high concentrations H$_2$S promotes inflammation, administration routes (intravenous infusion or bolus) may also cause opposite effects. Another
important factor is the releasing rate: the slow releasing of H$_2$S inhibited inflammation whereas the fast release of H$_2$S increased the synthesis of proinflammatory factors$^{304}$. Finally, animal species (rats vs. mice and others), inflammation models (regional vs. systemic inflammation), and the organ origin of H$_2$S (brain vs. pancreas, etc.) also influence the role of H$_2$S in inflammation.

**H$_2$S and cell proliferation**

H$_2$S is involved in modulating cell proliferation and apoptosis in a variety of cells$^{195}$. H$_2$S promotes a number of cellular signals that regulates metabolism, cardiac function and cell survival. Overexpression of CSE in human aortic SMCs or treatment with NaHS and other endogenously produced H$_2$S inhibited cell growth and induced cell apoptosis. The antiproliferative and/or proapoptotic effect of H$_2$S treatment may be related to activation of MAPK/ERK pathway and its downstream factor caspase-3$^{195}$. H$_2$S also induces stabilization of p53 coupled with the induction of downstream proteins such as p21, p53AIP1, induces translocation of proapoptotic protein Bax from cytoplasm to mitochondria and release of cytochrome c from the mitochondria without altering the antiapoptotic protein Bcl-2$^{243; 305}$. In addition, H$_2$S act as a cyclin dependent kinase (cdk) inhibitor in cell cycle progression in the rat smooth
muscle cells\textsuperscript{305}, human lung fibroblast\textsuperscript{306-307} and oral epithelia\textsuperscript{1252} cells by the up-regulation of key cell cycle proteins such as cyclin-E and CDC-6 (cell division cycle-6) and by decrease cyclin D1 and p27 involved in cell growth. However, H\textsubscript{2}S inhibit cell proliferation at G1 phase only at the early time points (12 and 24 h) such cells might be eliminated by the process of apoptosis as indicated by a higher incidence of DNA content in the sub-G1 phase, whereas at later time point (48 h)\textsuperscript{306} follows a partial recovery in cell survival. The anti-proliferative and/or pro-apoptotic effect of H\textsubscript{2}S may be of importance for the prevention of cell proliferation in disorders such as atherosclerosis, vascular graft occlusion, and neointimal hyperplasia leading to restenosis after angioplasty\textsuperscript{308}. In neuroblastoma cell line (SH-SY5Y) NaHS, at concentrations lower than 300 $\mu$M, suppress rotenone-induced apoptosis by inhibition of p38- and c-Jun NH\textsubscript{2}-terminal kinase (JNK), phosphorylation of MAPK, normalization of Bcl-2/Bax levels, and reduction of cytochrome c release, caspase-9/3 activation and poly(ADP-ribose) polymerase cleavage\textsuperscript{309}. The same anti-apoptotic effect of NaHS has also been observed on rat pheochromocytoma cells (PC12)\textsuperscript{310}. The opposite effects of H\textsubscript{2}S on apoptosis are cell type-specific and also depends H\textsubscript{2}S concentration used.
**H₂S and cancer**

Given the complexity of DNA damage and repair, controversy exists on the role of H₂S in cancer cell proliferation. In colon epithelial cancer cell line NaHS (50–200 µM) treatment killed the cancer cells stimulating the phosphorylation of ERK and p38 MAPK and butyrate also induces apoptosis stimulating CBS and CSE expression and H₂S production\(^{311}\). In another colon cancer cell line, HCT 116 cells, indeed NaHS (20 µM) promoted their proliferation increasing AKT and ERK phosphorylation and reducing activity of cyclin dependent kinase inhibitor p21 (Waf1/Cip1)\(^{312}\). H₂S-releasing NSAIDs have also showed to be effective and safe in chemoprevention in colon cancer and other types of cancers. The mechanism of action of these drugs may include inactivation of Nrf2 via sulhydration of Keap1, induction of glycolysis within cancer cells and induction of apoptosis of cancer cells associated whit inhibition of COX-2 activity\(^{313}\). Recently, CBS has emerged as a potential therapeutic target in both colon cancer and ovarian cancer\(^{314-315}\). In contrast a latest study demonstrates that H₂S increases nicotinamide phosphoribosyltransferase (Nampt) and ATP levels in cancer cells (HepG2, MDA-MB-231, MDA-MB-435S) inducing intensified glycolysis and rapid proliferation. In this way H₂S-Nampt pathway protects
cells from drug induced damage and it may responsible for resistance to therapy\textsuperscript{316}.

**Other biological effects**

The opening of the $K_{ATP}$ channel also underpins the relaxant effect of $H_2S$ in colonic\textsuperscript{317} and eye\textsuperscript{318}, but not bronchial smooth muscle\textsuperscript{319}. Because of the crucial role of $K_{ATP}$ channels in the regulation of pancreatic insulin secretion, multiple studies have examined the effect of $H_2S$ on $\beta$-cells, where $H_2S$ opens the $K_{ATP}$ channels and inhibites insulin secretion promoting hyperglicemia\textsuperscript{320}.

$H_2S$ is produced also in the lung and airway tissues, via the actions of CSE and CBS express in SMCs, vascular SMCs, and vascular endothelial cells\textsuperscript{321-323}, where it participates in the regulation of contractility of airway smooth muscles and lung circulation. It has been suggested that decreased CSE expression and $H_2S$ levels in pulmonary tissues are related to the pathogenesis of asthma. In fact, in an animal model of asthma administration of NaHS reduces airway inflammation, the number of eosinophils and neutrophils in bronchoalveolar lavage fluid airway (BALF), the expression of inflammatory genes, and also increased peak expiratory flow (PEF) indicating alleviation of airway obstruction\textsuperscript{324}. 


2.5 \textit{H}_2\textit{S} donors

With the myriad of purported biological actions of \textit{H}_2\textit{S} there is growing interest in more precise delivery of this volatile gas to target tissues in the form of \textit{H}_2\textit{S} “donating” compounds. Pharmacologically useful donors should be soluble in aqueous media, should not be toxic, should not metabolize quickly and should release \textit{H}_2\textit{S} in vivo slowly over a period of time.

\textit{H}_2\textit{S} donating compounds can be divided into three general classes:

- Sulphide salts;
- Natural compounds (mainly in foods);
- Synthetic compounds.

\textbf{Sulfide salts}

Sodium hydrosulfide (NaHS), sodium sulfide (Na\textsubscript{2}S) and calcium sulfide (CaS) are fast delivering compounds. They form HS\textsuperscript{-} and \textit{H}_2\textit{S} immediately upon solvation in physiological buffers. These have the advantage of the ready availability of high pure \textit{H}_2\textit{S} concentration, but because \textit{H}_2\textit{S} is rapidly lost from solution by volatilization in laboratory conditions their effective residence time in tissues is relatively short. Therefore it’s difficult to achieve the desired final concentration of \textit{H}_2\textit{S} and maintain that at constant level over a relatively long period. \textit{H}_2\textit{S} can be released from the
solution in a temperature- and concentration-dependent manner. At 37°C or below, the concentration of H₂S of the bath solution was relatively stable. At concentration below 1 mM, within 30 min H₂S amounts decreases of 15% in the solution. Also in vivo, the bolus injection of the animals with NaHS/Na₂S stock solution creates, immediately, a great surge of H₂S level, but thereafter, a much lower and declining level of H₂S is encountered and declining. These properties limits their therapeutic potential.

NaHS is the most used H₂S donor for its convenient preparation. In solution (water or ethanol) NaHS dissociates into Na⁺ and HS⁻ that in presence of H⁺ forms H₂S. In physiological condition (pH 7.4 and temperature of 37°C) NaHS solution will yield about one-third of the undissociated H₂S and the other two-third remains as HS⁻. Some effects of NaHS cannot be fully explained by H₂S. Also the altered Na⁺ concentration or ionic strength as well as the redox potential may be associated with NaSH effect, especially at high end of the concentrations used.

Na₂S (or IK-1001) is a salt water soluble. In solution, it dissociates and generates H₂S. Administration of Na₂S increased blood sulfide and thiosulfate levels and that exhaled H₂S could be detected.
Natural compounds

In our daily life, many dietary elements directly and indirectly supply \( \text{H}_2\text{S} \) to our body so that the advantage of this gasotransmitter can be realized, in many cases, without conscious thought.

Garlic: Polysulfides and S-ally-L-Cysteine

Garlic (\textit{Allium sativum}) has antioxidant, anti-inflammatory and anticarcinogenic functions. In particular, garlic consumption has been correlated with the reduction in multiple risk factors associated with cardiovascular diseases such as increased reactive oxygen species, high blood pressure, high cholesterol and platelet aggregation\textsuperscript{328}. Garlic is also effective against bacterial, viral, fungal, and parasitic infections and stimulates the immune system\textsuperscript{329}. The National Cancer Institute (NCI) set garlic on the top of a vegetable pyramid, representing potency in cancer prevention\textsuperscript{330}. The benefice pharmacological effects of garlic derive from \( \text{H}_2\text{S} \) production by the numerous organosulfur compounds present such as allicin, allyl sulfides, ajoene, polysulfides, as diallyl disulfide (DADS) and diallyl trisulfide (DATS); \textit{S-alk(en)yl-L-cysteine} sulfoxides and \textit{S-allyl cysteine} (SAC)\textsuperscript{331}. 
Organic polysulfides can interact with either exofacial membrane protein thiols or intracellular thiols, such as GSH, to form H$_2$S. Benavides et al. demonstrate the capacity of garlic extract (DADS and DATS) to produce H$_2$S measuring H$_2$S production in real time with a polarographic sensor. In particular the release of H$_2$S from DATS is 3 times that from DADS$^{236}$. One of the nonenzymatic mechanisms have been proposed to explain the chemical conversion of garlic-derived organic polysulfides to H$_2$S is the conversion of S-allyl-glutathione and allyl perthiol to allyl-GSSG and to H$_2$S through a sequence of reactions. Another mechanism is the direct thiol/disulphide exchange during the interaction of polysulfides and GSH$^{332-333}$. Additional evidences of the H$_2$S production from garlic derive by in vivo studies. For example, infusion of diallyl disulfide 1.8 mg/kg/min in rats increases exhaled H$_2$S$^{334}$. Moreover, in an acute myocardial infarction (AMI) rat model, pretreatment of the animals with S-allylcysteine protected partially the heart from ischemia damage and saved the animals from mortality by about 11% and these effects were antagonized by PPG inhibition of CSE$^{335}$. Direct administration of allicin (8 mg/kg) to rats also led to lowered systolic blood pressure and triglycerides.
Other examples of the therapeutic potential of garlic compounds are the reduction of β-amyloid fibril formation in Alzheimer's disease transgenic model\textsuperscript{337}; inhibition of angiotensin converting enzyme\textsuperscript{338}, prevention of vascular remodeling\textsuperscript{339} and suppression of L-NAME-induced hypertension\textsuperscript{340}.

**Broccoli and Sulforaphane**

Broccoli (Brassica oleracea) as well as other cruciferous vegetables tends to release strong smell of H\textsubscript{2}S when cooked or became rotten. Previous studies have reported the health beneficial effects of broccoli on the prevention and treatment of hypertension and atherosclerotic changes in the SHR stroke-prone rats\textsuperscript{341}. These effects of broccoli are largely ascribed to the action of sulforaphane, an isothiocyante compound that exhibits anticancer properties. It is rapidly absorbed by humans, reaching peak concentrations at 1 h and declining thereafter with a half-life of 1.8 hours\textsuperscript{342}. Sulforaphane protects vascular smooth muscle cells and endothelial cells from oxidative and inflammatory stress and suppresses angiogenies\textsuperscript{343-344}. In addition, it has neuroprotective and anti-inflammatory actions mediated in part through activation of heme oxygenase-1 and it provides some protection against ischemia reperfusion injury, hemorrhage
and serotonin-induced toxicity\textsuperscript{345}. Sulforaphane induces death of PC-3 cells (a human prostate cancer cell line) in a dose-dependent manner by the activation of p38 MAPK and JNK, and this effect is reversed scavenging of free H\textsubscript{2}S with methemoglobin or oxidized glutathione\textsuperscript{346}.

Another proof of the release of H\textsubscript{2}S is that sulforaphane in human breast cancer MCF-7 cells induces upregulation of thioredoxin reductase 1 (EC 1.8.1.9) that reduces thioredoxin that is fundamental for H\textsubscript{2}S synthesis from 3-MST.

A related isothiocyanate compound, erucin, is found in high levels in rocket salad species (Eruca sativa).

\begin{center}
\begin{tikzpicture}
\node[draw, inner sep=0, text height=1cm, text depth=0.25cm] (sulforaphane) at (0,0) {\textbf{sulforaphane}};
\node[draw, inner sep=0, text height=1cm, text depth=0.25cm] (erucin) at (2,0) {\textbf{erucin}};
\node[above] at (sulforaphane) {$\begin{array}{c}
\text{H}_3\text{C} \\
\text{S} \\
\text{N=\text{C=S}} \\
\end{array}$};
\node[above] at (erucin) {$\begin{array}{c}
\text{H}_3\text{C} \\
\text{S} \\
\text{N=\text{C=S}} \\
\end{array}$};
\end{tikzpicture}
\end{center}

**Other H\textsubscript{2}S-food**

Durian (Durio zibethinus Murray) is a notably flavorful or pungent fruit, of the Southeast Asia. Durian is rich in sulfur compounds, mostly dialkyl polysulfides\textsuperscript{347} that are responsible for its health benefits\textsuperscript{348}. Thousand-Year Egg is a Chinese delicacy made from duck, quail, or chicken eggs preserved in a mixture of clay, ash, lime, salt, and rice straw that is a source of H\textsubscript{2}S\textsuperscript{349}.
Red meat, cheese, milk, fish, soybeans, preserved bean curd (stinky tofu), breads, beers, sausages and dried fruit are all high-protein foods rich in sulfur amino acids generating H$_2$S in the colon following fermentation by colonic bacteria or by sulfate-reducing bacteria$^{350}$.

**Synthetic compounds**

In the last years have been developed several H$_2$S slow-releasing compounds to allow a long-term sustained therapeutic supply of H$_2$S.

**GYY4137**

GYY4137 (morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate) is a water-soluble molecule that releases H$_2$S slowly and steadily, either in aqueous solution or administered to the animal (intraperitoneal or intravenous).

One mmol/l of GYY4137 releases ~40 μmoles of H$_2$S in the first 10 min and then releases approximately 40 μmoles of H$_2$S for hour for the ensuing 80 min when
dissolved in acidic (pH 3.0) phosphate buffer whereas in buffer at pH 7.4 or 8.5 releases minus of 3 μmoles of H$_2$S in the same period. Injection of 133 μmol/kg either intraperitoneally (ip) or intravenously (iv) into rats, increases plasma H$_2$S levels from ~32 μmol/l to ~80 μmol/l in 30 min and this remains elevated (50 μmol/l) for 3 hours. H$_2$S concentrations quickly increases in the liver and heart for the enduring 20 min, which is even longer in the kidney. The slow-releasing property of GYY4137 results in slow vasorelaxation without affecting heart rate or myocardial contraction of rats. This effect is in part mediated by K$_{ATP}$ channels. Furthermore, GYY4137 exhibits antihypertensive activity in a NG-nitro-L-arginine methyl ester (L-NAME)-evoked rat hypertension model$^{235}$. GYY4137, administered 1 or 2 hours after LPS stimulation, significantly decreases secretion of many of the markers of LPS-induced inflammation in cultured RAW 264.7 cells including nitrate/nitrite, PGE2, TNF-α, IL-1β, IL-6, NF-κB, expression of inducible nitric oxide synthase (iNOS), myeloperoxidase activity and COX-2$^{351}$. Anti-cancer effects of GYY4137 have been observed in vitro and in vivo. Five-day treatment with GYY4137 (400 μmol/l) significantly reduced proliferation of breast adenocarcinoma (MCF-7), acute promyelocytic leukemia (MV4-11) and myelomonocytic leukemia (HL-60) cells; and at 800 μmol/l GYY4137 kills also human cervical
carcinoma (HeLa), colorectal carcinoma (HCT-116), hepatocellular carcinoma (Hep-G2), osteosarcoma (U2OS), without affecting survival of non-cancer human diploid lung fibroblasts (IMR90 and WI-38). This antiproliferative effect of GYY4137 is due to induction of apoptosis and cell-cycle arrest in the G2/M. Daily i.p. injection of GYY4137 also decreased the growth of HL-60 and MV4-11 tumors subcutaneously transplanted in immunodeficient mice\textsuperscript{352-353}.

**Cysteine analogs**

The cysteine analogs act as potential substrates for endogenous cysteine-metabolizing enzymes increasing H\textsubscript{2}S production at least two-fold. They are: S-propyl cysteine (SPC), S-allyl cysteine (SAC, also a derivative of garlic), S-propargyl cysteine (SPRC) and N-acetyl cysteine (NAC) \textsuperscript{354}.

[SAC (50 mg/kg/day for 7 days)](image) has been shown to low mortality and to reduce infarct size in an acute myocardial infarction rat model. Inhibition of CSE with PPG eliminated
the beneficial effects of SAC, increased infarct size, and significantly elevated blood pressure\textsuperscript{355}. Moreover, daily i.p. injection of cysteine analogs significantly protected the hearts from ischemia reperfusion injury and was associated with preserved superoxide dismutase (SOD), glutathione peroxidase activity, and tissue GSH levels, while reducing lipid peroxidation products\textsuperscript{354}. SPRC attenuated LPS-induced deficits in spatial learning and memory in rats and restored the LPS deficit in hippocampal H\textsubscript{2}S levels\textsuperscript{356}. SPRC and SAC have also anti-cancer activity. SPRC (1 \textmu M-30 mM) inhibited growth of cultured human gastric cancer cells (SGC-7901) in a dose dependent manner, stimulating apoptosis and inducing cell cycle arrest at the G1/S phase. In male nude mice with SGC-7901 tumors implanted in the flank, SPRC and SAC (50 and 100 mg/kg) increased plasma H\textsubscript{2}S concentration, reduced tumor volume, increased apoptotic tumor cell number and increased protein expression of Bax and p53 while decreased Bcl-2\textsuperscript{357}. SPRC reduced LPS-induced inflammatory response of rat by inhibiting NF-\kappa B activation and ERK1/2 phosphorylation and reducing intracellular ROS production\textsuperscript{358}. SPRC effects were more pronounced than SAC but both act not only as a substrate for H\textsubscript{2}S by CSE but they also up-regulate the enzyme. SPRC and SAC have been conjugated with leonurine, an alkaloid in Chinese motherwort, which make them more effective to induce H\textsubscript{2}S
production. Leonurine-SPRC has a cardioprotective effect in hypoxic neonatal rat ventricular myocytes by increasing cell viability, decreasing LDH leakage, decreasing MDA and ROS.

**H₂S-releasing hybrid compounds**

These molecules are created by combining a H₂S-releasing moiety with another parent compound with known molecular structure and biological functions. The purpose to create such a new compound is to enhance the functionality and safety of both compositing compounds and reduce potential side effects of each part. Anethole dithiolethione (ADT) and its main metabolite (ADTOH; 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione) have been used extensively as a donor of H₂S and its ease of esterification with other therapeutics has led to a considerable variety of H₂S “donating” drugs.

![Chemical structures of ADT and ADT-OH](image)

**ADT**  **ADT-OH**  **ACS 1**
ACS14 (S-ASPIRIN)

It is a hybrid between ADTOH and aspirin that conserves the cardioprotective efficacy with reduced gastric damage. Infact, ACS14 inhibited thromboxane synthesis, similar to aspirin, but caused much weaker gastric reactions than aspirin did$^{360}$. This could be due to the antioxidant effect of H$_2$S, released from the hybrid. Another related compound with S-aspirin is ACS21, S-salicylic acid. Both ACS14 and ACS21, limited the development of metabolic syndrome in rats induced by GSH depletion (including hypertension, endothelial dysfunction, and insulin resistance), and protected the heart from I/R damage limiting gastric lesion$^{361\text{-}362}$.

ACS6 (S-SILDENAFIL)

It is a hybrid between ADTOH and sildenafil. ACS6 like sildenafil is an effective and selective inhibitor of type 5 phosphodiesterase (PDE5) and relaxe cavernosal smooth muscle, but it is more potent in inhibiting the formation of superoxide and expression of p47(phox) and PDE5 than
sildenafil\textsuperscript{363}. Normally increased oxidative stress level and NOX expression compromise the efficiency of sildenafil in treating erectile dysfunction. This hybrid will take the advantage of sildenafil-maintained cGMP activity and H\textsubscript{2}S-activated K\textsubscript{ATP} channels and suppressed oxidative stress in penile tissues, leading to an enhanced potency in relaxing penile cavernosal muscles. This hybrid may also find its application in the treatment of abnormalities in male reproductive system and urinary system.

\textbf{ACS84 (S-DOPAS)}

It is a hybrid between H\textsubscript{2}S-donating dithiolthione (ACS1) or allyldisulfide and L-DOPA methyl ester. L-DOPA (Levodopa) is extensively used for treatment of Parkinson’s disease but it has the adverse effect to induce apoptosis of substantia nigra dopaminergic neurons. The presence of H\textsubscript{2}S moiety allows to protect neurons from apoptosis via its antioxidant and anti-inflammatory effects. Moreover, ACS84 reached the brain is metabolized as early as 1 h after
administration to rats intravenously, causing more than 2-fold increase in brain dopamine and 1.4-fold increase in GSH than L-DOPA did\textsuperscript{564}. In fact, ACS84 has a better permeability to blood brain barrier than L-DOPA. With cultured human microglia, astrocytes, SH-SY5Y neuroblastoma cells, and humanTHP-1 U373 cell lines, ACS84 increased intracellular H\textsubscript{2}S levels.

\textbf{ACS67 (S-LATANOPROST)}

It is a hybrid between H\textsubscript{2}S-donating dithiolthione (ACS1) and latanoprost. Latanoprost is a synthetic derivative of the natural prostaglandin F2 and it is used in the treatment of glaucoma because it reduces intraocular pressure (IOP) but latanoprost does not protect retina from ischemia damage and its tolerance by patients is low. ACS67, instead, attenuated the death of cultured retinal ganglion cells by increasing GSH levels and decreasing H\textsubscript{2}O\textsubscript{2} toxicity\textsuperscript{365}. In glaucomatous (carbomer model) pigmented rabbits intravitreal injection of ACS67 achieved a greater
anti-IOP effect than latanoprost preventing, also, ischemia
damage thanks to the neuronal protective effect of H$_2$S$^{366}$.

H$_2$S-NSAIDs

Another line of research that is producing new molecules and exploiting the H$_2$S concept is aimed at reducing the toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs). H$_2$S donors were shown to reduce the severity of NSAID-induced damage in the rat stomach. Moreover, NSAIDs decreased endogenous H$_2$S synthesis$^{367-368}$. ACS15 (S-DICLOFENAC or ATB337) it is an hybrid between ADTOH and diclofenac. Diclofenac is one of the NSAIDs that inhibiting inflammation and eliciting analgesia but also may cause GI toxicity among other side effects. This undesired side effect of NSAIDs has been shown to be minimized by H$_2$S donors in the rat stomach$^{367}$. Therefore ACS15 is more potent than diclofenac for anti-inflammatory activity and reduces the common side effects of diclofenace, such as neutrophil infiltration, leukocyte adherence, and GI
lesion\textsuperscript{234, 369}, as well as pancreatitis-related lung injury\textsuperscript{370} and has no effect on haematocrit.

With the same principle and expectations, other H\textsubscript{2}S-NSAIDs have been made:

ATB-429 is a derivative of mesalamine a drug commonly used in the treatment of inflammatory bowel disease (IBD). ATB-429 has been shown to be significantly more effective than mesalamine in animal models of Crohn's disease (CD) and ulcerative colitis (UC). Moreover, there is also considerable preclinical evidence that it is significantly more effective in treating the visceral pain associated with IBD\textsuperscript{371, 372}.

ATB-346 is a derivative of naproxen, widely used in the control of pain in osteoarthritis. However, like other NSAIDS,
naproxen carries a significant risk of serious gastrointestinal bleeding and cardiovascular effects (e.g., heart attacks, elevated blood pressure). In preclinical studies, ATB-346 exhibited increased effectiveness over its parent drug and a remarkable reduction in the gastrointestinal and cardiovascular toxicity\textsuperscript{373}. In addition, ATB-346 has been shown to have chemopreventive effect in colon cancer\textsuperscript{374}.

![Chemical structure of ATB-346]

Others are ATB-343 (derivative of indomethacin) and ATB-345. All these H\textsubscript{2}S -NSAIDs, they are metabolized by carboxylesterases in the body to slowly generate H\textsubscript{2}S.
**AIM**

Melanoma is the deadliest form of skin cancer and it is rising in incidence. Although in the past 3 years have been made notable advances in the understanding the molecular pathogenesis of melanoma and its treatment, melanoma therapy is still a challenge. Therefore, it is critical to identify other important potential targets in melanoma development and progression that are amenable to pharmacological inhibition.

In the last few years, numerous physiological and pathophysiologic effects have been proposed for the gasotransmitter hydrogen sulphide, even its role in cancer molecular mechanisms is still unclear. Recently, it has been shown that CSE enzyme is expressed in melanoma cell lines\textsuperscript{375} and that CBS enzyme plays a key role in colon and ovarian cancer\textsuperscript{314-315}.

Starting from these evidence, the aim of my PhD project was to investigate the role of the metabolic H\textsubscript{2}S pathway in human melanoma.

To address this issue we:

(i) evaluated the role of H\textsubscript{2}S on human melanoma cell proliferation and explained its possible mechanism of action;
(ii) evaluated the role of the endogenous pathway of H$_2$S in melanoma development and progression;

(iii) defined the clinical relevance of the H$_2$S donors in order to propose them as new therapeutic agents.
METHODS

3.1 Cell culture and reagents

NHEM was purchased from Lonza (Walkersville, MD, USA) and grown in melanocyte growth medium 2 (Lonza). The melanoma cell lines B16/F10, Sk-Mel-5 and Sk-Mel-28 were purchased from IRCCS AOU San Martino – IST (Genua, Italy), A375 from Sigma–Aldrich (Milan, Italy). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 μmol/l nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1 mmol/l sodium pyruvate (all from Sigma–Aldrich). Cells were grown at 37°C in a humidified incubator under 5% CO₂. The cell line PES 43 was isolated from a lung metastases of a patient from the National Cancer Institute, G. Pascale Foundation (Scala et al., 2006), and cultured in Iscove’s modified Dulbecco’s medium (Cambrex Bioscience, Verviers, Belgium) supplemented with heat-inactivated 10% fetal bovine serum, penicillin and streptomycin (100 units/ml each).

DATS (LKT Laboratories) was diluted in DMSO to produce a stock solution of 10 mM for in vitro experiments; GYY4137 (Cayman Chemical, Ann Arbor, MI, USA) was solubilized in PBS; PAG, CHH, 3-MP and NaHS (Sigma–Aldrich) were solubilized in H₂O; thioglycine and L-
thiovaline, a kind gift of Prof. Andreas Papapetropoulos, were solubilized in PBS. Vemurafenib (PLX4032) (Selleckchem) was solubilized in DMSO.

3.2 Proliferation assays

Cell proliferation was measured by the 3-[4,5-dimethyl]2,5-diphenyl tetrazolium bromide (MTT) assay or by the 5-bromo-2'-deoxy-uridine assay following the manufacturer’s instructions (Roche, Milan, Italy). Briefly, for the MTT assay the human melanoma cells (A375, Sk-Mel-5, Sk-Mel-28 and PES 43) and the NHEM cells were seeded onto 96-well plates (2x10³ cells/well) a day earlier and successively treated with vehicle or various concentrations of different H₂S-releasing compounds for 24, 48 and 72 h before adding 25 µl MTT (Sigma–Aldrich; 5 mg/ml in saline). Cells were then incubated for an additional 3 h at 37°C. After this time interval, cells were lysed and dark blue crystals were solubilized with a solution containing 50% N,N-dimethyl formamide and 20% sodium dodecylsulfate with an adjusted pH of 4.5. The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340), equipped with a 620-nm filter. None of the compounds tested under the same experimental conditions had a cytotoxic effect on NHEM.
3.3 Preparation of cellular extracts

A375 cells (1×10⁶) were seeded in 6-well plates and were treated with DATS 100 μM for 1, 3 and 6 h or with GYY4137 1 mM for 3, 6 and 24 h. To obtain citosolic or nuclear extracts, the cell pellet was resuspended in 100 μL of ice-cold hypotonic lysis buffer (10 mmol/L Hepes, 10 mmol/L KCl, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mmol/L benzamidine, 0.5 mmol/L dithiothreitol) and incubated on ice for 15 minutes. The cells were lysed by rapid passage through a syringe needle 5 times and centrifuged for 10 minutes at 13 000g. The supernatant containing the cytosolic fraction was removed and stored at −80°C. The nuclear pellet was resuspended in 30 μL of high-salt extraction buffer (20 mmol/L Hepes pH 7.9, 10 mmol/L NaCl, 0.2 mmol/L EDTA, 25% v/v glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mmol/L benzamidine, 0.5 mmol/L dithiothreitol) and incubated at 4°C for 30 minutes with constant agitation. The nuclear extract was then centrifuged for 10 minutes at 6000g, and the supernatant was aliquoted and stored at −80°C.
Whole-cell extracts of A375 cells were prepared after lysis in extraction buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]/HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail tablets, Roche). The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy).

3.4 Western blot analysis

Equal amounts of protein (40 µg/sample) from whole or nuclear cell extracts were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDSPAGE) and blotted onto a nitrocellulose membranes (Trans-Blot Turbo Transfer Starter System, Bio-Rad). The membranes were blocked for 2 h in 5% low-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature. Then the filters were incubated with the following primary antibodies: IκBα (sc-1643 Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:200); Bcl-2 (2876, Cell Signaling, Beverly, MA, USA; diluted 1:1000); caspase 3 (9662, Cell Signaling; diluted 1:1000); PARP (9542, Cell Signaling; diluted
1:1000); p44/42 MAPK (Erk1/2) (9102, Cell Signaling; diluted 1:1000); phosho-p44/42 Erk MAPK (Erk1/2, Thr202/Tyr204) XP (4370, Cell Signaling; diluted 1:2000); AKT (9272, Cell Signaling; diluted 1:1000); phosho-AKT (Ser473) XP (4060, Cell Signaling; diluted 1:2000); c-FLIP (06-864, Millipore; diluted 1 μg/ml); XIAP (R&D Systems, Minneapolis, MN, USA; 1 lg/ml); NF-κB p65 (F-6) (sc-8008 Santa Cruz Biotechnology; diluted 1:200); b-actin (Santa Cruz Biotechnology; diluted 1:1000) overnight at 4°C. The membranes were washed three times with PBST and then incubated with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology; diluted 1:2000) for 2 h at room temperature. The immune complexes were visualized by the ECL chemiluminescence method and acquired by the Image Quant 400 system (GE Healthcare).

3.5 Flow cytometry

Apoptosis was detected with an annexin V-FITC (BD Pharmingen, San Diego, CA, USA) according to manufacturer’s instructions. A375 cells were seeded in 35-mm culture dishes and allowed to attach overnight. The cells were treated with DATS (100 μM) for 3, 6, 24 and 48 h, collected, and washed twice with PBS. Samples were then taken to determine baseline and drug-induced apoptosis by Annexin V-FITC/propidium iodide (PI) (Beckman Coulter,
Brea, CA, USA) double staining or PI staining and flow cytometry analysis using a FACSCanto II 6-color flow cytometer (Becton Biosciences, San Jose, CA, USA). To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in annexin V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 10⁶ cells/ml. Subsequently, 5 µl of FITC-conjugated Annexin V and 5 µl of PI were added to 100 µl of the cell suspension (10⁵ cells). The cells were incubated for 15 min at room temperature in the dark. Finally, 400 µl of annexin V-binding buffer was added to each tube. A minimum of 50 000 events for each sample were collected and data were analyzed using FACSDIVA software (Becton Biosciences).

3.6 Cell cycle

Cells were plated in six-well plates at a density of 5x10⁵ cells/well. Cells were then treated with DATS 100 µM for 3, 6 and 24 h. Cells were collected, washed twice with ice-cold PBS buffer (pH 7.2–7.4), fixed with 70% alcohol overnight and stained with PI (1 mg/ml) in the presence of RNAse A (1 mg/ml) for at least 30 min prior to analysis by flow cytometry. The flow-cytometric analysis was performed using a FACSCanto II and the cycle analysis was performed with the MODFIT LT software (Verity Software House).
3.7 Electrophoretic mobility shift assay (EMSA)

Aliquots of total extracts (12 µg protein/sample) in 0.1% Triton X-100 lysis buffer were incubated with $^{32}$P-labeled κB DNA probes in binding buffer for 30 min. DNA-protein complexes were analyzed using non-denaturing 4% polyacrylamide gel electrophoresis. Quantitative evaluation of NF-κB-κB complex formation was done using a Typhoon-8600 imager (Molecular Dynamics Phosphor-Imager, MDP, Amersham Biosciences, Piscataway, NJ, USA) and IMAGEQUANT software (Amersham Biosciences) (MDP analysis). For control of equal loading, NF-κB values were normalized to the level of the nonspecific protein-DNA complex in the same lane.

3.8 RNA purification and qPCR

Total RNA was isolated from cells using the TRI-Reagent (Sigma–Aldrich, Milan, Italy) according to the manufacturer’s instructions, followed by spectrophotometric quantization. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥1.7. Isolated mRNA was reversetranscribed by iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Milan, Italy). qPCR was carried out in CFX384 real-time PCR detection system (Bio-
Rad) with specific primers using SYBR Green master mix kit (Bio-Rad). Samples were amplified simultaneously in triplicate in a one-assay run with a non-template control blank for each primer pair to control for contamination or primer-dimers formation, and the ct value for each experimental group was determined. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the ct values, using the $2^{-\Delta\text{Ct}}$ formula.

3.9 Transfection

A375 cells were seeded onto 96-well plates ($2 \times 10^3$ cell/well) and transfected the next day with the human CSE/pIRES-EGFP, the human CBS cDNA/pCMV-SPORT6 or the human 3-MST/pCI-HA using Lipofectamine 2000 (Life Technologies, Milan, Italy). Forty-eight hours after transfection, cell proliferation was evaluated by MTT assay (see Proliferation assays). Western blot analysis of CSE, CBS and 3-MST expression was carried out on whole cell extracts to confirm effective gene overexpression (anti-CSE, clone 2E12-1C10, mouse, 1:500, Novus Biologicals, Ltd; anti-CBS, clone H-300, rabbit, 1:500, Santa Cruz Biotechnology); anti-3-MST (clone NBP1-54734, rabbit 1:500, Novus Biologicals). For the silencing experiments, A375 cells were seeded onto 96-well plates ($2 \times 10^3$ cell/well) and transfected the next day, according to the manufacturer’s instructions, with CSE
Silencer® Select Pre-designed siRNA (Ambion, Carlsbad, CA, USA) (sense strand 5’-CUAUGUAUUCUGCAACAAt-3’, antisense strand, 5’-UUUGUUGCAGAAUACAUAGaa-3’); human 3-MST siRNA SMART pool siGENOME (Thermo-Fisher Scientific, Hudson, NH, USA) (GGAGAAGAGCCCUGAGGAG, CCGCCUUAUCAAGACCUA, CCACCCACGUGCUGGAUCUA, AGAAAGUGGACCUCUUA); human CBS siRNA SMART pool siGENOME (GGAAGAAGUUCGGCCUGAA, GGACGGUGGUGCAAGUG, CACCACCUGUGAGAUC, AGACGGAGACAGACACCUA). Forty-eight hours after transfection, cell proliferation was evaluated by MTT assay (see Proliferation assays).

### 3.10 Generation of Vemurafenib-resistant human melanoma cells

To generate Vemurafenib-resistant human melanoma subline, exponentially growing cell A375 were treated with increasing concentrations of vemurafenib\textsuperscript{145}. After attaining confluence, the cells were repassaged and retreated with vemurafenib. Every three passages, the vemurafenib sensitivity was ridetermine (see Proliferation assays), and the process was repeated with higher dose of vemurafenib until to obtain vemurafenib-resistant clone cells. Cells with the ability to grow in 1μM of vemurafenib were obtained ~6 months after the initial drug exposure. Resistant lines were
maintained in culture medium in presence of 1μM vemurafenib, supplemented every 72h. Then, A375 vemurafenib-resistant (A375rVem) cell were treated with DATS at different concentrations (10μM - 30μM - 100 μM) for 24, 48 and 72 h and cell proliferation was evaluated by MTT assay (see Proliferation assays).

3.11 Animals

Animal care was in accordance with Italian and European regulations on the protection of animals used for experimental and other scientific purposes. Mice were observed daily and humanely euthanized by CO₂ inhalation if a solitary subcutaneous tumor exceeded 1.5 cm in diameter or mice showed signs referable to metastic cancer. All efforts were made to minimize suffering. Female C57BL/6 mice (18-20 g) were from Charles River Laboratories, Inc. Mice were housed at the Animal Research Facility of the Department of Pharmacy of the University of Naples Federico II.

3.12 Induction of subcutaneous B16 lesions

Mice were subcutaneously (s.c.) injected in the right flank with B16-F10 cells (1x10⁵/0.1 ml). When tumors reached an average diameter of 2-4 mm, L-cysteine (300 or 600 mg/kg), DATS (50 mg/kg) or a combination of L-
cysteine (600 mg/kg), + PAG (10 mg/kg) or L-cysteine (600 mg/kg) + CHH (10 mg/kg) was given orally. Control mice received only vehicle. Tumor size was measured using a digital caliper, and tumor volume was calculated using the following equation: tumor volume = \( \frac{\pi}{6} (D_1 \times D_2 \times D_3) \)
where \( D_1 = \text{length}; D_2 = \text{width}; D_3 = \text{height} \) and expressed as cm\(^3\).

3.13 Induction of metastatic melanoma

Mice were intravenously (i.v.) injected with \( 5 \times 10^5 \) B16-F10 melanoma cells, in the caudal vein. Starting on the 1st day after tumor cell inoculation, DATS (50 mg/kg) or L-cysteine (600 mg/kg), was given orally every day for 14 days. Control mice received only vehicle. After 14 days the lungs were removed and was calculated the percentage of metastatic area using ImageJ software.

3.14 Patients and specimens

In all, 102 patients of the National Cancer Institute 'Giovanni Pascale' of Naples have been included in this study from 2004 to 2012. All patients were caucasians, and all gave their written informed consent according to the institutional regulations. This study was approved by the ethics committee of the National Cancer Institute 'G. Pascale'.

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3.15 Immunohistochemistry analysis

Tissue samples were retrieved from the paraffin blocks of the National Cancer Institute of Naples archives. Hematoxylin & eosin staining of a 4-μm section was used to verify all samples. Immunohistochemical analysis was performed on 4-μm sections from formalin-fixed, paraffin-embedded tissues in order to evaluate the expression of CSE and CBS. Negative control slides without primary antibody were included for each staining. Paraffin slides were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in 0.01 M citrate buffer (pH 6.0) for CSE antibody and 1 mM EDTA buffer, pH 8.0 for CBS and 3-MST antibodies, in a bath for 20 min at 97°C. After antigen retrieval, the slides were allowed to cool. The slides were rinsed with TBS and the endogenous peroxidase inactivated with 3% hydrogen peroxide. After protein block (BSA 5% in PBS 1X ), every slide was incubated with specific primary antibody: anti-CSE (clone 2E12-1C10, mouse, 1:1200, Novus Biologicals, Ltd, Cambridge, UK; anti CBS (clone H-300, rabbit, 1:500, Santa Cruz Biotechnology) anti 3-MST (clone NBP1-54734, rabbit 1:500, Novus Biologicals). The sections were rinsed in TBS and incubated for 20 min with Novocastra biotinylated secondary antibody (RE7103), a biotin-conjugated
secondary antibody formulation that recognizes mouse and rabbit immunoglobulins. Then the sections were rinsed in TBS and incubated for 20 min with Novocastra streptavidin-HRP (RE7104). Finally, peroxidase reactivity was visualized using 3,3’-diaminobenzidine (DAB) and the sections counterstained with hematoxylin and mounted. Results were interpreted using a light microscope. CSE, CBS and 3-MST expression was evaluated in stained tissue sections by two pathologists (G.B, A.A.) in a blinded manner. In each sample the number of positive cells was evaluated in 10 non-overlapping fields using 9400 magnification. Data are expressed as the percentage of positive cancer cells over the total number of cancer cells. The obtained median value was used as cut off for that specific marker (CSE, CBS, 3-MST). Therefore in order to be defined as negative, a sample has to be equal or below the median value.

3.16 Statistical analysis

Data from all in vivo experiments are reported as mean±SEM unless otherwise noted. Data were analyzed and presented using GRAPHPAD PRISM software (GraphPad). Significance was determined using Student’s 2-tailed t-test. Results were considered significant at P values less than 0.05 and are labeled with a single asterisk. In addition, P
values less than 0.01 and 0.001 are designated with double and triple asterisks, respectively.
4.1 Hydrogen sulfide donors inhibit human melanoma cell proliferation

To explain the role of H$_2$S in human melanoma, we assessed the effect of several H$_2$S-donors on A375 melanoma cell proliferation. All the compounds, but sodium hydrosulfide monohydrate (NaHS), inhibited the growth of A375 cells in a concentration-dependent manner (Table 4). Among all the H$_2$S donors tested, the most potent in inhibiting melanoma cell proliferation was DATS, with an IC$_{50}$ of 89 µM.

Similar results were obtained with all the other cell lines: Sk-Mel-5, Sk-Mel-28 and PES 43 whereas didn’t affect NHEM proliferation (Table 5). To better confirm that the anti-proliferative effect elicited by the H$_2$S-donors was not due to an artefact consequent to the hydrogen sulfide-induced reduction of mitochondrial respiration (MTT assay) we performed another proliferation assay, the 5-bromo-20-deoxy-uridine assay. Results obtained with this assay show a greater anti-proliferative effect of DATS (100 µM; –73% P < 0.001) and of GYY4137 (1 mM; –39%, P < 0.001; Figure 11). On the basis of the results obtained with both assays, DATS was selected for all subsequent experiments.

To further prove that the anti-proliferative effect of DATS was due to release of H$_2$S we had used haemoglobin, an H$_2$S
scavenger with whom it forms an inactive complex. Results obtained by MTT assay demonstrate a significantly reversion of anti-proliferative effect of DATS in the presence of haemoglobin (10 μM) (Figure 12).

Table 4. Effect of H₂S-donors on cellular proliferation.

Growth inhibition was measured using the MTT assay and is expressed as OD values at 48h. All the compounds used, but NaHS, inhibited the growth of A375 cells. Experiments were run in triplicate (*P<0.05; **P<0.01; ***P<0.001 vs CTL).
Table 5. Effect of H\textsubscript{2}S-donors on cellular proliferation.

Growth inhibition was measured using the MTT assay and is expressed as OD values at 48h. Both DATS and GYY4137 inhibited the growth of PES 43 (A), SkMel 5 (B) and SkMel 28 (C) cells. Experiments were run in triplicate (*P<0.05; **P<0.01; ***P<0.001 vs CTL).
Figure 11. Effect of hydrogen sulfide donors on A375 cell proliferation.

Cell proliferation was measured by using the 5-bromo-2’-deoxy uridine assay and it is expressed as OD values at 48h. Both DATS and GYY4137 inhibited the growth of A375 cells. Each experiment (n=3) were run in quadruplicate (***P<0.001 vs CTL).

Figure 12. Reversion of the anti-proliferative effect of DATS

Growth inhibition was measured using the MTT assay and is expressed as OD values at 72h. DATS inhibited the growth of A375 cells but in the presence of haemoglobin (10 µM) its anti-proliferative effect was significantly reversed. Experiments were run in triplicate (***P<0.001 vs CTL; °°° P<0.001 vs DATS).
4.2 Hydrogen sulfide donors cause cell cycle arrest and induce apoptosis of human melanoma

In order to clarify whether the antiproliferative effect exhibited by DATS was due to a necrotic or apoptotic process, A375 human melanoma cells were treated with DATS and apoptosis was determined by annexin V/propidium iodide (PI) staining, which detects the externalization of phosphatidylserine (PS). This dual staining distinguishes between unaffected cells (unlabeled; quadrant 3 Figure 13A), early apoptotic cells (annexin V-positive; quadrant 4, Figure 13A), late apoptotic cells (annexin V-positive, PI-positive; quadrant 2, Figure 13A), and necrotic (PI-positive; quadrant 1; Figure 13A). Treatment of A375 cells for 3, 6, 24 and 48 h with DATS (100 μM) resulted in a time-dependent induction of apoptosis. In particular, at 48 h almost all cells (93%) exhibited markers of late apoptosis (Figure 13B). This effect was accompanied by a time-dependent cleavage of caspase 3 and of its substrate poly(adenosine diphosphate-ribose) polymerase (PARP) (Figure 13C). In addition, DATS treatment of A375 cells induced time-dependent accumulation of G0/G1-phase populations (Figure 14). As expected, in DATS-treated cells, a reciprocal reduction of cell ratio in S and G2/M phases was also observed (Figure 14). Therefore, by donating hydrogen
sulfide, DATS induces apoptosis and cell cycle arrest of human melanoma cells.

**Figure 13. Induction of apoptosis by the H₂S-releasing donor DATS.**

(A) Cells were treated with DATS (100 μM) at different time points and apoptosis was determined by annexin V/propidium iodide (PI) staining. This dual staining distinguishes between unaffected cells (unlabeled; quadrant 3, Q3), early apoptotic cells (annexin V positive; quadrant 4, Q4), late apoptotic cells (annexin V positive, PI positive; quadrant 2, Q2), and necrotic (PI positive; quadrant 1, Q1). Treatment of A375 cells for 3-6-24-48 h with DATS (100 μM) resulted in a time-dependent induction of apoptosis. (B) Quantitative analysis of DATS-induced A375 apoptosis at various time points showing that at 48 h almost all cells (93%) exhibit markers of late apoptosis. (C) Western blot analysis of caspase 3 and PARP in A375 whole-cell lysates. A375 cells were incubated with DATS 100 μM for 1-3-6 hours and a time-dependent cleavage of caspase 3 and of its substrate PARP was observed. Actin was detected as loading control.
Figure 14. Inhibition of cell cycle progression of human melanoma cells by DATS.

DNA histogram shows the accumulation of G0/G1-phase cells induced by DATS in A375 cells and cell cycle arrest by the H2S-releasing donor DATS. Quantitative analysis of cell cycle distribution of A375 control-treated or DATS-treated at various time points. DATS treatment of A375 cells induced a time-dependent accumulation of G0/G1-phase populations (▬). As expected, in DATS treated cells, a reciprocal reduction of cell ratio in S (grey □) G2/M-phases (□) was also observed.
4.3 Hydrogen sulfide donors inhibit NF-κB activation and down-regulate NF-κB-dependent anti-apoptotic genes

Nuclear factor-κB (NF-κB) proteins are normally sequestered in the cytoplasm in an inactive form closely associated with the inhibitory protein inhibitor of kappa light chain gene enhancer in B cells-alpha (IκBα). Several reports have shown that in melanoma the constitutive activation of NF-κB confers tumor survival capacity and avoidance of apoptosis\textsuperscript{45}.

Western blot analysis carried out on the cytosolic extracts obtained from A375 cells treated with DATS 100 μM for 15, 30 and 60 min showed an inhibition of IκBα degradation at the earliest time points (Figure 15A). To investigate the effect of hydrogen sulfide on NF-κB activity, A375 cells were treated with DATS (100 μM) at different time points (1, 3 and 6 h; Figure 15B). The A375 cell line was found to display a constitutively high NF-κB DNA binding activity, as compared with NHEM, which was reduced in a time-dependent manner by DATS. In fact, treatment with DATS (100 μM) for 3 and 6 h caused a significant (P < 0.001) inhibition of NF-κB-DNA binding activity by 30 and 76%, respectively (Figure 15C). The major NF-κB band in A375 cells consisted of the p50 and p65 subunits. Following on, we assessed the expression of three anti-apoptotic proteins, X-
chromosome-linked inhibitor of apoptosis protein (XIAP), FLICE-inhibitory protein (c-FLIP) and B cell lymphoma gene-2 (Bcl-2), whose expression is modulated by the transcriptional activity of NF-κB. Western blot experiments showed that DATS markedly decreased the expression of all the anti-apoptotic genes considered (Figure 16), confirming NF-κB involvement.

**Figure 15. Melanoma cells constitutively express activated NF-κB to promote anti-apoptotic and pro-survival signaling.**

(A) Western blot analysis carried out on the cytosolic extracts obtained from A375 cells treated with DATS 100 μM for 15, 30, and 60 min shows an inhibition of IκBα degradation at the earliest time points. (B) Nuclear extracts from control-treated and DATS-treated A375 cells collected at 1-6 h were analyzed by EMSA for NF-κB activation. The A375 cell line displayed a constitutively high NF-κB DNA binding activity, as compared to NHEM, that was reduced in a time-dependent manner by DATS. These data are expressed as arbitrary units and shown in panel C, (**P<0.001 vs CTL).
Figure 16. Hydrogen sulfide donors down-regulate NF-κB-dependent anti-apoptotic genes

Western blot analysis and relative densitometry of c-FLIP, XIAP and Bcl-2 carried out on A375 cells treated with DATS 100 μM for 1-3-6 h. The hydrogen sulfide donor markedly decreased the expression of all the anti-apoptotic genes analyzed; ***P<0.001; **P<0.01; *P<0.05 vs CTL. Actin was detected as a loading control.
4.4 Expression of CSE, CBS and of 3-MST in human melanoma cell lines

In order to clarify the involvement of the endogenous pathway of H$_2$S in melanoma we performed a quantitative real-time PCR (qPCR) analysis of the expression levels of CSE, CBS and 3-MST genes in NHEM and in a panel of four distinct human melanoma cell lines (A375, Sk-Mel-5, Sk-Mel-28 and PES 43). As shown in Figure 17, the expression of the three genes was always very low in NHEM as opposed to melanoma cell lines, where CSE, CBS and 3-MST were differentially expressed. In particular CSE, but not CBS or 3-MST, was upregulated in all the cell lines considered, although the difference was not statistically significant (Figure 17).
mRNA expression levels of CSE, CBS and 3-MST in NHEM, A375, Sk-Mel-5, Sk-Mel-28 and PES 43 cells evaluated by qPCR analysis. The expression of the three genes was very low in NHEM. In melanoma cell lines CSE, CBS and 3-MST were differentially expressed. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the ct values. Each column is the mean ±S.E.M. of at least four independent determinations each performed in quadruplicate. ***P<0.001 vs NHEM
4.5 Overexpression of CSE in A375 cells modifies cell proliferation

On the basis of the results obtained and to better elucidate the role of CSE in melanoma cells proliferation, we chose, as a tool, the cell line with the lowest CSE expression levels, the A375 melanoma cells. The CSE, CBS and 3-MST genes were transiently overexpressed using an hCSE cDNA/pIRES2-EGFP, an hCBS cDNA/pCMVSPORT6 or a 3-MST/pCI-HA construct. Identical empty vectors lacking a cDNA insert were used as control. Transfection of the cells with CSE cDNA, CBS cDNA, 3-MST cDNA or with the empty vectors did not change the morphological characteristics of the cells. To determine whether the CSE overexpression affected the proliferation of human melanoma cell line, a MTT proliferation assay was carried out. Proliferation of the CSE-overexpressing cells was inhibited by about 30% (P < 0.001) as compared with control cells (Figure 18). Treatment with hemoglobin, a H₂S scavenger, reversed the anti-proliferative effect elicited by CSE overexpression, confirming the specificity of the effect. When A375 CSE-overexpressing cells were grown in the presence of L-cysteine (1 mM) the CSE substrate, the anti-proliferative effect was not significantly increased. CBS overexpression caused only a small inhibition (P < 0.05), which was not further increased when the cells were grown in the presence
of L-cysteine. 3-MST overexpression also slightly reduced cell proliferation and this effect was significantly increased (P < 0.05) in the presence of the enzyme substrate 3-mercaptopiruvate (3-MP) (10 µM) (Figure 18). These data show a major role for CSE in melanoma. We also performed silencing experiments on the A375 cell line. Cells were transiently transfected for 48 h with CSE Pre-designed siRNA, human CBS siRNA SMART pool or human 3-MST siRNA SMART pool. The negative control siRNA was used as internal control. As shown in Figure 19, silencing of the genes did not modify cell proliferation.
CSE over-expression inhibited proliferation of human melanoma cells, as demonstrated by the MTT proliferation assay *** P<0.001 vs Control (CTL). CBS over-expression only slightly (10%, *P<0.05 vs CTL) inhibited proliferation of human melanoma cells that was not further reduced by the addition of L-cysteine (1mM). 3-MST over-expression inhibited cell proliferation of about 7% that following the addition of the enzyme substrate 3-MP (10 µM) reached 15% (*P<0.05 vs CTL). Hemoglobin (10 µM) reversed the anti-proliferative effect induced by enzyme over-expression. Each column is the mean ±S.E.M. of 6 independent experiments each performed in quadruplicate.

CSE, CBS and 3-MST genes silencing did not modify proliferation of human melanoma cells, as demonstrated by the MTT proliferation assay. Each column is the mean ±S.E.M. of 3 independent experiments each performed in quadruplicate.
4.6 Hydrogen sulfide inhibits the growth of melanoma cutaneous in vivo in mice

To better elucidate the role of H$_2$S in melanoma development, we used a well-known murine model of melanoma, which is induced by subcutaneously injecting B16-F10 murine cells in C57BL/6 mice. L-Cysteine (300 and 600 mg/kg), the CSE substrate, was administered orally to mice. At day 14 after tumor implantation, a significantly, dose-dependent, reduction in tumor volume and weight was observed in L-cysteine-treated mice (0.240 ± 0.09 cm$^3$ mean tumor volume L-cys 300mg/kg vs control mice 0.365 ± 0.04 cm$^3$ mean tumor volume, $P < 0.05$ and 110 ± 9 mg mean tumor weight vs control mice 221 ± 30 mg mean tumor weight $P < 0.01$; 0.168 ± 0.03 cm$^3$ mean tumor volume L-cys 600mg/kg vs control mice 0.365 ± 0.04 cm$^3$ mean tumor volume, $P < 0.001$ and 65 ± 2 mg mean tumor weight vs control mice 221 ± 30 mg mean tumor weight, $P < 0.001$) (Figure 20). To verify the specificity of the effect of L-cysteine we used two selective inhibitors of the enzymes CBS and CSE, DL-propargilglicina (PAG) (CSE inhibitor) and the acid amminoossiacetico (CHH) (inhibitor of CBS), both at a dose of 10mg/kg. These inhibitors were administered alone or together with L-cysteine (600 mg/Kg).

As shown in Figure 21, the L-cysteine alone reduced by 51% the volume of the tumor mass (0.134 ± 0.03 cm$^3$ vs 0.348 ±
0.03 cm$^3$ control mice, $P < 0.001$) and 57% of the weight (220.3 ± 48.3 mg vs 532.8 ± 53.5 mg control mice, $P < 0.01$). This protective effect was significantly reduced by the PAG (0.320 ± 0.05 cm$^3$ vs 0.134 ± 0.03 cm$^3$ L-cysteine alone, $P < 0.001$; 497 ± 93.3 mg vs 220.3 ± 48.3 mg L-cysteine alone, $P < 0.01$). In contrast, CHH did not alter the effect of cysteine (0.211 ± 0.05 cm$^3$; 214.7 ± 32 mg). When administered alone both PAG and CHH didn’t significantly alter tumor growth (data not shown).

The tumor growth inhibition obtained by the endogenous-CSE-derived hydrogen sulfide was mimicked by the exogenous hydrogen sulfide delivered to mice following administration of DATS. In fact, DATS (50 mg/kg) significantly ($P < 0.001$) inhibited tumor growth by 67% (0.07 ± 0.001 cm$^3$ mean tumor volume; 88 ± 15.3 mg mean tumor weight) as compared with control mice (0.210 ± 0.004 cm$^3$ mean tumor volume; 358 ± 37.3 mg mean tumor weight)(Figure 22).
Figure 20. Hydrogen sulfide inhibits tumor growth in vivo.

L-cysteine (L-cys 300 mg/Kg, □) and (600 mg/Kg, ▲), were given orally to mice; control mice (○) received vehicle only. Tumor volume was monitored on the indicated days. The average tumor volume with standard error is plotted against the days after tumor implant. L-cysteine significantly reduced tumor volume in dose-dependent manner (*P< 0.05 vs control; ***P< 0.001 vs control, n=8; day 14). The average tumor weight with standard error shows that L-cysteine significantly reduced tumor weight in dose-dependent manner (**P< 0.01 vs control; ***P< 0.001 vs control, n=8; day 14).
Figure 21. Hydrogen sulfide inhibits tumor growth in vivo.

L-cysteine (L-cys 600 mg/Kg, ▲), L-cys+PAG (10 mg/Kg) (▼) or L-cys+CHH (10 mg/Kg) (♦) were given orally to mice; control mice (●) received vehicle only. Tumor volume was monitored on the indicated days. The average tumor volume with standard error is plotted against the days after tumor implant. L-cysteine significantly reduced tumor volume (**P< 0.01 vs control, n=8; day 14); the inhibitory effect of L-cysteine was abolished by PAG (***P<0.001 vs L-cys, n=8) but CHH did not alter the effect of cysteine (*P< 0.05 vs control vs control, n=8; day 14). The average tumor weight with standard error shows that L-cysteine significantly reduced tumor weight (**P< 0.01 vs control, n=8; day 14); the inhibitory effect of L-cysteine was abolished by PAG (*P<0.05 vs L-cys, n=8) but not CHH (**P< 0.05 vs control vs control, n=8; day 14). When administered alone both PAG and CHH didn’t significantly alter tumor growth (data not shown).
Figure 22. Hydrogen sulfide inhibits tumor growth in vivo.

The H₂S-donor DATS (50 mg/Kg, ▲) were given orally to mice; control mice (●) received vehicle only. Tumor volume was monitored on the indicated days. The average tumor volume with standard error is plotted against the days after tumor implant. DATS significantly inhibited tumor volume and tumor weight (***P< 0.001 vs control, n=8; day 14).
4.7 Effect of hydrogen sulfide donors on MAPK/ERK and PI3/AKT pathways

Two of the most frequently deregulated pathways in melanoma are mitogen-activated protein kinase (MAPK)/ERK and phosphoinositide 3-kinase (PI3K)/AKT. These two pathways play an important role in melanoma development and progression and are involved in the mechanism of resistance to targeted therapy. Therefore, we wanted to investigate whether H₂S was able to adjust these two important "pathways". Western blot experiments showed that DATS (100 μM) significantly reduced both the intensity of the band phospho-AKT and phospho-ERK in a time-dependent manner, indicating an inhibition of their activation (Figure 23). Thus, the proapoptotic effects of hydrogen sulfide on human melanoma involve a reduced activation of the AKT/p-AKT and ERK/p-ERK signaling pathways.
**Figure 23. Hydrogen sulfide donors down-regulate AKT and ERK pathways**

Western blot analysis and relative densitometry of phospho- and total AKT and ERK in A375 cells treated with DATS 100 μM for 1-3-6h. Both p-AKT and p-ERK band intensity was time-dependently reduced following treatment with DATS (100 μM); ***P<0.001; **P<0.01 vs CTL. Actin was detected as a loading control. Experiments (n=3) were run in triplicate.
4.8 Hydrogen sulfide inhibits A375rVem cell proliferation

To assess whether H$_2$S is efficacy also in conditions of resistance to therapy, we generated vemurafenib-resistant A375 (A375rVem) melanoma cells. A375 cells harbor the BRAF V600E mutation and are sensitive to vemurafenib treatment (100 nM). Drug-resistant A375 cells were obtained through treatment with increasing concentrations of vemurafenib (Figure 24).

Treatment with DATS inhibited significantly the growth of A375rVem cells in a concentration- and time- dependent manner (Figure 25).
Figure 24. Serial dose-response curve showing generation of acquired vemurafenib resistance in A375 cells.

Cells were cultured in increasing dose of vemurafenib (from 3nM to 1.3 μM) for several passages. Growth inhibition was measured using the MTT assay and is expressed as OD values at 72h. In parenteral cells A375 vemurafenib inhibited the growth significantly in concentration-dependent manner (**P<0.001 vs CTL). In the resulting vemurafenib-resistant cells vemurafenib didn’t inhibit cell growth.

Figure 25. Effect of hydrogen sulfide donors on A375rVem cell proliferation.

Growth inhibition was measured using the MTT assay and is expressed as OD values at 24h, 48h and 72h. DATS significantly inhibited the growth of A375rVem cells in concentration and time-dependent manner. Experiments were run in triplicate (**P<0.01; ***P<0.001 vs CTL)
4.9 Hydrogen sulfide inhibits metastatic melanoma in vivo in mice

To investigate the role of H$_2$S in melanoma progression, we performed a murine model of metastatic melanoma which is induced by injecting $5 \times 10^5$ cells B16/F-10 into the tail vein of mice C57BL/6. Through the circle cells reach the lungs, where they are visible as metastatic foci rich in melanin$^{377}$. The mice were treated with DATS (50 mg/kg) or with L-cysteine (1000 mg/kg) while control mice received only the vehicle, by orally administration. After 14 days the lungs were removed and was calculated the percentage of metastatic affected area, using the software Image J. Results show that treatment with L-cysteine reduced of about 65% the development of lung metastases compared to control mice (metastatic area: L-cys 4.4% vs control mice 70%). Similar effects are shown by DATS which reduced of about 67% the metastatic area (metastatic area: DATS 3.3% vs 70% control mice) (Figure 26).
Figure 26. Hydrogen sulfide reduces lung metastasis growth in vivo.

The H$_2$S-donor DATS (50 mg/Kg) and L-cysteine (1000 mg/Kg) were given orally to mice; control mice received vehicle only. L-cysteine reduced of about 65% the metastatic area; DATS reduced of about 67% the metastatic area (A) Gross examination of representative lungs (B) Graphical representation of the number of lung metastases in treated mice. Image J software was used for quantitative analyses.
### 4.10 CSE, CBS and 3-MST expression in human nevi

Expression of CSE, CBS and 3-MST was evaluated in six compound nevi, four junctional nevi and four dysplastic nevi. As shown in Table 6, all the dysplastic nevi (100%) were positive for CSE, negative for CBS and variable for 3-MST. Representative immunohistochemical-positive expressions of CSE, CBS and 3-MST in nevi and in melanomas, but not in lymph node metastases, are shown in Figure 27.

### 4.11 CSE, CBS and 3-MST expression in human primitive melanoma and metastases.

All specimens of primary melanoma analyzed by immunohistochemistry (n = 4) were positive for CSE expression (Table 7). CBS and 3-MST displayed a variable, but always very low, expression level in primary tumor (pT) (Table 7). A total of 72 melanoma metastases were analyzed. Metastases obtained were either lymph node metastases (n = 14) or tissue metastases (n = 58). Tissue metastases were distributed as follows: skin (22/58), lung (25/58), liver (6/58) and intestinal metastases (5/58). CSE-positive metastases were 39% (28/72); among these positive cases, 93% (26/28) were no lymph node metastases. CBS-positive metastases were 30% (22/72); among these positive cases
91% (20/22) were no lymph node metastases. 3-MST-positive metastases were 28% (20/72) none of which were lymph node metastases.

Figure 27. CSE, CBS and 3-MST immunohistochemical analysis.

Representative immunohistochemical positive expression of CSE, CBS and 3-MST in nevi (A, B, C; 40X); in melanomas (D, E, F; 40X); in no lymph nodes metastases (G, H, I; 40X).
### Table 6. CSE, CBS and 3-MST immunohistochemical analysis

Contingency table of CSE, CBS and 3-MST expression in nevi. In rows the frequencies are indicated in absolute values. The percentage is reported in brackets. Abbreviations: "pT" primary tumor; (-) no/low expression; (+) high expression.

<table>
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<tr>
<th>Nevi</th>
<th>CSE</th>
<th>CBS</th>
<th>3-MST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Compound</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Junction</td>
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<td>2 (50%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Dysplastic</td>
<td>0</td>
<td>4 (100%)</td>
<td>4 (100%)</td>
</tr>
</tbody>
</table>

### Table 7. CSE, CBS and 3-MST immunohistochemical analysis

Contingency table of CSE, CBS and 3-MST expression in primitive melanoma and metastases. All specimens were analyzed by immunohistochemistry. The frequencies are indicated in rows in absolute values. The percentage is reported in brackets. 'pT' primary tumor; (-) no/low expression; (+) high expression.

<table>
<thead>
<tr>
<th>Metastasis sites</th>
<th>CSE</th>
<th>CBS</th>
<th>3-MST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>4 (100%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>4 (100%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>4 (100%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4 (100%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Metastasis sites</td>
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<td>32 (55.2%)</td>
<td>26 (44.8%)</td>
<td>38 (65.5%)</td>
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<tr>
<td>Lymph Node</td>
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<td>2 (14.3%)</td>
<td>12 (85.7%)</td>
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</tbody>
</table>
DISCUSSION

Melanoma, the deadliest form of skin cancer, is among the most aggressive and treatment-resistant human cancers. The incidence of malignant melanoma is rising faster, indeed only from 1980 to 2009 the incidence has increased fivefold. Each year, more than 132,000 patients are diagnosed with this type of cancer. It is becoming clear that melanoma constitutes a heterogeneous group of tumors with different patterns of oncogenic mutation, overexpression and genomic amplification. The mutation rate of melanoma is higher than other aggressive tumors, probably due to the involvement of ultraviolet (UV) radiation in the genesis of superficial cutaneous melanoma. The most frequent driver mutations in melanoma involve genes as BRAF, NRAS, KIT, GNAQ, and GNA11. Constitutive upregulation of the MAPK pathway by a BRAFV600 mutation occurs in about 50% of melanomas. This leads to increased oncogenic properties such as tumor cell invasion, metastatic potential, and resistance to apoptosis.

Multiple cellular pathways have been implicated in melanomagenesis, ranging from signal transduction to developmental and transcriptional pathways and cell cycle.
The interactions between the various signalling molecules involved in melanoma progression are best viewed as a series of links between a number of interconnected nodes.

If melanoma is diagnosed at an early stage surgical excision is curative in approximately 99% of patients. However, later stages have poor prognosis due to lack of responsiveness to traditional chemotherapeutics. Over the last 30 years, no drug or combination of drugs have demonstrated significant impact to improve patient survival for long time. From 1995 to 2000, the use of cytokines such as interferon and interleukin became treatment options. In 2011, new drugs were approved by the U.S. Food and Drug Administration, including peginterferon alfa-2β for patients with stage III disease, vemurafenib for patients with metastatic melanoma with the BRAF V600E mutation, the MEK1/2 inhibitor trametinib and the monoclonal antibody ipilimumab, directed to the CTLA-4 T lymphocyte receptor. Other trials for new additional agents and combinations targeting the MAPK, PI3K/AKT/mTOR (PI3K), c-kit, are currently in progress. Individualized targeted therapy has showed response rates, in terms of overall survival, that have never been described in melanoma before. However, early development of resistance is the major drawback of this therapy. It is known that about 50% of patients treated with BRAF or MEK inhibitors have disease progression within 6–
7 months after the initiation of treatment due mostly to reactivation of the MAPK pathway. So, metastatic melanoma is generally still incurable. Therefore, it is critical to identify other important potential targets in melanoma development and progression that are amenable to pharmacological inhibition.

In the last few years, several physiological and pathophysiological roles have been proposed for the gasotransmitter hydrogen sulfide, along with a plethora of cellular and molecular targets\textsuperscript{222}. CSE produces H\textsubscript{2}S from L-cysteine alone in the presence of pyridoxal 5'-phosphate, whereas CBS generates H\textsubscript{2}S by condensation of L-cysteine and other thiols, such as L-homocysteine. Sequential reaction by the PLP-dependent enzyme, CAT, and a PLP-independent enzyme, 3-MST, has also been proposed as a potential H\textsubscript{2}S-producing pathway. CAT forms 3-mercaptopyruvate from L-cysteine and \(\alpha\)-ketoglutarate, and 3-MST subsequently catalyzes the formation of H\textsubscript{2}S from 3-mercaptopyruvate in the presence of reducing agents.

A number of studies have investigated the role of H\textsubscript{2}S in inducing cell death and evidence has been presented that this gas can exert both pro- and anti-apoptotic activity in cultured cells\textsuperscript{250,379}. It has been shown previously that CSE is expressed in melanoma cell lines\textsuperscript{375} but the role of the hydrogen sulfide pathway has never been investigated.
Conversely, a role for this pathway has been proposed in colon and ovarian cancer, where it is mainly driven by CBS\textsuperscript{314-315}.

The aim of my PhD project was to evaluate the role of the metabolic H$_2$S pathway in human melanoma.

To address this issue we:

(iv) evaluated the role of H$_2$S on human melanoma cell proliferation and explained its possible mechanism of action;

(v) evaluated the role of the endogenous pathway of H$_2$S in melanoma development and progression;

(vi) showed the clinical relevance of the H$_2$S donors in order to propose them as new therapeutic agents.

Controversy exists on the role of H$_2$S on cancer cell proliferation because of the complexity of DNA damage and repair. The activation of the H$_2$S pathway has been shown to be able to exert both pro- and anti-apoptotic activity in cultured cells\textsuperscript{250; 379}. These studies have been conducted on immortalized cell lines mainly using NaHS as the exogenous source of hydrogen sulfide. The use of NaHS as donor has two main drawbacks: (i) the fast kinetics of hydrogen sulphide release and (ii) the high concentrations used, often in the mM range. These two issues suggest that discrepancies (e.g. pro- and anti-apoptotic effect) may lie in the choice of the hydrogen sulfide donors used. Sulfide salts
such as NaHS and sodium sulfide (Na$_2$S) on addition of water generate a burst of hydrogen sulfide within a short period as opposed to the organic donors$^{352}$. Since cell culture takes place over a period of hours or days, it is likely that little, if any, hydrogen sulphide is present in medium within a short time of adding either NaHS or Na$_2$S. It is therefore necessary to perform repeated challenge or to use high (mM) doses. In a recent comparative study showing differences between fast (NaHS) and slow-release (GYY4137) H$_2$S donors, it has been demonstrated that only GYY4137 caused a concentration-dependent killing of several human cancer cell lines$^{352}$. Therefore, in our study we used both fast (NaHS) and slow releasers (DATS, GYY4137, thioglycine and L-thiovaline). All the compounds used, except NaHS, although with different IC50, inhibited cellular proliferation. The most active among the H$_2$S-donors tested was DATS and it was selected for the molecular studies since its hydrogen sulfide-delivering property had been well characterized$^{236}$. Cytofluorimetric studies demonstrated that the anti-proliferative effect of DATS was due to its ability to induce apoptosis and cell cycle arrest in G0/G1-phase. Caspase-3 is the main ‘effector’ caspase in the apoptotic pathway, which acts through cleavage of its well-known substrate PARP. The finding that DATS induces caspase 3 activation further confirmed that H$_2$S triggers apoptosis. Many
chemotherapeutic agents cause cell-cycle arrest but not apoptosis and this is one of the mechanisms leading to chemoresistance. In fact, growth arrest allows cells to repair their DNA and limits the efficacy of chemotherapy. Thus, by overriding tumor resistance to apoptosis, agents such as hydrogen sulphide donors are of great potential value. The apoptotic machinery can be controlled, at least in part, by NF-κB, which regulates transcription of the Bcl-2 family members. Constitutive activation of NF-κB has been described in melanoma. The activation of NF-κB promotes the multiple steps in melanoma progression such as transformation, initiation, promotion, angiogenesis, invasion and enhanced metastatic potential of malignant melanoma cells. Moreover, NF-κB activated would be responsible of resistance of cancer cells to chemotherapeutic agents.

Here we show that hydrogen sulphide donors inhibit IkBα degradation and this effect was associated to the inhibition of NF-κB nuclear translocation and activation. The issue of whether exogenous hydrogen sulfide inhibits or activates NF-κB signaling pathway has been a matter of debate. Indeed, in the relevant literature diametrically opposite views exist on the roles of this gas in NF-κB activation. However, the different experimental settings (in vitro versus in vivo) coupled to the different stimuli as well as to the protocols used do not allow a clear conclusion to be
drawn. It has been shown that the slow-releasing hydrogen sulfide donor derivatives of diclofenac and aspirin also inhibit NF-κB activation. In addition, it has recently been shown that hydrogen sulfide inhibits NF-κB activation in ox LDL-induced macrophage inflammation through both sulfhydration of free thiol group on cysteine 38 in p65 subunit and inhibition of IκBα degradation. In our study and experimental settings we found that hydrogen sulfide inhibits IκBα degradation, leading to inhibition of NF-κB nuclear translocation.

Thus our results are in line with the inhibitory effect of hydrogen sulfide on NF-κB activation. The fact that NF-κB is inhibited by H₂S is also supported by the finding that treatment with hydrogen sulfide donors greatly decreased the expression of the anti-apoptotic proteins c-FLIP, XIAP and Bcl-2. Indeed, these proteins are known to be transcriptionally regulated by NF-κB. To evaluate the involvement of the hydrogen sulfide pathway in melanoma, we analysed the expression of the enzymes involved in H₂S synthesis, CBS, CSE and 3-MST, in several melanoma cell lines, widely used in the relevant literature. All the melanoma cells selected expressed CSE, CBS and 3-MST differently. Whereas CSE expression was higher in all melanoma cells analysed as compared with NHEM, CBS expression was enhanced in A375 cells only, and
3-MST showed a variable pattern of expression among all cell lines.

Thus, to obtain further insights into the role of these enzymes in melanoma we perform a molecular modulation study selecting as a tool the A375 cell line. Overexpression of CSE in A375 inhibited cellular proliferation (50%). Treatment with hemoglobin, a hydrogen sulfide scavenger, reversed the anti-proliferative effect elicited by CSE overexpression, confirming the specificity of the effect. CBS overexpression caused only a small inhibition of cell proliferation (about 10%) that was not further increased by the addition of L-cysteine. The same magnitude of effect, about 8%, was observed following 3-MST overexpression. Addition of 3-MP, the enzyme substrate, increased the anti-proliferative effect up to 15%. Therefore, these results underline the primary role of CSE versus CBS and 3-MST. On the other hand, the silencing of all three enzymes did not modify cellular proliferation, implying that reduction of the basal endogenous level of H$_2$S production does not affect cell survival, or a compensatory mechanism may intervene.

To support these results, we developed a translational in vivo pre-clinical approach. For this purpose we used an animal model that recapitulates human cutaneous melanoma progression, the spontaneous C57BL/6-derived B16 melanoma cell line$^{376}$ because it is well-established and
is a widely used tumor model. We demonstrated that L-cysteine, the natural substrate of CSE, significantly reduced tumor volume and this effect was completely abolished by PAG, the selective CSE inhibitor, but not CHH, the CBS inhibitor. To further support our in vitro findings we also used DATS in vivo. DATS significantly inhibited tumor volume development proving that the tumor growth inhibition obtained by the endogenous-CSE-derived hydrogen sulfide was mimicked by the exogenous hydrogen sulfide delivered to mice following administration of DATS. To better define the mechanism by which this critical anti-tumor effect is achieved, we investigated on the possible involvement of the MAPK/ERK and the PI3K/AKT pathways. These pathways are the two most frequently deregulated pathways in melanoma and are also involved in primary and secondary resistance to BRAF inhibitors. We have shown that DATS inhibited the phosphorylation-activation of AKT and ERK. Our results are in agreement with other reports showing that exogenous hydrogen sulfide downregulates both AKT and MAPK pathways. Starting from evidence that MAPK and PI3K/AKT pathways are involved in melanoma progression (abnormal proliferation, angiogenesis, invasion and metastasis) and resistance to therapy with BRAF inhibitor, we have
investigated the role of H$_2$S in these conditions mimicking these clinical situations.

Acquired resistance to vemurafenib is a major factor limiting successful, long term targeted therapy for patient with malignant melanoma\textsuperscript{142}. For this purpose we have generated vemurafenib-resistant A375 melanoma cells, harbor the BRAFV600E mutation, and we have demonstrate that DATS is able to inhibit their proliferation. Therefore, DATS overcomes resistance to vemurafenib \textit{in vitro}.

Otherwise, we have performed a murine model of metastatic melanoma demonstrating that both L-cysteine and DATS, significantly, inhibited lung metastasis formation.

Finally to give another clinical relevance to this study and to obtain further insights into the role of the hydrogen sulphide pathway in melanoma we analysed the expression of CSE, CBS and 3-MST in human tissue samples. The immunohistochemical analysis performed on more than 100 human samples demonstrated that CSE expression increased from nevi to primary melanoma, decreased in tissue metastases and was absent in lymph node metastases. Conversely, analysis of CBS expression revealed that this enzyme was absent in dysplastic nevi. Positive CBS expression was found in only 25\% of the primary melanomas analyzed. Therefore, as opposed to other types of cancer\textsuperscript{314,315} CBS does not appear to play an important
role in human melanoma. 3-MST expression was always extremely variable in the human specimens analyzed (from nevi to metastasis). The finding that CSE expression is higher in primary melanoma than in distant metastatic melanoma suggests the involvement of this pathway in the progression of melanoma. These data support our hypothesis that CSE derived hydrogen sulfide plays a major role in melanoma. Our results are in line with a previous study showing that overexpression of CSE in HEK-293 results in the inhibition of cellular proliferation and DNA synthesis\textsuperscript{195}.

Our study demonstrates that in A375 cells, CSE but not CBS overexpression induces spontaneous apoptosis and exogenous hydrogen sulfide induces apoptosis. This apoptotic effect involves: (i) the suppression of pro-survival pathways associated to NF-κB transcription activity as demonstrated by the decreased expression of c-FLIP, XIAP and Bcl-2 and (ii) the inhibition of the activation of AKT and ERK1/2 downstream signalling pathways.

Moreover, hydrogen sulphide inhibiting simultaneously both the MAPK and PI3K/AKT pathways, is effective in the treatment of both cutaneous and metastatic melanoma \textit{in vivo} and in inhibiting growth of vemurafenib melanoma resistant cells.

All these data confirm the involvement of hydrogen sulfide in melanoma progression.
In conclusion we have demonstrated, thanks also to the evidence acquired with human specimens, the involvement of the L-cysteine/CSE/hydrogen sulfide pathway in human melanoma. Our study provides proof-of-principle toward establishing this pathway as a diagnostic tool or a potential therapeutic target in human melanoma.


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