A Thesis for the Doctor of Philosophy

A novel anticancer coumarin derivative triggers metabolic disruption by targeting STAT3 in KRAS mutant lung cancer cells

천연물 유래 Coumarin 유도체의 STAT3 세포신호전달 조절 및 대사 교란을 통한 KRAS 변이 비소세포성 폐암 표적 항암 기전

February, 2018

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A novel anticancer coumarin derivative triggers metabolic disruption by targeting STAT3 in KRAS mutant lung cancer cells

By

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ABSTRACT

Coumarins are natural compounds that exert antioxidant, anti-inflammatory, and anti-cancer activities and are known to modulate inflammatory pathways. Non-toxic biscoumarin OT52 strongly inhibited the proliferation of non-small cell lung cancer cells with KRAS mutations and stem-like characteristics through the reduction of aldehyde dehydrogenase expression, and abrogated spheroid formation capacity. These cytostatic effects were characterized by cell cycle arrest and the onset of senescence, which was concomitant with endoplasmic reticulum and Golgi stress, and led to metabolic alteration. Mechanistically, this cellular response was associated with the novel capacity of biscoumarin OT52 to inhibit STAT3 transactivation and the expression of its target genes linked to proliferation. These results were validated by the computational docking of OT52 to the STAT3 DNA-binding domain. The combination treatments of OT52 with subtoxic concentrations of Bcl-xL and Mcl-1-targeting BH3 protein inhibitors triggered synergistic immunogenic cell death, which was validated in vitro in colony formation assays and in vivo by zebrafish xenografts.

Keywords: KRAS mutation; non-small cell lung cancer; coumarin; STAT3; BH3 mimetics; immunogenic cell death

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LIST OF ABBREVIATIONS

NSCLN  Non-small cell lung cancer
TKIs   Tyrosine kinases inhibitors
EGFR   Epidermal growth factor receptor
EML4-ALK Echinoderm microtubule-associated protein like 4 anaplastic lymphoma kinase
NCI    National Cancer Institute
CCD    Coiled-coil domain
DBD    DNA binding domain
NH2    Amino-terminated domain
SH2    Src homology 2
TAD    Transcriptional activation domain
STAT   Signal transducer and activator of transcription
IFN    Interferon
SHP    Src homology region 2 domain-containing phosphatase
PP     Protein phosphatase
SOCS   Suppressor of cytokine signaling
PIAS   Protein inhibitor of activated STAT
ER     Endoplasmic reticulum
UPR    Unfolded protein response
PERK   Protein kinase-like ER kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRE</td>
<td>Inositol-requiring enzyme</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>CHOP</td>
<td>c/EBP homologous protein</td>
</tr>
<tr>
<td>GRP</td>
<td>Glucose-regulated protein</td>
</tr>
<tr>
<td>BIP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>ICD</td>
<td>Immunogenic cell death</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelocytic leukemia</td>
</tr>
<tr>
<td>Bcr-Abl</td>
<td>Break point cluster–Abelson</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EsSOCS</td>
<td><em>Eriocheir sinensis</em> suppressors of cytokine signaling</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JfGPH</td>
<td>Japanese flounder glycoprotein 130 homologue</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor protein 53</td>
</tr>
<tr>
<td>RbSTAT4</td>
<td>Single transducer and activator of transcription 4 family identified from rock bream</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SD</td>
<td>Sarcodiol</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressors of cytokine signaling 3</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WSSV</td>
<td>White spot syndrome virus</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma virus</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
</tbody>
</table>
Chapter I

Literature review
1. Cancer

1.1 Overall characteristics of cancers

From a traditional perspective, cancer arises from a loss of cellular control. In normal tissue, when new cells start to grow, old cells start to die to maintain proper homeostasis. However, in cancer, this balance is disrupted, which results in uncontrolled cellular growth and the loss of apoptotic programmed cell death functions. Moreover, normal cells grow in a monolayer and experience contact inhibition; however, cancer cells grow in multiple layers, piled up on each other, with the loss of contact inhibition.

In 2000, Hanahan and Weinberg defined the hallmarks of cancer, in which they organized the complex relationships in cancer biology into six characteristics: self-sufficiency in proliferating signals, unlimited replicative potential, evasion of programmed cell death, insensitivity to growth suppression, angiogenesis, and tissue invasion and metastasis [1].

In 2011, they updated their previous study to be more relevant to current cancer biology by the inclusion of four more characteristics: tumor-promoting inflammation, metabolic functional reprogramming, genome instability and mutation, and the deregulation of immunogenic functions [2].

1.2 Non-small cell lung cancer and targeted therapies

In 2017, non-small cell lung cancer (NSCLC) remains the leading cause of cancer mortality worldwide, accounting for more than one million deaths each year [3].
Despite the large number of anti-cancer drugs developed during the last decade, the median survival rate in patients with NSCLC is still disappointing. Approximately 85% of the total number of diagnosed lung cancers are NSCLC, including approximately 60% of adenocarcinomas, 25–30% of squamous cell carcinomas, and 10–15% of large cell carcinomas [4]. In clinical applications, these histological characterizations are very useful for the selection of therapeutic strategies [5]. Moreover, improvements in the specific classification of NSCLCs have led to the identification of the genetic backgrounds, which has led to significant development in targeted drugs and knowledge of the clinical implications for chemotherapeutic sensitivity in various tumors; this information has provided the second-generation paradigm for anti-cancer drug development.

Genetic mutations have been identified in the adenocarcinomas, which are the most common type of NSCLC: the predominant mutations are found in KRAS (25%) and epidermal growth factor receptor (EGFR; 23%), in addition to other unknown or minor mutations. These oncogenic mutations may phosphorylate tumorigenic intracellular signaling, including the RAS, RAF, ERK, AKT, and STAT3 signaling pathways, which are extensively linked to carcinogenic functions such as tumor formation, angiogenesis, and metastasis.

Genetic discovery has promoted the targeted drug development, clinical evaluation, and achieved FDA approval protein of tyrosine kinases inhibitors (TKIs), which target EGFR mutations and echinoderm microtubule-associated protein like 4 anaplastic lymphoma kinase (EML4-ALK) translocation [6]. In addition, target drugs to EGRF mutations, erlotinib and gefitinib, are currently
considered as the best therapeutic options for patients with NSCLC. However, EGFR and KRAS mutations are mutually exclusive and EGFR-TKIs drugs are resistant to patients with NSCLC harboring KRAS mutations. Unlike the targeting of EGFR to TKIs, there is lack of information on drugs targeting the KRAS mutation, even though the presence of KRAS mutations in NSCLC was first discovered more than 20 years ago.
2. Natural compound and drug development

2.1 Natural compounds and anti-cancer agents

According to the classical pharmacology statements by Pedanius Dioscorides in 78 A.D., *De Materia Medica*, western people in ancient times used thousands of natural substances as medicinal herbs; the medicinal materials demonstrated in this pharmacopeia are still very useful in modern drug industries and therapeutic applications [7]. Over the last 40 years, naturally derived materials have remained the mainstay of chemotherapies for various diseases [8].

These natural materials provide numerous distinctive structural forms and are crucial as basic templates for the development of drugs that exert specific biological properties. Indeed, most of these research areas have been a combination of biology, pharmacology, and chemistry. These combination studies have led to the creation of novel bioactive naturally derived products and enabled great progress in sensitive techniques for the purification and characterization of the therapeutic capabilities of these bioactive molecules. In 1960, as a follow-up program, the National Cancer Institute (NCI) commenced a very large-scale screening project of anti-cancer targets [9, 10]. As a result, thirty-five thousand herb samples were screened in cancer cell lines; *Taxus brevifolia*-derived Taxol, which was developed in this project from 1960 to 1982, has been approved by the FDA as an anti-cancer agent to ovarian cancer, breast cancer, lung cancer, and gastric adenocarcinoma [11-13].

Although numerous scientists and clinicians are involved in the attempts to
overcome cancer, the mortality rate of the disease is still high and no drugs provide the perfect therapeutic treatment. However, drug development industries have recently been highly motivated towards the development of naturally-derived therapeutic compounds. In the statistical data gathered by the WHO, over 80% of the global population depends on plant-derived herbal medicines for their healthcare, especially in developing countries [14]. In the global pharmacologic research industry, over 50% of market of the anti-cancer drug market is derived from natural resources and their secondary synthetic metabolites, including plant, insect, and marine sources [15, 16]. Among these, almost 60% of naturally derived anti-cancer drugs have been approved for therapeutic applications [17, 18]. Fruits, vegetables, and herbs are basic resources, which have been developed as anti-oxidant and anti-aging therapies in healthcare research for many years, owing to their various constituent vitamins, fibers, phenolics, and carotenoids. These are not only present in food, but therapeutic drugs developed many years ago, such as penicillin, aspirin, and cyclosporine, which are derived from natural plant chemicals and soil organisms. Thus, these types of study are still of great interest and are highly promising in the present day [7, 19].

2.2 Naturally derived-coumarins and their therapeutic functions

Amongst the numerous structures with potential therapeutic activity, coumarin/benzopyran-2-one-based compounds have attracted much attention for their medicinal effects. The pyran-2-one ring is a characteristic of several
biologically active components that are widespread in natural or synthetic drugs. Coumarins contain an outstanding structural diversity of natural products, with more than one thousand derivatives isolated from over 800 plant species at present. For example, Citrus plants such as bergamot orange (*Citrus bergamia* Risso) are known to contain elevated levels of bergamottin [20]. Coumarins are widely used in pharmacological research owing to their potential therapeutic activities including antiseptic, antiviral, anti-coagulation, and anti-inflammatory effects [20-23]. Importantly, the biscoumarin derivative used here shares the 4-hydroxy-2H-chromen-2-one motif that is found in vitamin K antagonists such as the anticoagulant warfarin. Another biscoumarin, dicoumarol, originates from various plants and fungi and has also been described as anticoagulant drug [24]. In 1964, Michaels reported that anticoagulants, including bishydroxycoumarin, ethyl biscoumacetate, or acenocoumarol, led to a reduced incidence of cancer and mortality in patients undergoing anticoagulant therapy for thrombo-embolic disease. Previous investigations demonstrated the anti-cancer effects of the compound on pancreatic cells via the inhibition of NADPH quinone oxidoreductase-1. At present, no information regarding the ability of coumarin and its derivatives to disrupt metabolic homeostasis in KRAS-mutant lung cancer has been reported [25, 26].
2.3 Marine derived-compounds and their therapeutic potential

Marine-derived natural products offer numerous advantages to the anti-cancer drug industry. Although most of the earth’s surface is covered by oceans, there are still huge unexplored marine resources for therapeutic development. The oceans account for 70% of the earth’s surface, with 95% of oceans extending to more than a thousand meters deep; this depth offers huge possibilities for the development of bioactive materials [16, 27-29]. Over many years, a great diversity, abundance, and distribution of marine life has been discovered, with approximately 250,000 known species. It is speculated that the number of marine organisms may reach at least one million globally, with hundreds of millions of microbial organisms predicted. Many previous studies have elucidated that ocean is an unprecedented region of biological diversity. These various organisms in deep oceans endure extreme conditions, with a lack of oxygen and light, and high pressures; consequently, they have development physiological and biological adaptations necessary for survival. These adaptations cause modifications to the metabolic pathways and genetic regulation, which are proposed to of high value to human beings. According to many researchers, approximately 70% of deep-sea microorganisms, gorgonians, and sea sponges possess anti-cancer activity [30].

Furthermore, there are many FDA-approved drugs (for example, the leukemia drug, cytarabine (Ara-C), eribulin mesylate (E389), which affect metastatic breast cancer, and herpes simplex virus medicine Viabine (Ara-A)) derived from sponges. Additionally, omega-3-acid ethyl ester from fish affects hypertriglyceridemia and brentuximab vedotin (SGN-35) from
mollusks/cyanobacteria affects anaplastic large T-cell systemic malignant lymphoma and Hodgkin’s disease [31].

In the 1950s, several marine drug compounds derived from *Cryptotethya crypta* (a Caribbean sponge) were demonstrated to have anti-viral effects; subsequently, based on these structures, Ara-C (cytosine arabinoside) was developed in 1959 and is currently an FDA-approved drug [32]. Since then, marine derived natural chemical compound research and industries have made extensive progress. Sarcodictyins and eleutherobin were first isolated from marine coral in 1987 and 1994, respectively. These were the first attempts to isolate compounds from marine coral and the extracts showed anti-cancer activities that were 50 times more efficient than Taxol.

In 1985, NCI commenced a new project in which compounds from marine-derived microorganisms, plants, and animals were evaluated in sixty species of human cell lines, including lung, skin, ovary, breast, brain, prostate, kidney, and colon cancer, and leukemia [33]. In 2014, marine-derived natural compound researchers and drug development have increased in prominence. According to Mayer’s statement to Midwestern University, seven marine-derived compounds have FDA approval as pharmaceutical drugs, two compounds are in phase III trials, which is the final step of clinical pipeline, five compounds are in phase II, three compounds are in phase I/II, and 13 compounds are in phase I; furthermore, the investigations span a wide range of conditions, including cancer, Hodgkin’s disease, and chronic pain.
<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Disease model</th>
<th>Cell lines</th>
<th>Effect observed</th>
<th>STAT protein</th>
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<tr>
<td>Cytarabine (Ara-C)</td>
<td>Leukemia</td>
<td>K562</td>
<td>Inhibition of STAT5 tyrosine phosphorylation</td>
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<td>DMXBA (GTS-21)</td>
<td>Preadipocyte</td>
<td>3T3-L1</td>
<td>Reduction ASP-mediated chemokine MCP-1 secretion</td>
<td>STAT3</td>
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<tr>
<td>Bryostatin 1</td>
<td>Blood cancer</td>
<td></td>
<td>Induction of tyrosine phosphorylation, DNA binding of STAT1</td>
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<td>(15S,25S,3E,7E,11E)</td>
<td>Colon cancer</td>
<td>HT-29</td>
<td>Disruption of mitochondrial membrane potential, ROS generation, Cell cycle arrest, De-phosphorylation of STAT3</td>
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</tr>
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<td>Cembratetraen-17,2-olide (LS-1)</td>
<td>Colon cancer</td>
<td>HT-29</td>
<td>Disruption of mitochondrial membrane potential, ROS generation, Cell cycle arrest, De-phosphorylation of STAT3</td>
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<tr>
<td>Sarcodiol (SD)</td>
<td>Skin cancer</td>
<td>B16F10</td>
<td>Inhibition of de novo DNA Synthesis, Induction of DNA fragmentation, Inhibition of STAT3</td>
<td>STAT3</td>
</tr>
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<td>Apratoxin A, Breast</td>
<td>Osteosarcoma, Breast cancer</td>
<td>U2OS, MCF7</td>
<td>Inhibition of STAT3 tyrosine phosphorylation, Gp130 degradation</td>
<td>STAT3</td>
</tr>
<tr>
<td>GV-c9, and GV-c10</td>
<td>Macrophage</td>
<td>Raw 264.7</td>
<td>Inhibition of inflammatory markers (IL-6, TNF-alpha, and nitric oxide)</td>
<td>STAT1</td>
</tr>
</tbody>
</table>

**Table 1.** Overview of the effects of marine natural compounds on STAT family proteins.
3. Signal transducer and activator of transcriptions (STATs)

3.1 Structure of STATs

Sequence comparison and crystallographic analysis have identified several conserved functional domains in the STAT family of proteins. Structurally, STATs have well-conserved functional domains, including a coiled-coil domain (CCD), a DNA binding domain (DBD), an amino-terminated domain (NH$_2$), a linker, and SH2 phospho-tyrosine activation domain; each has specific function. The DBD defines the specificity of DNA binding and regulates distinguishable signal to specific ligands. The Src homology 2 (SH2) domain is a well-conserved domain that plays a crucial role in the binding capacity of phospho-tyrosine specific motifs, as well as the mediation of interactions between STAT and JAK proteins, STATs and receptors, and STAT and STAT proteins. The carboxyl terminus transcriptional activation domain (TAD) is quite divergent in the sequence and size among different STATs. This transcriptional activation domain plays a role in the modulation of the transcriptional activity and the functional characteristics of STAT proteins [34, 35].

Transcriptional activity and their functional roles are required for STAT dimerization through phosphorylation of a particular residue, which promotes the formation of phosphorylated complexes. Among several residues, tyrosine 705 is the predominant one for STAT phosphorylation, DNA binding activity, nuclear translocation, and leads to the induction of cytokine-responsive genes. The tyrosine residue represents a tyrosine-specific phosphorylation site approximately
700 residues in the c-terminal domain from the n-terminus, which controls the DNA binding activity of STAT proteins. This phosphorylation is regulated by STAT dimerization through a reciprocal combination with the SH2 domain [36, 37].

STAT proteins were initially discovered as interferon (IFN)-regulated genes in the early 1990s. The STAT family comprises seven structurally distinguishable members in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [38]. These molecules are cytoplasmic transcription factors, for cytokine, hormone, and growth factor signal transduction. STAT proteins also function as downstream effectors, able to modulate various biological cellular processes including fetal development, organogenesis, apoptosis, growth, differentiation, immune activity, and inflammation.

3.2 Overview of STAT3 signaling pathway and cancer

Signal transducer and activator of transcription 3 (STAT3) is a critical mediator of functional responses and specificity in cytokine signaling. At the receptor complex, STAT3 proteins become phosphorylated on a conserved tyrosine 705 residue, which induces their dimerization, nuclear translocation, and DNA binding, and leads to the induction of cytokine-responsive genes. Moreover, STAT3 is regulated by EGFR signaling, which is overexpressed in approximately 40–80% of patients with NSCLC [39]. STAT3 can be activated by non-receptor tyrosine kinases, such as Src and c-Abl, and is negatively regulated by
phosphatases including Src homology region 2 domain-containing phosphatase (SHP)1 or protein phosphatase (PP)2A. In healthy cells, activated STAT3 is quickly dephosphorylated by phosphatases. However, in cancer, STAT3 remains constitutively activated. Hence, therapies that directly or indirectly target STAT3 could provide novel anti-cancer therapeutic approaches. Furthermore, negative regulators of STAT3, which include suppressor of cytokine signaling (SOCS) 3 and protein inhibitor of activated STAT3 (PIAS3), could also be potential molecules through which STAT3 can be targeted.
Figure 1. Schematic model for JAK/STAT cell signaling pathway and the inhibition by JAK/STAT targeting anticancer drugs.

4.1 Endoplasmic reticulum (ER) and Golgi stress

The drug-mediated disruption of cancer cell homeostasis leads to cell cycle arrest, cell death or senescence, and endoplasmic reticulum (ER) stress. The ER plays a key role in cellular signaling, such as stress response, lipid synthesis, calcium homeostasis, and post-translational modifications, by conferring proper folding to functional proteins. Under oxidative stress or DNA damage, the cells accumulate misfolded or unfolded proteins and trigger the ER stress response or the unfolded protein response (UPR) signaling to prevent the pathological accumulation of misfolded proteins in the ER lumen [40, 41]. ER stress stimulates three signaling pathways through the ER transmembrane sensors, including protein kinase-like ER kinase (PERK), Ser/Thr protein kinase/endoribonuclease inositol-requiring enzyme (IRE) 1, and activating transcription factor (ATF) 6. Under ER stress, activated PERK phosphorylates eukaryotic initiation factor (eIF) 2a, which subsequently activates c/EBP homologous protein (CHOP) to induce ER stress-mediated apoptosis [42]. Moreover, ATF6 is also one of the major ER-stress transmembrane transcription factors that activate 78 kDa glucose-regulated protein (GRP78/binding immunoglobulin protein (BIP)). ATF6 and GRP78/BIP complexes are dissociated in response to ER stress, and the loss of GRP78/BIP binding correlates with cleaved ATF6 translocation to the Golgi apparatus, which eventually stimulates CHOP and ER chaperone proteins, including GRP78/BIP. Moreover, ER stress is directly linked to immunogenic cell death (ICD), which is characterized by calreticulin translocation and HMGB1 release that lead to
immune modulation and the activation of macrophages and dendritic cells [42-44].

4.2. Cellular senescence and senolytic effects

Previously, cellular senescence has been defined as the process of cell cycle arrest experienced by cells after the intrinsically programmed number of divisions determined by telomerase activity in vitro [45]. However, it is more recently described through extrinsic mechanisms, such as in vitro culture environment in normal tissue, which leads to premature induction with an unclear senescence phenotype; it has also become one of the major targets for anti-cancer research [46].

Senescence is associate with the self-defense mechanism against cellular stress, which forces cells out of the control of cell cycle regulation and their entrance into irreversible replicative arrest. This systematic mechanism is irreversible and cells cannot divide any longer, despite sufficient growth conditions. This may usually occur either by telomere-dependent senescence (telomere shortening replicative senescence) or by telomere-dependent senescence (DAN damage, ER, and ROS stress, stress-induced senescence) [45-47]. Both senescence states are characterized by the following: an enlarged cell morphology and distinctive flattening; an increase in senescence-associated β-galactosidase activity; irreversible growth arrest; and overexpression of cell cycle inhibitors.

In cancer, pro-survival senescence cells were predominantly characterized by the
induction of replicative arrest, resistance to apoptosis, and pro-inflammatory pathways [48]. For this reason, many cancer researchers have focused on anti-cancer agents that promote the characteristics of stress-induced senescence through the induction of multiple cellular stresses. Moreover, anti-apoptotic cells and mice showed drug sensitivity as a result of the stress-induced senescence, cell organelle dysfunction, and non-apoptotic induction of cell death. Moreover, senescence acts as a natural barrier against cancer promotion through evolution, as cells that harbor mutations in tumor promoting functions are decreased [49, 50].

Several agents have been proven as senescence inducing anti-cancer drugs, which makes them susceptible to the pro-apoptotic microenvironment. In 2015, the first senolytic agents were developed to selectively eradicate cell death-resistant senescence cells through the stimulation of the pro-apoptotic pathways. Although several senolytic agents are in clinical trials, the potential beneficial effects, including lower pro-apoptotic levels and sub-toxic concentrations for senescence induction, are requirements for cytostatic characteristics [51, 52]. Our cytostatic hydroxyl bis-coumarin derivative OT52 is an interesting compound for senolytic drug design.
5. Zebrafish as a promising animal model for anti-cancer drug discovery

Drug discovery has recently begun to adopt highly technologic approaches. Experimental approaches, such as whole genomic analysis, microarrays, computational assays, and robotics, have enhanced the rapid design of robust drug screening methods. Moreover, high throughput drug screening has generated a random screening library for a huge number of chemical candidates to selective targets in various disease models [53, 54].

Nevertheless, the process of drug discovery has slowed; after drug screening, animal validation study are essential to reveal drug solubility, absorbance, toxicity, and metabolic stability prior to clinical trial. The biggest issue is failure of animal studies, owing to the stricter ethical criteria and social welfare required for animal models. Previously, mammals such as mice were considered the most relevant animal model for drug screening and are representative animal models for research. However, mammals are limited for large-scale drug screening because of cost, logistical difficulties, and technical support challenges. For these reasons, smaller size animal models are proposed for new large-scale drug screening tools and have demonstrated high fecundity and cost-effectiveness for drug development. In addition, small animal validation would provide some clues to the next step in the validation in a larger mammalian model [53, 55].

In the early 1980s, *Danio rerio*, the zebrafish was established as an animal model for genetic evaluation and biological research. However, it has recently been considered as a target for anti-cancer drug screening and has been utilized in many
cancer studies. Moreover, zebrafish embryos are widely used to investigate the cell behavior of human-derived cancer xenotransplant models, such as angiogenesis and metastasis. These embryo models are most useful for drug screening as they reveal the permeability of small molecule drug compounds. The morphologic abnormalities and toxicities observed between human and Zebrafish models are very similar. Moreover, many researchers have used a human cancer cell xenograft model for the in vivo validation of tumorigenic parameters focused on angiogenesis and migration. The anti-cancer drug efficacy for angiogenesis and migration is easily evaluated owing to their transparent body and early development of blood vessels. In addition, the cost of a zebrafish facility is less than 1% of that required for mouse maintenance over a one-week experimental period. Thus, zebrafish may provide an optimal vertebrate animal model for drug screening with high cost- and time-efficiency. Furthermore, zebrafish can be grown in microplates, which minimizes the required drug quantities. In addition, the blood circulation and heart pulsation are visible under a microscope, which enables simple evaluation of cardiotoxicity [54, 56].
References


Chapter II

Cytostatic hydroxycoumarin OT52 induces ER/Golgi stress and STAT3 inhibition triggering non-canonical cell death and synergy with BH3 mimetics in lung cancer
1. Abstract

Coumarins are natural compounds with antioxidant, anti-inflammatory and anti-cancer potential known to modulate inflammatory pathways. Here, non-toxic biscoumarin OT52 strongly inhibited proliferation of non-small cell lung cancer cells with KRAS mutations, inhibited stem-like characteristics by reducing aldehyde dehydrogenase expression and abrogated spheroid formation capacity. This cytostatic effect was characterized by cell cycle arrest and onset of senescence concomitant with endoplasmic reticulum and Golgi stress, leading to metabolic alterations. Mechanistically, this cellular response was associated with the novel capacity of biscoumarin OT52 to inhibit STAT3 transactivation and expression of its target genes linked to proliferation. These results were validated by computational docking of OT52 to the STAT3 DNA-binding domain. Combination treatments of OT52 with subtoxic concentrations of Bcl-xL and Mcl-1-targeting BH3 protein inhibitors triggered synergistic immunogenic cell death validated in colony formation assays as well as in vivo by zebrafish xenografts.
2. Introduction

Lung cancer is the leading cause of cancer-related death with non-small cell lung cancer (NSCLC) being the most abundant form, accounting for more than 80% of all cases [57]. Recently, the paradigm of chemotherapeutic research shifted from cytotoxic chemotherapy to more targeted treatments. However, NSCLC patients harboring a Kirsten rat sarcoma (KRAS) mutation present resistance against such EGFR TKI drugs. Unlike EGFR targeting therapies, there is no chemotherapeutic strategy against KRAS mutations discovered in NSCLC more than 20 years ago [58].

KRAS target gene Signal transducer and activator of transcription (STAT) 3 is a critical transcriptional regulator of cell proliferation, differentiation, cell death, and immune function [59]. STAT3 is constitutively activated in various tumors, it is constitutively activated in over 50% of lung cancer, and is considered as a novel molecular target for cancer therapy [60]. STAT3 and its activator Janus activated kinase (JAK) are controlled by cytokines including interleukin (IL)-6, EGF or interferon (IFN) [38, 61]. Moreover, STAT3 is regulated by EGFR signaling which is overexpressed in about 40 to 80% of NSCLC patients [39]. Hence, a therapy targeting directly or indirectly STAT3 could provide novel anticancer therapeutic approaches. Furthermore, negative regulators of STAT3 including suppressor of cytokine signaling (SOCS) 3 and protein inhibitor of activated STAT3 (PIAS3) could also contribute to target STAT3.

Drug-mediated disruption of cancer cell homeostasis leads to cell cycle arrest, cell
death or senescence as well as endoplasmic reticulum (ER) stress. The ER plays a key role in cellular signaling such as stress response, lipid synthesis, calcium homeostasis, and post-translational modifications by providing proper folding to functional proteins. Under oxidative stress or DNA damage, cells exceeding protein folding capacity accumulate misfolded or unfolded proteins and trigger ER stress response or unfolded protein response (UPR) signaling preventing pathological accumulation of misfolded proteins in the ER lumen [40, 41]. Moreover, ER stress is directly linked to immunogenic cell death (ICD) which is characterized by calreticulin translocation and HMGB1 release leading to immune modulation and activation of macrophages and dendritic cells.

Natural compounds are an essential source of molecular scaffolds required for the development of anticancer drugs [19, 59, 62, 63]. Coumarins are widely used in pharmacological research due to their potential therapeutic activities including antiseptic, antiviral, anti-coagulation, and anti-inflammatory effects [20-23]. Importantly, the biscoumarin derivative used here shares the 4-hydroxy-2H-chromen-2-one motif, also characterizing vitamin K antagonists like warfarin, an anticoagulant [64]. Another biscoumarin, dicoumarol originated from different plants and fungi [25] and was also described as anticoagulant drug. Interesting, Michaels published in 1964 that anticoagulants including bishydroxycoumarin, ethyl biscoumacetate, or acenocoumarol led to a reduced cancer incidence and mortality in patients undergoing anticoagulant therapy for thrombo-embolic disease [26].

We elucidated here the molecular mechanisms and in vivo potential of the bis (4-
hydroxycoumarin) derivative 3, 3’-[3-(2-hydroxy-4-methoxyphenyl)-3-oxopropane-1, 1-diyl] bis (4-hydroxy-2H-chromen-2-one), OT52 triggering cell cycle arrest and senescence, and consecutive multiple cellular stress mechanisms including ER and Golgi stress, accompanied by metabolic alterations. We also identified that OT52 inhibited anchorage-independent cellular growth. Furthermore, cytostatic compound OT52 sensitized lung cancer cells against inhibitors selective for Bcl-xL/Mcl-1 to induce non-canonical cell death pathways. Altogether our results provide first pre-clinical in vitro and in vivo anti-cancer effects of coumarin derivative OT52 in KRAS mutant non-small cell lung cancer.
3. Materials and methods

3.1 Chemistry

The reaction of 4-hydroxycoumarin 1 with ω-formyl-4’-methoxy-2’-hydroxyacetophenone 2 catalyzed by 4-pyrrolidinopyridine (4-PPy) in refluxing chloroform, has led to the desired 3,3’-[3-(2-hydroxy-4-methoxyphenyl)-3-oxopropane-1,1-diyl]bis(4-hydroxycoumarin) OT52 in 76% yield.

3.2 Cell culture and treatment

The non-small cell lung cancer cell lines A549 (American Type Culture Collection, ATCC, Manassas, VA, USA), H460, and H1650 (SNU Korea Cell Bank, Seoul, Korea) were cultured in RPMI medium (Lonza, Basel, Switzerland) according to standard procedures. Pharmacological inhibitors or inducers used in the manuscript are detailed in Supplementary Table 1.

3.3 Analysis of glycolysis and mitochondrial stress

The alteration in the extracellular acidification rate (ECAR) was measured in response to the sequential injection of glucose, oligomycin (H+-ATP-synthase inhibitor), and 2-deoxy-D-glucose (2DG) (hexokinase inhibitor) to detect non-glycolytic acidification, glycolysis, maximal glycolytic capacity, and glycolytic reserve using a Seahorse XF24 with a glycolysis flux analyzer kit (Agilent Technologies, Santa Clara, California).
For mitochondrial stress analysis, we evaluated the oxygen consumption rate (OCR) as OXPHOS measurement. Sequential injection of oligomycin, FCCP, rotenone and antimycin (mitochondrial complex I and III inhibitors) allowed to quantify basal respiration, proton leak, maximal respiration, non-mitochondrial respiration, ATP production, and spare respiratory capacity using a Seahorse XF24 with a mitochondrial stress analysis kit (Agilent Technologies, Santa Clara, California).

3.4 In situ analysis of endoplasmic reticulum and Golgi

Cellular localization of ER and Golgi were visualized by ER/Golgi Cytopainter (Abcam, Cambridge, MA, USA) by confocal microscopy. Images of the ER and Golgi were observed using a fluorescence filter of the excitation/emission wavelength of 480/534 nm after staining with the Cytopainter Golgi/ER staining kit (Abcam, Cambridge, MA, USA) according to the manufacturer’s protocol and analyzed with a confocal microscope (Carl Zeiss, Oberkochen, Germany).

3.5 STAT3 TransAM

A549 cells were seeded at 15,000 cells/cm². After 24 h, cells were treated with OT52 for 24 h followed by activation with IL6 (50 ng/mL) for 10 min. Subsequently, nuclear proteins were extracted from the cells according to
manufacturer's protocol (Active Motif, Nuclear extract kit, Carlsbad, CA, USA). Nuclear protein extracts were used for TransAM assays, which were conducted according to the manufacturer’s instruction (Active Motif, TransAM STAT3, Carlsbad, CA, USA). The luminescent signal was measured with a Luminometer (Berthold Technologies, Bad Wildbad, Germany).

3.6 Cell proliferation and viability assays

Cell proliferation and viability were measured by using the trypan blue exclusion assay (Lonza, Basel, Switzerland). The number of cells per ml was counted, and the fraction of trypan blue-positive cells was estimated by using a Malassez cell counting chamber (Marienfeld, Lauda-Königshofen, Germany).

3.7 Cell cycle distribution

For cell cycle analysis, cells were collected and fixed in 70% ethanol. DNA was stained with a propidium iodide (PI) solution (1 μg/ml, Sigma-Aldrich, St. Luis, Missouri, USA) in 1x PBS, supplemented with RNase A (100 μg/ml; Roche, Basel, Switzerland). Samples were analyzed by flow cytometry using a FACS Calibur™ system, Becton Dickinson (BD) Biosciences (San Jose, CA, USA). Data were recorded statistically (20,000 events/sample) using the Cell Quest software (BD Biosciences) and analyzed by Flow-Jo 8.8.5 software (Tree Star, Inc., Ashland, OR, USA).

3.8 Senescence-associated β-galactosidase (SA-β-gal) staining
A549 cells were plated in 9.6 cm\(^2\) plates and treated with indicated concentrations of OT52 for three days. SA-β-gal staining was performed according to the manufacturer's instructions (Cell Signaling Technology).

### 3.9 Morphological analysis

A549 cells were washed in 1x PBS, fixed and stained with the Diff-Quik stain kit (Dade Behring S.A., Brussels, Belgium) according to the manufacturer's procedure. Images were acquired using a microscope (Nikon, Tokyo, Japan). 3D surface plot graph was generated by Interactive 3D surface plot plugin using the Image J 1.8.0 software.

### 3.10 Transmission electron microscopy (TEM)

Cells were pelleted and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, U.S.A) diluted in 0.1 M sodium cacodylate buffer, pH 7.2 (Electron Microscopy Sciences, U.S.A) overnight. Cells were then rinsed with sodium cacodylate buffer twice and postfixed in 2% osmium tetroxide for 2 h at room temperature. Samples were washed with distilled water and then stained with 0.5% uranyl acetate at 4°C overnight. After 24 h, samples were dehydrated through a graded series of ethanol solutions to water followed by propylene oxide, and then infiltrated in 1:1 propylene oxide/Spurr’s resin. Samples were kept overnight embedded in Spurr’s resin, mounted in molds and left to polymerize in an oven at 56 °C for 48h. Ultrathin sections (70–90 nm) were obtained with ultramicrotome, EM UC7 (Leica, Germany). Sections were stained with uranyl acetate and lead
citrate and subsequently examined with a JEM1010 transmission electron microscope (JEOL, Japan).

3.11 Spheroid formation assays
For the ultra-low adherence method, 10,000 cells were seeded in 100 µl per well at indicated concentrations of OT52 in 96-well round bottom microplates (Corning, NY, USA). Cells were incubated at 37 °C and 5% of CO₂ for three days to form spheroids. For the hanging drop method, cells were suspended in growth media at the concentration of 5,000 cells/20 µl. Drops of prepared cell solution (20 µl/ drop) were then patterned on a lid of a 60-mm tissue culture plate and incubated for 7 days to form spheroids.

3.12 Clonogenic assays
For colony formation assays, cells (10⁴ cells/ml) were grown in semi-solid methylcellulose medium (Methocult H4230, StemCell Technologies Inc., Vancouver, Canada) supplemented with indicated OT52 concentrations. Colonies were detected after 10 days of culture by adding 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reagent (Sigma) and were scored by Image J 1.8.0 software (U.S. National Institute of Health, Bethesda, MD, USA).

3.13 Immunofluorescence
After blocking in PBST (0.1% Triton X-100 in PBS) containing 3% bovine serum
albumin (BSA, Bovogen, Keilor East, Victoria, Australia) overnight at 4°C and washing in PBST (2 × 15 min), spheroids were incubated with primary antibodies diluted in PBST on a gently rocking rotator at 4°C for 24 h and rinsed in PBST (4 × 30 min). Spheroids were then incubated with Alexa Fluor-conjugated secondary antibodies for 24 h and analyzed with a confocal microscope (Carl Zeiss, Oberkochen, Germany).

3.14 ROS quantification
Intracellular ROS levels were measured using 2′,7′-dichlorofluorescein diacetate (H$_2$DCFDA; Invitrogen) by fluorescence microscope (Nikon, Tokyo, Japan). Hydrogen peroxide (H$_2$O$_2$; 100 μM; Sigma-Aldrich) was used as a control. The relative H$_2$DCFDA fluorescence intensity (corrected for background fluorescence) was measured using the Image J 1.8.0 software (U.S. National Institute of Health, Bethesda, MD, USA).

3.15 Protein extraction and western blotting
Whole cell extracts were prepared using M-PER® (Thermofisher, Waltham, Massachusetts, USA) supplemented with 1x protease inhibitor cocktail (Complete EDTA-free, Roche, Basel, Switzerland) according to the manufacturer's instructions. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Little Chalfont, UK). Membranes were incubated with selected primary antibodies (Supplementary Table 2). Chemo-luminescence signal was detected with the ECL Plus Western Blotting Detection System (GE
Healthcare, Little Chalfont, UK) and quantified by ImageQuant LAS 4000 mini system (GE Healthcare). Results are represented as histograms corresponding to the mean ± SD of the quantification of three different blots using samples from three independent treatments and reported to the control of each blot.

### 3.16 Luciferase reporter gene assay

STAT3 Luciferase Reporter Hela stable cell line (Signosis, Santa Clara, CA, USA) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (GE Healthcare, Hyclone) supplemented with 10% (v/v) fetal calf serum (Biowest, Nuaille, France) and 1% (v/v) antibiotic–antimycotic (Lonza, Basel, Switzerland) at 37 °C and 5% CO₂. Cells were treated with OT52 at indicated concentrations for 24 h, followed by IL-6 activation (50 ng/mL) for 7 h. After incubation, 75 µL of Dual-Glo™ Luciferase Reagent (Promega, Madison, Wisconsin, USA) was added to 75 µL of the cellular suspension for a 10 min at 22 °C before luciferase activity measurement. Luminometer (Berthold Technologies, Bad Wildbad, Germany) was used to measure luciferase activity.

### 3.17 Evaluation of apoptosis

The percentage of apoptotic cells was quantified as the fraction of cells showing fragmented nuclei, as assessed by fluorescence microscopy (Nikon, Tokyo, Japan) after staining with Hoechst 33342 (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich). Enzymatic activity of caspases-3/7 was determined using the Caspase-Glo 3/7 Assay (Promega, Madison, Wisconsin, USA).
3.18 Immunogenic cell death markers

For ectopic calreticulin (ERp60) expression, 15,000 cells/cm² were cultured and treated with indicated concentrations of OT52 for 24 h. Cells were collected, washed twice with PBS and fixed in 0.25% paraformaldehyde in 1x PBS for 5 min. After washing twice in cold PBS, cells were incubated for 30 min with the primary antibody (Abcam), diluted in cold blocking buffer (2% FBS in 1x PBS), followed by washing and incubation with the Alexa488-conjugated monoclonal secondary antibody in a blocking buffer (for 30 min). Each sample was then analyzed by FACS and fluorescence microscopy to identify cell surface calreticulin. Isotype-matched IgG antibodies were used as a control.

Quantification of HMGB1 release in the supernatants was assessed by enzyme-linked immunosorbent assay kit from Shino-Test-Corporation (Jinbocho, Chiyoda-ku, Tokyo, Japan) according to the manufacturer’s instructions.

3.19 Docking studies

Structures of SH2 domain in STAT3 were acquired from the protein data bank (PDB ID: 4E68) and structure of OT52 was generated by GlycoBioChemPRODRG2 server. Docking simulation was created by Autodock Vina (version 1.1.2).

3.20 Zebrafish toxicity assay and xenografts

Wild-type zebrafish (Danio rerio) were obtained from the Zebrafish International Resource Center (ZIRC, University of Oregon, OR), maintained according to
SNU guidelines at 28.5°C with 10 h dark/14 h light cycles. For toxicity assays, embryos were treated with 0.003% phenylthiourea 14 h before the assay to remove pigmentation. 2 h before the assay, the embryo's shell was eliminated and then treated for up to 24 h with OT52 at indicated concentrations in 24 well plates. Ethanol (3%) was used as a positive control for toxicity. Viability and abnormal development were assessed after 24 h of treatment under light microscopy (Carl Zeiss Stereo microscope DV4, Seoul, Korea). Pictures were taken by fixing zebrafish embryos onto a glass slide with 3% methyl-cellulose (Sigma-Aldrich).

For cancer xenograft assays, after mating, fertilized eggs were incubated in Danieau's solution with 0.003% of phenylthiourea (PTU) at 28.5°C for 48 h. Micropipettes for injection and anesthesia were generated from a 1.0 mm glass capillary (World Precision Instruments, FL, USA) by using a micropipette puller (Shutter Instrument, USA). 48 h post fertilization (hpf), zebrafish were anesthetized in 0.02% tricaine (Sigma, MO) and immobilized on an agar plate. A549 cells were treated with indicated compounds for 22 h; then cells were stained for additional 2 h by 4 µM of cell tracker CM-Dil dye (Invitrogen). 100–200 of A549 cells were injected into the yolk sac by microinjection (PV820 microinjector, World Precision Instruments, FL, USA). Subsequently, zebrafish were incubated in 24-well plates containing Danieau's solution with 0.003% phenylthiourea (PTU) at 28.5°C for 72 h. Fishes were then immobilized in a drop of 3% methylcellulose in Danieau's solution on a glass slide. Pictures were taken by fluorescence microscopy (Leica DE/DM 5000B). Fluorescent tumors were quantified by Image J software (U.S. National Institute of Health, Bethesda, MD,
3.21 Statistical analysis

Data are expressed as mean ± SD and significance was estimated by using one-way or two-way ANOVA tests, as further detailed in figure legends. Post-hoc analyses were performed using Graph Pad Prism 7 software, (La Jolla, CA, USA). Statistical significances were evaluated at $p$-values below 0.05 and represented by the following legend: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; posthoc analyses Dunnett; Sidak). All histograms represent the mean ± SD of at least three independent experiments.
3.22 Pharmacological inhibitors or inducers used in this study (Table 1).

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* Duration indicates a pre-treatment
### 3.23 Primary antibodies information used in this study (Table 2).

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<td>sc-56053</td>
<td>1:1000 in 5% milk</td>
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<td>1:1000 in 5% milk</td>
</tr>
<tr>
<td>ERp57</td>
<td>abcam</td>
<td>Ab10287</td>
<td>1:500 in PBST</td>
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<tr>
<td>ALDH</td>
<td>BD Biosciences</td>
<td>611194</td>
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</tr>
<tr>
<td>ACTB</td>
<td>Sigma-Aldrich</td>
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</tr>
<tr>
<td>α-tubulin</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-8035</td>
<td>1:10000 in 5% milk</td>
</tr>
<tr>
<td>Lamin B</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-6216</td>
<td>1:10000 in 5% milk</td>
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</tbody>
</table>
4. Results

4.1 Structures of coumarin and synthetic bis coumarin derivatives OT52

Figure 1. Chemical structure of the natural product, dicoumarol (A) as a backbone of synthetic molecule, bis (4-hydroxycoumarin) OT52 (B).
4.2 Cytostatic activity of coumarin derivative OT52 in non-small cell lung cancer

To investigate the anticancer effect of OT52 on lung cancer proliferation and viability, we selected lung cancer cell lines with a differential mutational background (KRAS mutation: A549, H460; EGRF mutation: H1650) and treated them with various concentrations of OT52 treatment for 72 h (Fig. 1C-H). Our results showed GI\textsubscript{50} values of 33.3 µM in A549, 35.7 µM in H460, and 50.1 µM in H1650 at 72 h of OT52 treatment (Fig. 1C-E). Interestingly, OT52 induced a cytostatic effect without toxicity (Fig. 1F-H). As OT52 showed a most noticeable anticancer effect in A549 cells, we decided to analyze its effects mainly in this cell line.

Considering the growth inhibition potential, we then investigated the effect of OT52 on cell cycle distribution by flow cytometry at increasing concentrations after 72 h. Our results showed a significant dose-dependent increase of cells in G1 at 10-20 µM with a concomitant reduction of the S phase, whereas the G2 phase was less affected. (Fig. 1I). We used cyclooxygenase-2 inhibitor celecoxib as a \textit{bona fide} control [42]. We then investigated the effect of OT52 on cell cycle regulators and observed a decrease of cyclin D1 and c-Myc expression levels after 24 h (Fig. 1J-K) in agreement with the observed cell cycle inhibition.

We also validated the acute and cardiotoxicity of OT52 by using 24 h of post-fertilization (hpf) zebrafish embryos. Here we provide \textit{in vivo} evidence that OT52 did not induce acute toxicity nor affect the normal development of zebrafish.
larvae, as shown by the absence of abnormal morphology, maintained viability, even at the highest concentration used, after 24 h (Fig. 1L-N). Furthermore, OT52 did not trigger cardiotoxicity, even at the highest concentration, as the heartbeat range remained unaffected by the treatment, underlining the safe profile of this compound (Fig. 1O).

Figure 2. Effect of OT52 on proliferation and viability of non-small cell lung cancer cell lines.

A549 (A, and D), H460 (B, and E), and H1650 (C, and F) were treated with increasing concentrations of OT52. After 24, 48 and 72 h, proliferation (A-C) and viability (D-F) were determined.
Figure 3. Effect of OT52 on cell cycle arrest of non-small cell lung cancer cell line and cell cycle regulatory protein expressions.

(A) A549 cells were treated at increasing concentrations of OT52 and celecoxib (Cele) was used as a positive control. Cells were stained with propidium iodide, and DNA content was measured by flow cytometry to estimate cell cycle distribution. (B-C) A549 cells were treated with increasing concentrations of OT52 for 24 h; proteins were extracted and assessed for cyclin D1 (B) and c-Myc (C) expression levels by western blot analysis.
Figure 4. In-vivo acute toxicity and cardiotoxicity of OT52 on zebrafish larvae.

Zebrafish larvae were treated at 24 hpf by increasing concentrations of OT52 (A). At 24 hpf, viability (B), body length (C) and heartbeat frequency (D) were quantified and represented as a graph. Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Dunnett, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
4.3 Morphologic modifications and extracellular matrix remodeling by OT52

Even though OT52-treated lung cancer cell lines underwent cell cycle arrest without toxicity, we observed a rapid OT52-mediated alteration of the cellular morphology after Diff-quick staining (Fig 2A). Reduced cytoplasm, vacuolization and extensive induction of stress fiber formation were detected in OT52-treated cells in a time-dependent manner. To quantify these changes, we used a surface plot analysis to present graphically OT52-triggered stress alterations.

Considering both cell cycle arrest and morphological alterations, we then assessed senescence-associated β-galactosidase (SA-β-gal) staining (Fig. 2B). Our results showed that OT52 treatment significantly induced SA-β-gal positivity starting from 10 µM compared to DMSO-treated A549 cells (Fig. 2B). Quantification of SA-β-gal positive cells revealed a 3.9-fold increase at 50 µM of OT52 treated A549 cells compared to DMSO-treated cells (Fig. 2B). Doxorubicin-induced SA-β-gal staining was used as a positive control (Fig. 2B). These results suggested that OT52-triggered G1 phase cell cycle arrest led to cellular senescence, concomitant with morphologic alterations including stress fiber formation, vacuolization, and cytoplasmic shrinkage. Transmission electron microscopy (TEM) analysis revealed abnormal mitochondrial disruption, valvular dehiscence of the endoplasmic reticulum, Golgi body diffusion, lamellar body formation, autophagosome formation, increased lipid droplet formation, nuclear distortion, and chromatin condensation. Altogether these observations indicated increased stress levels triggered by OT52, in line with previous observations (Fig. 2C).
Figure 5. Morphologic alterations and extracellular matrix remodeling by OT52.

A549 cells were treated with 50 µM of OT52 or DMSO for the indicated time. Cells were stained with Diff-Quik staining reagent, and cellular morphology was analyzed. Surface plot analysis was achieved by Interactive 3D surface plot plugin using the Image J 1.8.0 software.
Figure 6. Cellular senescence and SA-β-gal activation by OT52.

A549 cells were treated with indicated concentrations of OT52 and doxorubicin, used as a positive control. Cells were stained with SA-β-galactosidase staining reagent. Quantification of SA-β-galactosidase positive cells was shown as a graph.
Figure 7. Alterations of organelle structures were detected by transmission electron microscopy (TEM).

A549 cells were treated with 50 µM of OT52 or DMSO for the indicated time. Alterations of organelle structures were detected by transmission electron microscopy (TEM): (1) mitochondria, (2) endoplasmic reticulum, (3) Golgi, (4) lamellar body, (5) autophagosome, (6) lipid droplet, (7) lysosome, (8) nuclear distortion (9) nuclear condensation.
4.4 OT52 inhibits spheroid formation and spheroid aldehyde dehydrogenase expression

Since spheroids were found to be enriched with cancer stem-like characteristics including enhancement of self-renewal capacity, invasion, metastasis, drug resistance, and increased aldehyde dehydrogenase (ALDH) activity, we investigated the effect of OT52 by spheroid and colony formation assays. Results show that OT52 notably abrogated spheroid forming capacity in all non-small cell lung cancers cell lines (Fig. 2D). To further validate OT52-induced spheroid inhibition, we assessed spheroid viability by measuring spheroid ATP production, and IC₅₀ values were calculated. OT52 dose-dependently inhibited ATP production in all NSCLC cell line spheroids tested with IC₅₀ values of 29.3 µM, 62.7 µM, and 107.4 µM, in A549, H460, and H1650 respectively (Fig. 2E-G). Interestingly, stemness indicator ALDH was significantly reduced in A549 spheroids after OT52 treatment (Fig. 2H-I).
Figure 8. Effect of OT52 on spheroid forming capacity and spheroid ATP levels.

(A) A549, H460, and H1650 cells were treated with indicated concentrations of OT52 in round-bottom cell culture plates to allow anchorage-independent growth. Representative bright field images of A549, H460, and H1650 spheroids. (B-D) Spheroid ATP production was measured by luminescent 3D Cell-titer Glo. Quantification of relative ATP levels of A549 (B), H460 (C), and H1650 (D) spheroids is shown as a graph.
Figure 9. Multiple analysis of OT52-treated A549 non-small cell lung cancer cell 3D spheroids.

Quantification of relative volume (A), area (B), density (C), and debris (D) was evaluated for A549 3D spheroid. A549 were treated at indicated concentrations of OT52 and spheroids were obtained by the u bottom ultra-law attachment incubation technique. Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Dunnett, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
Figure 10. Effect of OT52 on spheroid ALDH1 expressions.

A549 were treated at indicated concentrations of OT52 and spheroids were obtained by the hanging-drop technique after 7 days. Spheroid ALDH expression levels were evaluated by immunofluorescence, and picture was taken by confocal microscopy. Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Dunnett, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
4.5 OT52 induces metabolic alterations in the lung cancer cell lines

As induction of stress-induced senescence by OT52 treatment could also lead to metabolic alterations, we analyzed changes in glycolysis and oxidative phosphorylation (OXPHOS) activity, mitochondrial bioenergetics, and reactive oxygen species (ROS) generation in OT52-treated A549 cells. A global reduction of the extracellular acidification rate (ECAR) (Fig. 3A) was observed after OT52 treatment. After glucose injection, we detected a rapid increase of ECAR levels in untreated cells (40 mpH/min), but in contrast, OT52 completely abrogated this increase (Fig. 3A). Glycolytic flux reduction via hexokinase inhibitor 2-DG induced a strong depletion of the ECAR level in control A549, however, in OT52-treated cells we did not observe any significant changes (Fig. 3A).

From this metabolic profiling, specific parameters could be deduced, and our results show a systematic decrease of the metabolic functions of A549 cells after OT52 treatment in agreement with the onset of senescence. Our results show a 2.1-fold decrease of non-glycolytic acidification (Fig. 3B), 4.8-fold decrease of glycolysis (Fig. 3C), 3.4-fold decrease of maximal glycolytic capacity (Fig. 3D) and 3.6-fold decrease of the glycolytic reserve (Fig. 3E). Altogether results documented an inhibition of metabolic functions by OT52, triggering an energetic collapse in A549 lung cancer cells.

Next, to elucidate whether OT52-triggered glycolytic depletion escalated into mitochondrial stress, we evaluated the level of oxygen consumption rate (OCR) as OXPHOS measurement (Fig. 3F). We observed decreases of basal respiration...
(Fig. 3G; 2.1-fold), proton leak (Fig. 3H; 4.3-fold), maximal respiration (Fig. 3I; 30-fold), non-mitochondrial respiration (Fig. 3J; 4.4-fold), ATP production (Fig. 3K; 2.2-fold), and spare respiratory capacity (Fig. 3L; 16-fold) in response to OT52 treatment. The initial OCR level of OT52-treated A549 cells, is lower than control A549 cells, indicating a decline of total mitochondrial mass (Fig. 3F). According to subsequent injection of oligomycin revealed that ATP production-related mitochondrial respiration was notably restrained in control A549, but not in OT52-treated cells. Next, to evaluate maximal respiration, FCCP, a mitochondrial uncoupler, was injected to stimulate maximal mitochondrial respiration. In OT52-treated cells, however, no increase in mitochondrial respiration could be observed (Fig. 3F). Moreover, mitochondrial complex I and III inhibitors, rotenone and antimycin attenuated the level of OCR in control cells but remained without effect in OT52-treated A549. Altogether, these results showed that OT52-induced mitochondrial stress accompanied by the loss of glycolytic functions.

As OT52-mediated mitochondrial stress could lead to ROS accumulation, we next assessed the effects of OT52 on the intracellular ROS formation (Fig. 3M). As illustrated in Fig. 3N, ROS generation in response to OT52 treatment increased from 3 h to 9 h after treatment but then decreased by 30% from 12 h (Fig. 3N). ROS generation could be observed in 90% of OT52-treated A549 cells, but not in untreated cells (Fig. 3N). H₂O₂-treated A549 served as a positive control (Fig. 3N). OT52-mediated metabolic alteration of lung cancer cells, concomitant with a loss of glycolytic activity, increased OXPHOS, abrogation of mitochondrial...
mass, and eventual ROS generation reflected that mitochondria could a target of OT52 in cancer cells. Moreover, our results are in line with our previous data as OT52-induced disruption of intracellular metabolic homeostasis could directly trigger to the stress fiber formation, extracellular matrix (ECM) remodeling, and stress-induced senescence in lung cancer cells (Fig. 2).
Figure 11. Effect of OT52 on metabolic alteration in A549 lung cancer cells.

(A) A549 cells were treated with 50 µM of OT52 or DMSO for 9 h, then ECR was analyzed using a Seahorse XFp glycolysis stress test: for the determination of the OT52-mediated glycolytic alterations, glucose, oligomycin, and 2-DG were subsequently injected at indicated time points. (B-E) Each glycolytic function was shown as individual graphs including non-glycolytic acidification (B), glycolysis (C), glycolytic capacity (D), and glycolytic reserve (E).
Figure 12. Effect of OT52 on mitochondrial functional profiling in A549 lung cancer cells.

(A) A549 cells were treated with 50 µM of OT52 or DMSO for 9 h, then OCR was analyzed using a Seahorse XFp mito-stress analysis kit, and for detection of sequential events on mitochondrial reparation, oligomycin, FCCP, and antimycin A and rotenone were sequentially injected. F. Mitochondrial respiration profiling was acquired by a Seahorse XF24 analyzer. (B-G) OT52-triggered mitochondrial
respiration was shown as quantification graphs including basal level (B), Proton leak (C), maximal respiration (D), non-mito respiration (E), ATP production (F), and spare respiratory capacity (G).
Figure 13. Effect of OT52 on Reactive Oxygen Species (ROS) generation in A549 lung cancer cells.

(A-B) A549 cells were treated by increasing concentrations of OT52 or DMSO for indicated periods of time and H2O2 was used as a control. H2DCFDA fluorescence labeling was used for ROS detection by fluorescence microscopy. ROS generation after 9 hr of increasing concentrations of OT52-treated A549 cells (A). Quantification of ROS is shown as a graph (B). Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Dunnett, two-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
4.6 OT52-induced cytological alterations of ER and Golgi is concomitant with ER/Golgi stress regulatory protein expression

As our TEM results demonstrated that ER and Golgi structures were dilated by OT52 from 24 h after the treatment in lung cancer cells (Fig. 2C), we next verified the effect of OT52 on ER/Golgi stress. To assess ER/Golgi structural modifications and localization, we selectively stained ER and Golgi apparatus in red and green, respectively using ER/Golgi Cytopainter in OT52-treated or untreated A549 cells. As illustrated in Figure 4A, ER tubules were originally mainly localized in a perinuclear region in control cells whereas OT52 treatment triggered a dose-dependent cytoplasmic redistribution of the ER signal. Similarly, Golgi apparatus was strictly localized in a perinuclear area in control cells, whereas OT52 treatment again led to a cytoplasmic redistribution of the signal (Fig. 4A). Considering this subcellular reorganization of ER and Golgi, we examined whether ER/Golgi stress regulatory proteins were affected by OT52 treatment. Our results revealed that 50 µM of OT52 treatment increased ER-related protein expression levels including GRP78/Bip (3.8-fold), PERK (1.1-fold), p-eIF2α (2.4-fold), ATF4 (3.4-fold), ATF6 (6.9-fold), and CHOP (6.2-fold) (Fig. 4B and C). Altogether, OT52 induced ER/Golgi stress further contributing/explaining cell cycle arrest and senescence.
**Figure 14. Effect of OT52 on cytological alterations of ER and Golgi structures.**

A549 cells were treated with indicated concentrations of OT52 or DMSO for 24 h. Cellular localization of ER and Golgi were visualized by ER/Golgi cyto painter by confocal microscopy. Organelle probes were conjugated to the following fluorescent dyes: ER; Red, Golgi; Green, Nucleus; Blue.
Figure 15. Effect of OT52 on ER and Golgi stress related-protein expressions.

(A) A549 cells were treated at increasing concentrations of OT52, and Thapsigargin (Thap) was used as a positive control. (B) Quantification of
corresponding protein expression levels is shown as a graph for 78 kDa glucose-regulated proteins (GRP78/Bip), protein kinase-like ER kinase (PERK), eukaryotic initiation factor 2 (p-eIF2α), activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), and C/EBP homologous protein (CHOP). Asterisks indicate a significant difference compared to control negative as analyzed by Dunnett, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
### 4.7 OT52 abrogates STAT3 signaling

As shown in our previous results (Fig. 1 and 2), KRAS mutant lung cancer cells (A549, and H460) are significantly more sensitive to OT52 compared to EGFR mutant lung H1650 cells in response to OT52 treatment. To better understand the mechanism behind the anticancer activity of OT52 in KRAS mutant cell types, we next assessed the effect of OT52 on STAT3, as a major downstream signaling target of KRAS and responsible for transactivation of target genes involved in cell proliferation and survival.

To gain insight into the mechanistic interaction of OT52 with STAT3, we conducted a molecular docking study of OT52 to the STAT3 DNA binding domain (DBD). We found that OT52 allosterically interacted with the STAT3 DNA binding site near the SH2 domain with ΔG of -8.1 kcal/mol stabilization energy, and was committed in polar interactions with several key amino acids (Fig. 5A). The known coumarin scoparone [65] used as a control docked with a ΔG of -5.0 kcal/mol (Fig. 5B). Combinatory docking simulation with OT52 and scoparone revealed comparative results between two molecules (Fig. 5C).

IL-6-induced STAT3 phosphorylation was significantly abrogated by OT52 with an IC$_{50}$ of 29 µM (Fig. 5D). We next investigated the time-dependent effect of IC$_{50}$ of OT52 on STAT3 phosphorylation. STAT3 phosphorylation was reduced from 12 h of 30 µM OT52 treatment (Fig. 5E). To confirm the effect of OT52 on downstream transactivation, we analyzed DNA binding affinities of STAT3 using a TransAM binding assay which showed a significant inhibition of the binding.
activity from 30 µM of OT52 treatment with IC₅₀ value of 34 µM (Fig. 5F). Moreover, we determined the effect of OT52 by a luciferase reporter gene assay that showed significant reduction of IL-6-induced STAT3 reporter gene activity from 33 µM (Fig. 5G). Ruxolitinib (INC424), a selective JAK 1/2 inhibitor approved by Food Drug Administration (FDA), was used as *bona fide* control.
Figure 16. Computational Docking analysis of OT52 to the SH-2 domain.

(A) A549 cells were treated at increasing concentrations of OT52, and Thapsigargin (Thap)
Figure 17. Effect of OT52 on STAT3 regulation.

(A) A549 cells were treated with indicated concentrations of OT52 or DMSO for 24 h then IL-6 was added for 10 min. Total proteins were isolated and analyzed by Western Blot for STAT3 tyrosine phosphorylation, and total STAT3 levels. p-STAT3 (y705) was normalized by the expression level of total STAT3. (B) A549 cells were treated with 30 µM of OT52 or DMSO for indicated times, and before protein extraction, IL-6 was added for 10 min for STAT3 induction. Total proteins were isolated and analyzed by Western Blot for STAT3 tyrosine phosphorylation and total STAT3. p-STAT3 (y705) was normalized by the expression level of total STAT3.
Figure 18. Effect of OT52 on STAT3 transactivation.

(A) A549 cells were treated by increasing concentrations of OT52 or DMSO for 24 h, and IL-6 was added for 10 min for STAT3 induction. Graphs of relative STAT3 DNA binding affinity was obtained by STAT3 TransAM assay. (B) p-Stat3-Hela cells were treated by increasing concentrations of OT52 or DMSO for 24 h, and IL-6 was added for 7 h. STAT3 reporter gene activity was measured by an STAT3 luciferase assay. (C) IC50 values of p-Stat3 inhibition by OT52 were analyzed by GraphPad Prism7. Results correspond to mean ± SD of the quantification of three independent experiments.
4.8 OT52-triggered abrogation of STAT3 phosphorylation is regulated by SOCS3 and PIAS3

As OT52 inhibited STAT3 phosphorylation and transactivation potential in KRAS mutant lung cancer cell lines, we next evaluated protein expression levels of STAT3 regulators to elucidate the mechanism of OT52-mediated STAT3 inhibition. Before OT52 addition, cells were pretreated with tyrosine bisphosphate inhibitor, sodium orthovanadate (Na₃O₄). Results showed that Na₃O₄ did not prevent OT52-induced STAT3 inhibition (Fig. 5I) suggesting independence from phosphatase activation. Conversely, expression of STAT3-related phosphatases PP2A, SHP-1, and PTEN and kinases including Src, c-Abl were not affected by OT52 treatment (Fig. 5J and 5K). Next, we analyzed two major negative regulators of STAT3, suppressor of cytokine signaling 3 (SOCS 3) and protein inhibitor of activated STAT3 (PIAS 3), to further elucidate mechanisms of OT52-mediated STAT3 inhibition. Results show that 50 µM of OT52 increased SOCS3 and PIAS3 levels in the cytoplasm and nucleus respectively by 2.8-fold and 1.5-fold increasing (Fig. 5L and 5M). Altogether, OT52-induced SOCS3 and PIAS3 expression lead to STAT3 inhibition, independent of other enzymatic regulators.

Our results show inhibition of STAT3 signaling by OT52 in agreement with the previously shown inhibition of STAT3 target genes c-Myc and cyclin D1 (Fig. 1). As it is well-known that oncogenic KRAS upregulates expression of pro-survival Bcl-2 genes, we investigated the expression levels of another STAT3 target genes, Mcl-1 and Bcl-xL. Our results show that even though transcription of these genes
is under STAT3 control, no downregulation or accumulation could be observed (Supplementary Fig. S2) most likely contributing to the absence of cytotoxicity observed after OT52 treatment (Fig. 1). We hypothesized then that induction of cell death would require additional targeting of such anti-apoptotic Bcl-2 family members by BH3 protein inhibitors.

Figure 19. Effect of tyrosine phosphatase on OT52 triggering STAT3 inhibition.
(A) A549 cells were pre-treated with or without indicated concentrations of the tyrosine phosphatase inhibitor, sodium orthovanadate (Na3OV4) for 1 h and with or without 50 µM of OT52 was treated for 24 h. Then IL-6 was treated for 10 min for STAT3 stimulation. Total proteins were isolated and analyzed by Western Blot for STAT3 tyrosine phosphorylation, and total form of STAT3 and histogram of corresponding p-STAT3 (y705) expression was normalized by total form of STAT3. A549 cells treated by increasing concentrations of OT52 or DMSO vehicle for 24 h, and IL-6 was treated for 10 min for STAT3 induction. (B-C) Total proteins were isolated and analyzed by Western Blot for STAT3 regulator protein expressions including p-Src (y416), c-Abl, PP2A, SHP-1, and PTEN.
Figure 20. Effect of OT52 on major negative regulators of STAT3, SOCS3, and PIAS3.

(A-B) Cytoplasmic and Nuclear fraction were isolated and analyzed by Western Blot for SOCS3 and PIAS3 expressions. Protein extracts purity and loading quantification were verified by α-tubulin (cytoplasm) and lamin B (Nucleus). Histogram of cytoplasmic SOCS3 was normalized by α-tubulin (A), and Nuclear PIAS3 was normalized by lamin B (B). Results correspond to mean ± SD of the
quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Turkey, two-way ANOVA.

(* P < 0.05; ** P < 0.01; *** P < 0.001).
4.9 OT52 sensitized A549 cells against small-molecule BH-3 protein inhibitors and induced caspase-independent cell death

As OT52 triggered cytostatic effects without cytotoxicity and inhibited STAT3 signaling without affecting levels of anti-apoptotic Bcl-2 family proteins Mcl-1 and Bcl-xL, major anti-apoptotic genes expressed by A549 cells, which do not express Bcl-2, we first conducted viability and proliferation assays with Mcl-1 inhibitor A-1210477 and Bcl-xL inhibitor A-1331852 for 24 h. Results showed that A549 cells were sensitive to these targeted inhibitors from 30 and 20 µM for Mcl-1 and Bcl-xL inhibitors, respectively (Supplementary Fig. S3). To assess the combinatory effect of OT52 with these inhibitors, we then used subtoxic concentrations of these compounds in combination with OT52. Results show that 20 µM of A-1210477 or 10 µM of A-1331852 induced cell death of 40% and 70% respectively (Fig. 6A and 6B) when combined with OT52 at 50 µM, a nontoxic concentration, required to obtain a cytostatic effect when used alone (Fig. 1).

Then we further evaluated the expression levels of apoptosis-related proteins. Results showed that OT52 combined to A-1210477 induced Mcl-1 stabilization witnessing inactivation (Fig. 6C). Moreover, results showed that sub-toxic concentrations of A-1210477 alone lead to the appearance of a short isoform of Mcl-1 (32 kDa). OT52 combined to A-1331852 efficiently reduced Bcl-xL expression levels (Fig. 6D). Interestingly, procaspase-3 and -7 expression levels remained unaffected by combination treatments (Fig. 6C and 6D) as no cleavage was observed, and these results could be confirmed by luminescent caspase-3/7 assays. Furthermore, pan-caspase inhibitor z-VAD-FMK pre-treatment did not
affect the result of combination treatments of OT52 and A-1210477 or A-1331852 (Fig. 6E). Altogether, we concluded that OT52 sensitized lung cancer cells against A-1210477 and A-1331852 to trigger a form of caspase-independent cell death. We confirmed these data by Annexin-V/PI staining. Results showed that OT52 and A-1210477 or A-1331852 combination induced around 40% and 50% of cell death independent of z-VAD-FMK (Fig. 6F and 6G). Eventually, Hoechst/PI staining of cells treated with a combination of OT52 and A-1210477 or A-1331852 showed around 40%, and 60% of cell death mostly in late apoptosis, insensitive to z-VAD-FMK (Fig. 6H). Altogether, OT52 sensitized NSCLC cell against small-molecule BH3 protein inhibitors via caspase-independent cell death induction.
Figure 21. Effect of OT52 on anti-apoptotic protein expressions on NSCLC cells.

A549 cells were treated with indicated concentrations of OT52 or DMSO for 24 h. Total proteins were isolated and analyzed by Western Blot for Mcl-1 and total Bcl-xL.
Figure 22. OT52 sensitizes against selective BH3 protein inhibitor.

(A-B) A549 cells were treated with 50 µM of OT52 and 20 µM of A-1210477 (MI) or 10 µM of A-1331852 (BI) for 24 h in single or combination treatments. Cell viability (A) and proliferation (B) were assessed by the trypan blue staining.
Figure 23. Combinatory effect of OT52 and BH3 mimetics on apoptosis regulatory protein expressions.

(A-B) A549 cells were treated with 50 µM of OT52 and 20 µM of A-1210477 (MI) or 10 µM of A-1331852 (BI) for 24 h in single or combination treatments. Total proteins were isolated from combination OT52, and A-1210477 (A) and OT52 and A-1331852 (B) treated A549 cells. Western Blots for apoptosis-related protein expression. Etoposide (VP-16, 100 µM) was used as a positive control of apoptosis induction and caspase cleavage.
Figure 24. Combinatory effect of OT52 and BH3 mimetics on caspase3/7 activations.

A549 cells were pre-treated with or without 50 µM of pan-caspase inhibitor, z-VAD-FMK for 1 h, and then 50 µM of OT52, 20 µM of A-1210477 (MI), and 10 µM of A-1331852 (BI) were treated for 24 hours at indicated single or combination treatment conditions. Relative caspase 3/7 activity was measured by Caspase-Glo 3/7 assay.
Figure 25. OT52 sensitizes against selective BH3 protein inhibitor.

(A) Evaluation of cell death mechanisms by flow cytometer assay using Annexin-V-FITC/PI dual staining analysis with z-VAD-FMK, OT52, A-1210477 and A-1331852 single or combination treated A549 at indicated conditions. (B) Quantification of cell death modalities triggered by OT52 and A-1210477 and A-1331852 combination treatments.
Figure 26. OT52 sensitizes against selective BH3 protein inhibitors triggering non-canonical cell death induction.

Evaluation of cell death mechanisms by confocal microscopy using Hoechst/PI staining. Nuclear condensation of A549 cells treated at indicated conditions, stained with Hoechst/PI (Blue; Hoechst, Red; propidium iodide).
4.10 Induction of immunogenic cell death markers by OT52

Our previous results confirmed an increase of late apoptotic or necrotic cells by the combination of OT52 and BH3-protein inhibitors in a caspase-independent manner. Moreover, OT52 potently induced ER stress (Fig. 4). Interestingly, under those conditions, A549 cells released protein markers characterizing an immunogenic form of cell death. Cell surface calreticulin expression was indeed increased by OT52 in a dose-dependent manner up to 3.9-fold at 50 µM of OT52 (Fig. 6I and 6J). Moreover, the combination of OT52 with BH3 protein inhibitors triggered a significantly increased release of HMGB1 compared to OT52, A-1210477, or A-1331852 alone (Fig. 6K).
Figure 27. OT52 sensitizes against selective BH3 protein inhibitors triggering Immunogenic cell death induction.

A549 cells were treated with indicated concentrations of OT52 for 24 h, and oxaliplatin was used as a bona fide ICD inducer. Evaluation of ectopic calreticulin exposure by flow cytometry (A). Quantification of ectopic calreticulin exposure after OT52 treatment. A549 cells were treated with indicated concentrations of OT52, A-1210477, and A-1331852 for 24 h, alone or in combination (B). HMGB1 release levels were measured by enzyme-linked immunosorbent assay (C). Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control negative as analyzed by Turkey, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
Synergistic anti-cancer effects of a combination of OT52 with A-1210477 or A-1331852 in 3D cell culture assays and zebrafish in vivo xenografts

Our previous results confirmed synergistic effects of a combination of OT52 and BH3-mimetics in A549 lung cancer cells. To validate these results obtained in 2D culture, we assessed the effect of the drug combinations in anchorage-independent assays to demonstrate the anti-cancer potential under such conditions. Results showed that a single treatment by OT52 or by BH3 mimetics alone did not prevent A549 colony formation; however, their combination led to a complete abrogation of 3D colony formation (Fig. 7A). We observed a significant decrease of the total number of colonies (Fig. 7B), total area of colonies (Fig. 7C), and average size of colonies (Fig. 7D). Moreover, combination of OT52 and BH3 mimetics inhibited A549 spheroid formation in agreement with the results obtained with colony formation assays (Fig. 7E). We extended this investigation into an in vivo setting and demonstrated that combinations of OT52 with BH3 mimetics triggered complete abrogation of tumor formation whereas single treatments were unable to significantly reduce the volume of such tumors in zebrafish xenografted with pretreated A549 cells (Fig. 7F-G, Supplementary Fig. S4).
Figure 28. The synergistic anti-cancer effect of OT52 combined to BH3 protein inhibitors on colonogenic assay.

An in-vitro clonogenic assay shows the long-term effects of combination treatment of human lung cancer cells with OT52 and A-1210477 or A-1331852. A549 cells were treated with a combination of OT52 and A-1210477 or A-1331852 for 10 days under the anchorage-independent growth conditions. B-D) Multiple quantifications of the result of the clonogenic assay with: total number of colonies (B), total area of colonies (C), and average size of colonies (D). Results correspond to mean ± SD of the quantification of three independent experiments. Asterisks indicate a significant difference compared to control.
negative as analyzed by Turkey, one-way ANOVA. (* P < 0.05; ** P < 0.01; *** P < 0.001).
Figure 29. The synergistic anti-cancer effect of OT52 combined to BH3 protein inhibitors on in-vitro anchorage independent growth assay.

Synergistic effect on spheroid formation with drug combinations in A549. Suspended A549 cells were seeded with or without the drug combination in round bottom plates for 3 days. Pictures were taken by bright field microscopy.
Figure 30. The synergistic anti-cancer effect of OT52 combined to BH3 protein inhibitors on zebrafish in-vivo xenografts.

(A-B) Anti-cancer effect of drug combinations by a zebrafish in-vivo xenograft model. A549 cells were pretreated with or without compounds as indicated treatment for 24 h. Cells were then labeled by CM-Dil for 1 h and injected into zebrafish. After 72 h, fluorescent signal was detected by fluorescence microscopy. Images show ten fish used per condition (A). Fluorescence intensities are
represented as a graph. Results represent the mean ± SD of ten independent xenograft fish (B).
Figure 31. The synergistic anti-cancer effect of OT52 combined to A-1210477 or A-1331853 on zebrafish in-vivo xenografts.

Anti-cancer effect of drug combinations by a zebrafish in-vivo xenograft model. A549 cells were pretreated with or without compounds as indicated treatment for 24 h. Cells were then labeled by CM-Dil for 1 h and injected into zebrafish. After 72 h, fluorescent signal was detected by fluorescence microscopy. Images show ten fish used per condition.
Figure 32. Schematic diagram of a combination treatment of OT52 with BH3 mimetics.

Schematic diagram of a combination treatment of OT52 with BH3 protein inhibitors triggering cellular stress and immunogenic cell death markers. Combinations of OT52 and A-1210477 or A-1331852 induced synergistic cell death induction leading to exposure of immunogenic cell death markers and non-
canonical apoptosis. OT52-mediated ER stress synergistically triggered calreticulin exposure and HMGB1 release, two ICD markers. Concomitantly, non-canonical apoptosis was induced by ROS induction and glycolytic dysfunction in combination treatments.
5. Discussion

Natural coumarins and its derivatives are widely used for the development of novel molecules with therapeutic potential. Selected coumarin derivatives were previously proven to be outstanding anti-lung cancer molecules to overcome drug resistance in non-small cell lung cancer [26, 42]. Coumarin derivatives, osthole [7-methoxy-8-(3-methyl-2-butenyl) coumarin], Ferula-derived umbeliprenin, daphnetin (7,8-dihydroxycoumarin), 3-[4-(2-(dimethylamino)ethoxy)phenyl]-7-methoxy-4-phenyl-2H-chromen-2-one, and 7-methoxy-4-phenyl-3-[3-(pyrrolidin-1-yl)propoxy]phenyl]-2H-chromen-2-one, were discovered as anti-lung cancer agents in A549 to induce G2/M phase arrest, apoptosis, and ROS induction [43-45]. It also has been shown previously in a large population study, adjusting for cofactors as age and sex, that long term use of anticoagulants, could have a protective role on cancer incidence (HR 0.88, 95% CI 0.80-0.98, significant). The same trend could be observed in subgroup of respiratory tract cancers (0.78, 95% CI 0.57-1.07), even though the effect was not significant, as in most subgroups [46]. Considering that anticoagulants may play a role as anticancer agents, we intended to further explore this hypothesis in non-small cell lung cancer, an important group of respiratory tract cancer. Based on this therapeutic potential of coumarin backbone characteristics, we approached the cytostatic characteristic of a novel coumarin derivative, OT52, on non-small cell lung cancer cell models. In the present study, we demonstrated the cytostatic activity of OT52 in non-small cell lung cancer cells with KRAS or EGFR mutational background. All NSCLC cell lines were affected by OT52 treatment;
nevertheless, KRAS-mutant cells were more sensitive compared to EGFR mutated cell lines. Considering the absence of efficient drugs to target KRAS mutated NSCLC, OT52 could be excellent scaffold to target KRAS-mutated NCSLC. Cancer expressing KRAS mutations are characterized by uncontrolled cell proliferation whereas our results demonstrated that A549 cells were arrested in G1 by OT52 concomitant with morphological modifications and metabolic alterations. Therefore targeting the cellular metabolism could improve the response against cancer therapeutics [47] considering that most cancer cells show atypical metabolic characteristics such as Warburg glucose metabolism as well as cancer-specific metabolic alterations [47]. So far, metabolic alterations induced by coumarin derivatives largely remained unexplored. In this present study, we demonstrated for the first time how coumarin derivative OT52 triggers a metabolic switch and resulting cellular stress in lung cancer cells. We show that OT52 inhibited A549 ATP production by glycolytic dysfunction concomitant with ROS generation and abrogation of mitochondrial respiration. Overall this metabolic stress was accompanied by stress fiber formation, cell cycle inhibition, senescence induction, and ER/Golgi stress. Interestingly, previous research indicated that biscoumarol leads to fragmentation of the tubular-reticular non-compact zones of the Golgi apparatus. Mironov et al. [34] suggested this coumarin as a “tool for studies of Golgi structure and function”. Beyond this observation, we believe that the alteration of the Golgi homeostasis creates a stress situation in the cell contributing to the sensitization effect observed in our study. Moreover, both Golgi and ER stress could constitute an important mechanism also for other
compounds and a required step for the synergistic increase of the potential of BH3 mimetics and other anticancer drugs. Altogether, our results suggest that OT52 and related scaffolds play a potential role in the inhibition of lung cancer cellular activities via induction of cellular stress modalities. An overview of the different molecular events is shown in Fig. 13.

Cellular senescence is a crucial process leading to irreversible growth arrest, and is promoted by drug-mediated cellular stress and DNA damage, termed as stress-induced premature senescence (SIPS) [48]. Recently, the therapeutic use of SIPS inducers including resveratrol, shikonin, gefitinib, and immune modulators constitutes a novel approach to target lung cancer [48-50]. On the other hand, senescent cells secrete growth factors, proteases, and cytokines also called senescence-associated secretory phenotype (SASP) factors [51] which modulate autocrine and paracrine mechanisms to disrupt cellular homeostasis and promote cancer signaling and tumorigenic activities [52]. To overcome this critical therapeutic point, compounds leading to death of senescent cells i.e. triggering a senolytic effect are actively investigated. BH3 mimetic Navitoclax (ABT-263), and TW-37 showed a senolytic effect in lung fibroblast by targeting anti-apoptotic factors [53, 54]. In our study, OT52 induced SIPS through cellular stress induction such as cell cycle arrest, ROS, and ER stress. At the same time, we showed that IL-6-induced STAT3 phosphorylation was abrogated by OT52, and a combination treatment with OT52 and BH3 mimetics triggered synergistic effects to eventually activate a non-canonical cell death pathway. Altogether, we suggest OT52 as a promising molecule to trigger SIPS alone. Most importantly, a combination of
BH3 mimetics like A-1210477 and A-1331852 with OT52 not only triggers senescence but adds a strong senolytic potential.

As mentioned, coumarins present potential unwanted side effects linked to their inherent anticoagulant activity. Even though we did not assess the anticoagulant potential of OT52 in vivo, we underline that caution is required for the use of such agents. As an example, well-known coumarins, including bergamottin interfere with phase I drug metabolism by interaction with cytochrome P450 (CYP) 3A4 [55]. In the case of an anticancer use of coumarins, this potential side effect needs to be closely monitored.

In non-small cell lung cancer, EGFR-targeting TKIs like erlotinib (Tarceva) or gefitinib (Iressa) became well established in both biological and clinical NSCLC treatment but not in KRAS-mutated NSCLC [56, 57]. Additional molecular targets needed to be investigated in such cancer types including downstream signaling pathways including STAT3 inhibited by biscoumarin OT52. Moreover, STAT3 is a key metabolic regulator as mitochondrial proteins were down-regulated by STAT3 [58]. Inhibition of STAT3 tyrosine phosphorylation was connected to glycolytic dysfunctions before growth arrest, both in vitro and in vivo [59]. In our study, we found that coumarin derivatives inhibited IL-6-induced STAT3 phosphorylation by stimulation of SOCS3 in the cytoplasm. Moreover, our compounds concomittantly abrogated STAT3 DNA binding via nuclear PIAS3 accumulation. Taken together our results suggest that OT52 might be a novel compound which directly targets negative STAT3 regulators SOCS3 and PIAS3. Coumarin analog scoparone (6,7-dimethoxycoumarin) inhibited prostate cancer
cell by suppression of STAT3 binding to SH2 domain without other modulation of STAT3 regulators including JAK2 and Src [41].

A tumor microenvironment that favors anchorage-independent growth of cancer cells is a key aspect of the cancer phenotype increasing the metastatic potential [60]. Abrogation of colony and spheroid forming abilities are a crucial indicator allowing to predict the therapeutic potential of novel therapeutic applications [61]. Our results showed that OT52 efficiently abrogated A549 spheroid formation as well as ALDH expression in spheroids, which is the marker of cancer stemness. Moreover, we found that OT52 reduced microvilli formation thus reducing A549 cell motility and potentially metastatic capacity. Altogether our results suggested that OT52 is a potent nature-derived cytostatic agent, but devoid of cytotoxic potential. Moreover, our results show consistent expression levels of anti-apoptotic Mcl-1 and Bcl-xL proteins most likely contributing to survival of cells under OT52-induced growth arrest. Preclinical profiling of patient anti-apoptotic protein expression levels demonstrated that selected functional deficiencies in apoptosis signal transduction pathways trigger resistance mechanisms in various types of cancer [62, 63]. In lung cancer, up-regulation of Mcl-1 and Bcl-xL play a critical role in such resistance mechanisms, so that these proteins became interesting targets for novel and personalized treatments [64].

To achieve re-sensitization, a rational drug combination consists in the association of compounds with a cytostatic potential to targeted agents able to abrogate cell death resistance mechanisms. So far, clinical trials were conducted with Erlotinib in combination with gemcitabine and cisplatin in NSCLC [65]. Resistance due to
the secondary T790M mutation, acquired after EGFR TKI treatment, could be efficiently overcome by a combination of Bcl-xL and Bcl-2 inhibitors with Erlotinib in NSCLC [66-68]. Ruxolitinib (INCB018424) an FDA approved IL-6/STAT3 targeting drug was shown to overcome cisplatin resistance in NSCLC via STAT3 inhibition [69]. Here, considering that single treatments of OT52 did not reduce Mcl-1 or even induced Bcl-xL expression levels, then we hypothesized that targeting of both STAT3 and Bcl-2 family proteins (Bcl-xL or Mcl-1) in KRAS mutated lung cancers might provide a synergistic therapeutic effect. According to the previously published data, KRAS mutations can stimulate Bcl-xL expression through STAT3 activation to trigger drug resistance and anti-apoptotic signaling [70]. Moreover, sensitivity of lung cancer cells can be restored through Bcl-2 and Bcl-xL inhibitors leading to BIM accumulation [67, 68]. Consequently, clinical trials involving combination treatments of Bcl-2 inhibitor Navitoclax and the 3rd generation EGFR TKI osimertinib were initiated in NSCLC (NCT02520778) [68]. Recently in a peptide-based BH3 profiling as predictor of drug chemosensitivity, BH3-mimetic drugs, A-1331852 (Bcl-xL inhibitor), and A-1210477 (Mcl-1 inhibitor) were identified as cancer chemotherapeutic drugs inducing cell death [71] in hematologic and lung cancer. Our results showed that a combination with OT52 and A-1210477 or A-1331852 dramatically sensitized KRAS-mutated NSCLC cell line A549 to induce caspase-independent cell death. Interestingly, subtoxic concentrations of A-1210477 and A-1331852 alone were unable to trigger canonical apoptosis despite the targeting of well-known inhibitors of mitochondrial cell death.
Immunogenic cell death (ICD) aims to activate adaptive immunity after treatment by selected anticancer agents. It is believed that compounds triggering ICD will lead to improved therapeutic outcomes and complete remission. So far nor coumarins nor BH-3 mimetics were shown to trigger this cell death modality. Even though ICD induction remains a matter of intense research, a precise link between chemical scaffold and ICD induction remains elusive. Nevertheless multiple ICD markers such as calreticulin (CRT) and high-mobility group box 1 (HMGB1) are considered as molecular ICD hallmarks [72, 73]. Moreover, ER stress, autophagy or mitophagy were designated as cell stress reactions fostering ICD. Related to ER stress, eIF2 phosphorylation is recognized as a prototypical ICD biomarker, followed by CRT translocation from the ER lumen to the cell surface to allow activation of a cognate cancer specific immune response. Moreover, non-histone nuclear protein HMGB1, released after nuclear and plasma membrane permeabilization [74-77] is not only considered as a central mediator of SASP [78] and but it is also induced by the STAT3 inhibitor, Ruxolitinib [79]. In agreement with our data obtained with OT52, other known senescence inducers, including doxorubicin or oxaliplatin are discovered as the bona fide ICD inducers as well [73, 77, 80]. In this study, we proved that OT52 alone induced ER stress concomitant with senescence and STAT3 inhibition. Combination treatments of BH3-mimetics and OT52 led to late-apoptotic and necrotic cell death induction described to trigger HMGB1 release [81]. Based on our results, OT52 induced CRT exposure at 50 µM without releasing significant amounts of HMGB1 which only became significant after a co-treatment with BH3.
mimetics. Here the Bcl-xL inhibitor A-1331852 appeared more potent compared to A-1210477 inhibiting Mcl-1. These results should be considered as a proof of concept for induction of ICD markers after cellular stress including ER and Golgi stress as well as SIPS mediated ICD by OT52 and BH3-mimetics associated with STAT3 inhibition in NSCLC. In the future, in-depth investigation with functional assays and validation of ICD will further validate the therapeutic usefulness of such combination treatments. Altogether we show here for the first time a potentialization of the activity of BH3 mimetics, acquiring an immunogenic component after biscoumarin-induced non-toxic cellular stress.

We were also able to translate these cell-based assays into a 3D culture environment and in zebrafish xenografts by validating the synergistic anti-cancer effect of OT52 and Mcl-1 or Bcl-xL inhibitors on anchorage-independent cell growth assay, clonogenic and spheroid formations and in zebrafish xenograft in vivo. Altogether, we prove that the inhibitory potential of OT52 through sequential activation of cell stress leading to cell cycle arrest, metabolic catastrophe and senescence sensitized NSCLC cells to BH3-mimetics triggering an unexpected, yet efficient non-canonical cell death both in cells, maintaining efficiency in an in vivo setting.

In conclusion, we investigated the cytostatic anti-cancer mechanisms by which biscoumarin OT52 triggers cellular stress, including metabolic alterations, mitochondrial stress and ER/Golgi stress preceded by STAT3 inactivation. Moreover, we show here for the first time how biscoumarin OT52 is triggering sensitization against BH3-mimetics in NSCLC leading to immunogenic cancer...
cell death mechanisms. Comprehensive understanding of anti-cancer mechanisms in response to OT52 in NSCLC provides novel insight into chemotherapeutic strategy overcoming unexpected side effects and improving drug sensitivity of 1st and 2nd generation chemotherapeutic agents. Although additional validation of OT52-mediated immunogenic cell death pathways must be performed in clinical samples by further investigations to comprehensive understanding based on in vivo immune system. Also, its synergistic activity with BH3-mimetics may provide novel preclinical research avenues. Altogether, we suggest coumarin-derived molecules as novel candidates for future drug investigation in lung cancer.
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Conflict of interest

The authors declare no conflict of interest.
6. References


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Chapter III

Signal transducers and activators of transcription (STAT) regulatory networks in marine organisms: from physiological observations towards marine drug discovery
1. Abstract

Part of our ocean’s richness comes from its extensive history of supporting life, resulting in a highly diverse ecological system. To date, over 250,000 species of marine organisms have been identified, but it is speculated that the actual number of marine species exceeds one million, including several hundreds of millions of species of marine microorganisms. Past studies suggest that approximately 70% of all deep-sea microorganisms, gorgonians, and sea sponges produce secondary metabolites with anti-cancer activities. Recently, novel FDA-approved drugs derived from marine sponges have been shown to reduce metastatic breast cancer, malignant lymphoma, and Hodgkin’s disease. Despite the fact that many marine natural products have been shown to possess a good inhibition potential against most of the cancer-related cell signaling pathways, only a few marine natural products have been shown to target JAK/STAT signaling. In the present paper, we describe the JAK/STAT signaling pathways found in marine organisms, before elaborating on the recent advances in the field of STAT inhibition by marine natural products and the potential application in anti-cancer drug discovery.
2. Abbreviations

ALL: Acute lymphocytic leukemia; AML: Acute myelocytic leukemia; Bcr-Abl: Break point cluster–Abelson; CLL: Chronic lymphocytic leukemia DNA: Deoxyribonucleic Acid; EGFR: Epidermal growth factor receptor; EMEA: European Medicines Agency; EsSOCS: *Eriocheir sinensis* suppressors of cytokine signaling; INF: interferon; IL: interleukin; JAK: Janus kinase; JfGPH: Japanese flounder glycoprotein 130 homologue; JNK: c-Jun N-terminal kinase; NCI: National Cancer Institute; NF-κB: nuclear factor-κB; p53: tumor suppressor protein 53; RbSTAT4: Single transducer and activator of transcription 4 family identified from rock bream; ROS: reactive oxygen species; SD: Sarcodiol; SmSOCS3: *Scophthalmus maximus* suppressors of cytokine signaling 3; SOCS2: suppressors of cytokine signaling 2; STAT: Single transducer and activator of transcription; WHO: World Health Organization; WSSV: White spot syndrome virus.
3. Introduction

Cancer mortality rates remain high despite tremendous research efforts and innovative clinical trials with new drug candidates. Many of these compounds were from natural origins and, according to the World Health Organization (WHO), over 80% of the global population depends on plant-derived herbal medicines for their healthcare, especially in developing countries [14]. Worldwide, over 50% of the anti-cancer pharmaceutical market is based on derivatives from natural resources and their synthetic metabolites, including derivatives from plants, insects, and marine life [15, 16]. Among them, almost 60% of naturally-derived anti-inflammatory [17, 18] and anti-cancer drugs are approved for therapeutic applications [66]. Fruits, vegetables, and herbs are very basic resources used for their anti-oxidant and anti-aging properties by healthcare researchers for a long time [7], based on their contents of vitamins, fiber, antioxidants, phenolic and carotenoid compounds. Development of therapeutic drugs, such as penicillin, aspirin, and cyclosporine, which are derived from natural plant chemicals and soil organisms, dates back many decades. Thus, research and development of naturally-derived products is still highly interesting and promising [19].

Marine-derived natural products have also been found to be highly interesting for the anti-cancer drug industry even though not yet sufficiently exploited [8, 30, 67, 68]. Although oceans cover approximately 70% of our planet, there are still huge untapped marine resources available for the discovery of future therapeutic applications. Approximately 95% of all oceans reach depths of over a thousand
meters, and these depths hold promises for discovery and development of bioactive compounds [15, 16, 28, 29, 62]. Previous studies have shown that the ocean has yet unexplored regions of biological diversity with various organisms residing in extreme environments without oxygen, light and under high pressure [69]. To live under these conditions, organisms must have both physiological and biological adaptations for survival [68]. These adaptations often involve modification of metabolic pathways and methods of genetic regulation, and these condition-acclimatized products should prove to be very valuable for human health.

Furthermore, important FDA-approved drugs were already derived from sponges, for example, the leukemia drugs Cytarabine (Ara-C) (1) and eribulin mesylate (E389) (2), which reduce metastatic breast cancer as well as the herpes simplex virus drug, Vidarabine (Ara-A) (3). Other derivatives from fish like omega-3-acid ethyl ester (4) are active on hypertriglyceridemia. Brentuximab vedotin (SGN-35) (5) originally from mollusk associated cyanobacteria affects anaplastic large T-cell systemic malignant lymphoma, and Hodgkin’s disease [31].

Whereas marine drugs were shown to affect basically all hallmarks of cancer [28] and even to contribute to epigenetic reprogramming [62], selected cell signaling pathways remain to be further investigated. Many compounds were shown to affect nuclear factor-κB (NF-κB) signaling [70], less is known about the impact of natural compounds of STAT-signaling in cancer [71]. Moreover, it appears that STAT signaling in marine organisms is also an immersing field of research so that we suggest here to review both the impact of endogenous STAT signaling as an
essential function in marine organisms followed by the description of anticancer applications of selected anti-STAT marine compounds. This topic will be highlighted by recent advancements in marine compound drug discovery.

Figure 1. Chemical structures of Cytarabine (Ara-C), Eribulin mesylate (E389), Vidarabine (Ara-A), and omega-3-acid ethyl ester
4. Past and present of drug discovery in the field of cancer and marine influences

According to classical pharmacology statements by *Pedanius Dioscorides* in 78 A.D. in *De Materia Medica*, ancient western societies used thousands of natural substances from medicinal herbs, and these have been found to be very useful in industrial and therapeutic drug applications [7]. Nature-derived materials have been the starting point for the development of most chemotherapeutic drugs over the last 40 years [8].

These natural compounds come with outstanding structural diversity and act most likely as physiological regulators with often-unexplored functions. Nevertheless, they serve as basic templates for the development of drugs with biological properties, through interdisciplinary studies using ecology, biology, pharmacology, and chemistry. These interdisciplinary studies aim to create novel bioactive compounds that can then by synthesized, purified, and characterized for therapeutic bioactivities. In 1960, the National Cancer Institute (NCI) began a large-scale project aiming to discover novel anti-cancer compounds [9, 10]. As a result, 35,000 herb samples were screened in cancer cell lines, and *Taxus brevifolia*-derived Taxol (6) was developed as part of this project between 1960 and 1982 [72]. Taxol (6) is an FDA-approved anti-cancer agent to treat ovarian cancer, breast cancer, lung cancer, and gastric adenocarcinoma [11-13].

In the 1950s, several compounds derived from *Cryptotethya crypta* from Caribbean sponges showed initially anti-viral effects. Based on these bioactivity
assays, Ara-C (cytosine arabinoside) (1) was developed several years later and it became an FDA-approved drug [32]. Since then, marine-derived natural chemical compound research has come a long way. Sarcodictyins (7) and Eleutherobin (8) were first isolated from marine corals in 1987 and 1994 and were found to possess anti-cancer activities, with efficacies 50 times greater than Taxol (6).

In 1985, the NCI started a new project in which compounds from marine-derived microorganisms, plants and animals were tested on a panel of sixty human cell lines, including lung, skin, ovary, breast, brain, prostate, kidney, and colon cancer as well as leukemia [33]. By now, at least, seven marine-derived compounds are FDA-approved pharmaceutical drugs, and two compounds are undergoing phase III clinical trials. Five other compounds are in phase II clinical trials, three compounds are in phase I/II, and 13 compounds are on phase I. Targets of these derivatives include a wide range of diseases, including cancer and compounds will be described in the next section.

![Chemical structures of Taxol, sarcodictyin A, and eleutherobin](image)

**Figure 2.** Chemical structures of Taxol, sarcodictyin A, and eleutherobin
5. Clinical Trials of Marine-Derived Anticancer Drugs

Cytarabine (arabinosyl cytosine or cytosine arabinoside, Ara-C) (1), vidarabine (arabinofuranosyladenine or adenine arabinoside, Ara-A) (3), and ziconotide (Prialt) (9) are marine-derived drugs approved by the Food and Drug Administration (FDA) [73, 74].

Cytarabine (1) is a synthetic compound from Tethya crypta, a Caribbean sponge [8]. Cytarabine (1) arrests the cell cycle by working as an S-phase antimetabolite-like cytotoxic drug and it causes inhibition of Deoxyribonucleic Acid (DNA) polymerase and thus synthesis. Cytarabine (1) was developed from both Cytosar-U and Depocyt, which received FDA approval in 1969. Cytarabine (1) is used for treatment of meningeal leukemia, myelogenous leukemia, acute myelocytic leukemia (AML), and acute lymphocytic leukemia (ALL) [75, 76].

![Chemical structure of Ziconotide (Prialt)](image)

**Figure 3.** Chemical structure of Ziconotide (Prialt)
European Medicines Agency (EMEA) also recently approved Trabectedin (Yondelis-1, ET-743) (10), discovered in *Ecteinascidia turbinata* in the Mediterranean and Caribbean. Originally, this synthetic compound was developed from Safracin B (11) with over 90 steps of synthesis and multiple semisynthetic processes including spiro tetrahydroisoquinoline formation, esterification, Curtius rearrangement, carbinolamine formation, and Mannich bisannulation [77]. Trabectedin (10) is capable of arresting cell cycle progression in G2/M phase, and it induces p53-independent apoptosis [78]. This molecule, with the trade name Yondelis, was the first compound approved by the European Union as a marine-derived anticancer agent [27] and is effective in treating relapsed platinum-sensitive ovarian cancer [79]. Sobloidotin (Auristatin PE; TZT-1027) (12), Plitidepsin (Aplidin) (13) from tunicate and Tetrodotoxin (tectin) from puffer fish are in Phase III clinical trials in the USA, and plitidepsin (Aplidin) (13) is also in active clinical trials in the EU. Sobloidotin (Auristatin PE; TZT-1027) (12) is a synthetic compound derived from dolastatin and is a vascular disrupting agent, which targets vascular tumors through the inhibition of tubulin activation [80, 81]. Clinical trials for this molecule were conducted in USA, Japan, and EU, but after Phase I and II clinical trials, the licensing permissions were terminated.

Marine-derived natural compounds in phase II clinical trials include PM00104 (Zalypsis) (14), DMXBA (GTS-21) (15), Elisidepsin (Irvalec, PM02734) (16), Plitidepsin (Aplidin) (13), Plinabulin (NPI-2358) (17), and ILX-651 (tasidotin or synthadotin) (18).
Figure 4. Chemical structures of Trabectedin (Yondelis-1, ET-743) (10), Safracin B (11), Soblidotin (Auristatin PE; TZT-1027) (12), and Plitidepsin (Aplidin) (13).

DMXBA (GTS-21) (15) is derived from anabaserine extracted from nemertines, a phylum of carnivorous, mainly marine worms. DMXBA (GTS-21) (15) shows activation of anti-inflammation mechanisms that are modulated through effects on macrophage specific receptors [82]. Recently, studies demonstrated
improvement of cognition fBRC [83, 84] so that the drug is developed as a novel anti-Alzheimer’s disease compound. PM00104 (Zalypsyis) (14) is isolated from mucus of renieramycins and Jorunna funebris-derived from tunicates and marine sponges [85]. PM00104 (Zalypsyis) (14) induces S-phase cell cycle arrest and apoptosis through induction of DNA double strand breakage in various cancer cells [86] with powerful antitumor functions in prostate, breast, and renal cancer as well as in hematological diseases. This compound was developed as Zalypsyis and is undergoing phase II clinical trials.
Figure 5. Chemical structures of PM00104 (Zalypsins), DMBX (GTS-21), elisidepsin (Irvalec), plinabulin (NPI-2358), and ILX-651 (tasidotin or synthadotin)

Several marine natural compounds are in phase I clinical trials, including bryostatin 1 (19), Marizomib (NPI-0052, salinosporamide A) (20), and E7974 (hemiasterlin) (21), identified in marine sponges [87]. These induce tumor cell
apoptosis, through an antimitotic tubulin-based mechanism [87]. Although the tubulin-mediated agent binds β-tubulin, hemiasterlin predominantly binds to α-tubulin, and has been found to abrogate carcinogenesis in esophageal and prostate cancer [87].Marizomib (NPI-0052, salinosporamide A) (20) is a marine-derived compound from marine *Salinispora tropica* and is a selective proteasome inhibitor [88-90]. By inhibiting the proteasome, non-lysosomal proteins are degraded, which could be a target for the treatment of cancer. Marizomib (NPI-0052, salinosporamide A) (20) is also in phase I clinical trials for treatment of lymphomas, leukemia, multiple myeloma, and various solid tumors.
Figure 6. Chemical structures of bryostatin 1, Marizomib (NPI-0052, salinosporamide A), and E7974 (hemiasterlin)
6. STAT signaling in health and disease

6.1 JAK/STAT signaling pathways

Single transducer and activator of transcription (STATs) are critical mediators of functional responses and specificity in cytokine signaling. After activation of a receptor complex, STATs are phosphorylated on a conserved tyrosine residue, which induces dimerization, nuclear translocation, and DNA binding and leads to the induction of cytokine-responsive genes [36, 37]. The STAT pathway is activated by phosphorylation of cytokines such as interferon (IFN) and interleukin (IL) binding to their specific receptors on the cell surface. In addition to ILs or IFNs, diverse cytokines are now known to trigger STAT activation. These cytokines bind various cytokine receptors that are associated with the Janus kinase (JAK) family [34, 35]. There are four types of mammalian JAKs, tyrosine kinases 2 (TYK2), JAK1, JAK2 and JAK3. After ligand binding, receptor and JAK complexes are phosphorylated, which leads to the assembly and phosphorylation of STATs.

Transcriptional activity and their functional roles are required for dimerization of STATs through phosphorylation of particular tyrosine residues, which promote and mediate binding of regulatory receptors with the formed phosphorylated complex [91]. The c-terminal domain of STAT proteins contains the tyrosine-specific phosphorylation site while the n-terminus controls the DNA binding activity. Phosphorylation is regulated by STAT dimerization via SH2 domain interaction [36, 37, 92].
STAT proteins were initially discovered as interferon (IFN) regulated genes in the 1990s [36, 37, 92]. STATs are composed by seven structurally distinguished members in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [38]. These molecules comprise cytoplasmic transcription factors such as cytokine, hormone, and growth factor signal transduction. Additionally, STATs have functions on their downstream effectors [38, 93-98]. STAT proteins modulate various biological cellular processes including fetal development, organogenesis, apoptosis, growth, differentiation, immune system, and inflammation [93, 97, 99-106]. STAT proteins often exist as monomers in the cellular cytoplasm, but form dimers through SH2 interactions after tyrosine phosphorylation by ligand stimulation [91]. These complex molecules then translocate into the nucleus to promote transcriptional functions. In tumorigenesis, activated STATs are linked to constitutive activation of tyrosine kinases, including Jak, Break point cluster–Abelson (Bcr-Abl), Epidermal growth factor receptor (EGFR), Src. Selected natural compounds were shown to interfere with JAK/STAT signaling [71, 107-109].

6.2. JAK/STAT signaling in marine organisms

The Jak/Stat pathway is considered essential for immune and anti-inflammatory defense. Accordingly it is not astonishing to see this signaling pathway appearing together with adaptive immune system [110] during early vertebrate development. According to Vogl and coworkers, Stats exist in metazoans, choanoflagellates and
slime molds. The same authors describe Jaks in bilaterians but absent in mollusks, round- and flatworms. They conclude that “the Jak-Stat pathway evolved at the base of the bilaterians, but has been lost in some invertebrate groups” [111].

So far a number of studies reported about the physiological function and existence of an interferon/antiviral response in marine organisms. For instance, IFNs with structural and functional properties similar to mammalian type I IFNs, were cloned from various types of fish, including Atlantic salmon, channel catfish, pufferfish, zebrafish and other teleost fish [112]. An important question was about the physiological role of these genes and whether they were required for anti-viral defense of marine organisms related to JAK/STAT activation? Interestingly Santos et al. discovered a type-1 cytokine receptor (Japanese flounder glycoprotein 130 homologue; JfGPH) with JAK and STAT 3 binding motifs in the cytoplasmic region. These early results suggested a mediatory role for JAK/STAT signal pathway in fish, so that the authors hypothesized a role in immune response, and reproduction/development [113]. Later suppressors of cytokine signaling 2 (SOCS2) from Eriocheir sinensis (EsSOCS2) were shown to be inducible by bacteria so that Zhang et al. concluded that this pathway was most likely involved in the immune defense responses in E. sinensis [114]. In addition, the economically important large yellow croaker (Pseudosciaena crocea) suffers by outbreaks of marine bacteria including Aeromonas hydrophila. Here Mu et al. showed that inflammatory response might play an important role in the early stages of fish infection. The authors validated that signaling cascades such as the Toll-like receptor, JAK/STAT, and MAPK pathways to be regulated by A.
hydrophila infection and to play essential roles in large yellow croaker immune response to bacterial infection [115]. Similarly turbot *Scophthalmus maximus* SOCS homologue (SmSOCS3) is a cytokine-inducible suppressor of pro-inflammatory cytokine signaling in HK macrophages. Regulated expression of SmSOCS3 is essential for innate immune response against bacterial infection [116]. Li et al. published that the transcription of STAT in shrimp was regulated by White spot syndrome virus (WSSV) infection allowing the authors to hypothesize the existence of such a regulatory pathway in shrimp, which would be responsive to viral infection [117]. Many other marine invertebrates with economic importance were characterized concerning their JAK/STAT signaling components: SOCS-2 from pearl oyster *Pinctada fucata* plays a regulatory role against the stimulation [118]; a member of the STAT4 family was discovered in rock bream (*Oplegnathus fasciatus*) (RbSTAT4) and its regulation under pathological stimuli was investigated [119]; Janus kinase (designated as LvJAK) gene was cloned and characterized from whiteleg shrimp *Litopenaeus vannamei* [120].
Figure 7. Schematic models for JAK/STAT cell signaling pathway and the inhibitory actions for JAK/STAT target anticancer drugs.
These examples demonstrate the essential function of JAK/STAT like functions in marine invertebrates so that discovery of compounds interfering with these mechanisms also from marine organisms should be expected, considering the biochemical warfare described so far between marine organisms related to space and nutrients as we previously reported [67].

6.3. JAK/STAT-inhibiting anticancer drugs from marine organisms

A large number of natural anti-cancer drugs target control of cell signaling pathways involved in carcinogenesis, which is triggered by improper multiple cellular processes including PI3k/Akt, mTOR, NF-κB or JAK/STAT signaling. Even though not many compounds of marine origins were shown to possess activity against JAK/STAT signaling, several drugs targeting the regulation of these pathways are undergoing clinical trials for FDA approval. Bryostatin 1 (19) is isolated from Bugula neritina, a bryozoan. These molecules were tested in over 80 clinical trials for disease treatment and were shown to bind to protein kinase C without carcinogenesis promoting activity. According to Battle et al., bryostatin-treated chronic lymphocytic leukemia (CLL) cells show induction of protein kinase C and in turn activation of STAT1, which is essential for CLL differentiation. This research concludes that bryostatin 1 (19) promotes MAPK activation, which then induces the production of IFN-γ triggering JAK/STAT1 signaling [121].
Hong et al. observed that the marine cembrenolide diterpene (1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide (LS-1) \(^{(22)}\), possessed antiproliferative and cytotoxic potential in colon cancer cells \textit{via} a reactive oxygen species (ROS)-dependent mechanism. Treatment of HT-29 cells with LS-1 \(^{(22)}\) resulted in apoptosis induction \textit{via} the intrinsic mitochondrial pathway. This compound also induced phosphorylation of c-Jun N-terminal kinase (JNK) and dephosphorylation of STAT-3. These findings underline anticancer efficacy of LS-1 indirectly involving inhibition of STAT3 \(^{[122]}\).

Figure 8. Chemical structure of (1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide (LS-1)

Figure 9. Chemical structure of sarcodiol (SD) \(^{(23)}\)
Sarcodiol (SD) (23) is a semi-synthetic derivative of sarcophine, a marine natural product. The authors show that SD (23) inhibits the *de novo* DNA synthesis and enhances fragmentation of DNA. Interestingly, SD (23) inhibits the expression levels of STAT-3 and cyclin D1. SD (23) treatment also enhances cellular levels of tumor suppressor protein 53 (p53) and induces caspase-dependent cell death mechanisms [123]

![Sarcodiol (SD) (23)](image)

**Figure 10.** Chemical structure of apratoxin A (24)

Apratoxin A (24) is a cytotoxic marine natural product that rapidly inhibits STAT-3 phosphorylation. Apratoxin A (24) inhibits interleukin 6 induced activation of JAK/STAT signaling and prevents N-glycosylation of receptor tyrosine kinases triggering proteasomal degradation. Moreover the authors used proteomics to demonstrate down-regulation of proteins in the endoplasmic reticulum where N-glycoprotein are synthesized. By *in vitro* cell free systems, apratoxin A (24) was shown to prevent co-translational translocation of proteins of the secretory pathway [124].
Figure 11. Chemical structures of two enone fatty acids, GV-c9, and GV-c10 (25)

Two enone fatty acids, GV-c9, and GV-c10 (25) from Gracilaria verrucosa, a marine red alga with anti-oxidant and anti-cancer properties, inhibited NF-κB reporter activity by blocking NF-κB nuclear translocation as well as JAK/STAT (p-STAT1) signaling [125].

7. Conclusion and Perspectives

In the field of marine compound JAK/STAT inhibitors we feel that much remains to be discovered. Even though the number of bona fide JAK/STAT inhibitors remains modest we strongly believe that many inhibitors so far identified as NF-κB inhibitors of marine origins could as well act as JAK/STAT inhibitors considering the fact that many compounds from terrestrial plants showed important redundancy concerning the inhibition of both key inflammation pathways. Active research in this directing could generate additional hits. Finally
both NF-κB and STAT transcription factors are regulated by epigenetic post-translational regulatory mechanisms. Considering the important number of epigenetically active molecules also from marine origins, we speculate here about marine compounds that would confer epigenetic regulation whether at the level of DNA methylation, histone modifications or small regulatory RNA expression, and hypothesize that marine natural compounds could regulate the JAK/STAT regulatory pathways in such a way [62, 126, 127].
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Conflict of Interest

The authors declare no conflict of interest.
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Chapter IV

Preclinical assessment of the bioactivity of anticancer compounds by spheroids, colony formation assays, and zebrafish xenografts
1. Abstract

Pre-clinical screening of novel therapeutic agents both in vitro and in vivo are an essential tool for the cancer drug discovery process. It is well-known that human cancer cell lines do not respond to therapeutic compounds in 2D monolayer cell cultures as they do in vivo or 3D culture systems. Therefore, in recent years a paradigm shift was observed in pre-clinical research to validate potency of new molecules in 3D culture system which more precisely mimic the tumor microenvironment. These systems characterize the disease state in a more physiologically relevant manner and help to gain better mechanistic insight and understanding of the pharmacological potency of a given molecule. Moreover, with the current trend in improving in vivo cancer models, zebrafish has emerged as an important vertebrate model to assess in vivo tumor formation and study the effect of therapeutic agents [I].
2. Introduction

Cancer is caused by cellular mutations and as a consequence biochemical signaling pathways are disrupted triggering uncontrolled cell division and resistance to cell death leading to tumor formation. Tumors interfere with physiological functions of digestive, nervous, circulatory systems and subsequently neighboring tissues. Despite extensive research efforts, cancer remains the most prevalent life-threatening disease in the world [2].

One of the significant limitations associated with the development of new efficient targeted therapies is the failure of commonly used assays to simulate the exact biological outcome of drug exposure [3]. Cancer drug discovery still mostly relies on testing the efficacy of therapeutic agents in cancer cell lines cultured in 2D monolayer cultures which seldom align with the outcomes in clinical trials [4,5]. Therefore, there is a growing interest to develop better conventional cancer models which could mimic more intently the native features of tumors in vivo [6].

In recent years, the increasing interest in 3D culture models resulted in the development of improved methodologies to produce 3D tumor models.

Here, we present an approach with three different 3D cell culture techniques allowing to improve the understanding of the potency of therapeutic agents before more in-depth in vivo assays. Our method consists in combining colony and spheroid formation assays with an in vivo tumor formation test based on a zebrafish model to further validate the efficacy of novel compounds and monitor cancer progression in a living organism. Altogether, the three different approaches
described here allow a much more efficient pre-clinical screening of novel drug-like compounds, compared to 2D cell culture.

3. Materials and methods

3.1 Colony formation assays

3.1.1. K562 cells are cultured in RPMI media supplemented with 10 % FBS and 1 % antibiotics.

3.1.2. On the day of the experiment, cell concentration is determined, and cells are collected at a concentration of 50,000 cells in 100 µl of 1x sterile PBS in 1.5ml tubes.

3.1.3. 1.1 mL of methylcellulose is dispatched in 15mL tubes. For each condition, one tube is prepared.

3.1.4. 1.1 mL methylcellulose is supplemented with 10% FBS.

3.1.5. Seeding concentration for cells is 1000 cells/ml.

3.1.6. After adding the cells, tubes are vortexed for 1 minute holding them vertically on the Vortex (Vortex Genie 2) set at highest speed 10, to mix methylcellulose and cells well.

3.1.7. The test compound or DMSO is added. Depending on the concentration of compounds and the volume added for each condition, the maximum volume of DMSO is used for the control condition.
3.1.8. Tubes are again vortexed for 1 minute.

3.1.9. For each assay, 1 mL of the mixture is seeded in one center well of a 12-well cell culture plate.

3.1.10. Empty wells are filled with 1 mL of sterile water or 1xPBS to provide humidity.

3.1.11. Culture plates are incubated for 10 days in a 5% CO2 incubator at 37°C.

3.1.12. After 10 days, 200 µl of MTT solution from a stock solution of 5 mg/mL is dissolved in 1xPBS and added into each well.

3.1.13. After 10 to 15 minutes of incubation, viable colonies turn violet.

3.1.14. The image is captured using a LAS4000 camera (GE Healthcare Life Sciences) using the white background plate. Settings are: method: digitize; exposure type: precision; exposure time: manual, 1 second; sensitivity/resolution: high resolution. The image is stored in tiff format.

3.1.15. Quantification of viable colonies is done using Image J software. Command followed Image-Type-8 bit; Adjust-threshold (threshold is set to control and kept constant for all conditions); Analyze- analyze particle.

3.2 Spheroid formation assay by the hanging drop technique.

3.2.1. U373 cells are cultured at a concentration of 20,000 cells/cm² in RPMI media supplemented with 10% FBS and 1% antibiotics.
3.2.2. On the day of the experiment, Petri dishes are prepared by adding 15 mL of sterile 1x PBS per condition.

3.2.3. Drops for the spheroid formation experiment contain 1000 or 2000 or 3000 cells in 25 µL of media. The number of cells that form uniform spheroids with an intact outer boundary is considered adequate to form spheroids.

3.2.4. Pipet 25 µL of spheroid forming cell suspension by using a multi-channel pipet or regular pipet as single drops on the inner side of the Petri dish lid by keeping enough space between each drop.

3.2.5. Replace the petri-dish lid with the drops on top of the Petri dish filled with sterile 1x PBS.

3.2.6. Petri dishes with hanging drops are stacked together and kept in an incubator for 4 days at 5% CO2 at 37 °C.

3.2.7. After 4 days of incubation, aspirate spheroids and collect all spheroids in a six-well plate.

3.2.8. Capture images of the spheroids using a microscope at 4x magnification.

3.2.9. The viability of the spheroids is determined by using cell Titre glow 3D reagent from Promega corporation following manufacturer’s protocol.

3.2.10. Quantification of spheroid size and volume is conducted using Image J and ReVisp software. For Image J: “Polygonal Selection” to select the boundary of the spheroid; “Analyze-measure” to obtain the surface area of the spheroids.
For ReVisp software each spheroid is saved as a mask, software commands are followed, and volume is quantified as voxels.

3. 3 Zebrafish xenograft assay

3.3.1. Stock solution preparation

3.3.1.1. 30x Danieau’s medium: Add 1740 mM of NaCl, 21 mM of KCl, 12 mM of MgSO4.7H2O, 18 mM of Ca(NO3)2, and 150mM of HEPES solution to 1L of distilled water. Adjust to the pH to 7.6.

3.3.1.2. 1% (50x) tricaine solution: Add 20 mg of ethyl 3-aminobenzoate methanesulfonate (tricaine) to 2ml of distilled water.

3.3.1.3. 1% phenol Red-PBS solution: Add 2 µl of Phenol Red sodium salt solution to 198 µl of sterilized PBS solution.

3.3.1.4. 3% methylcellulose: Add 3 g of methylcellulose to 100 ml of 1x Danieau’s medium.

3.3.2. Zebrafish egg production and incubation

3.3.2.1. Place two female and one male zebrafish independently in a partition tank in the dark for 24 h before fertilization.

3.3.2.2. After 24 h of separation, fish of both genders are mixed to initiate mating.

3.3.2.3. Collect fertilized eggs in the Petri dish with fresh Danieau’s solution.
3.3.2.4. Wash eggs for three times with Danieau’s solution and incubate for 8 h at 28.5°C.

3.3.2.5. After 8 h of incubation in Danieau's solution, change to Danieau’s medium containing 0.03% 1-phenyl-2-thiourea (PTU) to inhibit pigmentation.

3.3.2.6. Sort out unfertilized and undeveloped embryos to prevent contamination.

3.3.3. Embryo incubation and dechorionation

3.3.3.1. 24 h post fertilization (hpf), dechorionate zebrafish embryos using sharp forceps.

3.3.3.2. Incubate hatching embryos in Danieau’s medium with 0.03% PTU at 28.5°C until 48 hpf.

3.3.4. Compound-treated cell preparation

3.3.4.1. Cells are seeded at the usual densities in a culture flask.

3.3.4.2. After 24 h of seeding, compounds are added at the required concentration and time in an incubator at 37°C. The treatment time is selected to maintain the viability of the cells but to commit towards cell death.

3.3.4.3. 2 h before treatment termination, cells are stained with 4 µM of fluorescence cell tracker CM-Dil dye at 37°C in a CO2 incubator.

3.3.4.4. After 2 h of incubation, cells are harvested (suspension cells) or trypsinized (adherent cells), and 106 cells are diluted in 50 µl of phenol Red-PBS solution.
3.3.5. Micro-injection of cancer cells for xenograft formation

3.3.5.1. Zebrafish are anesthetized in a 0.02% tricaine solution until they settle down on an agar plate.

3.3.5.2. A 1.0 mm (outside diameter, OD) glass capillary) is pulled by a micropipette puller (Setting: P: 300, Heat: 260, Pull: 60, VEL: 50, and Time: 200). The pulled capillary is cut using a syringe.

3.3.5.3. Microcapillaries are filled with 20 µl of cell solution including Phenol Red using PBS 20 µl of micro-loader.

3.3.5.4. Injection pressure and time are adjusted to dispense 2 nl per injection.

3.3.5.5. 100-200 cells are injected into the yolk sac by 6 to 10 nl for 3 to 5 injections.

3.3.5.6. After injection, fish are recovered in fresh Danieau’s medium containing PTU solution for 10 to 30 min.

3.3.5.7. Fish are dispatched in 24-well plates with Danieau’s medium containing PTU solution are and incubated for 72 h at 28.5°C.

3.3.5.8. After 72 h of incubation, fish are anesthetized in a 0.02% tricaine solution and are immobilized on a glass slide with a drop of 3% methylcellulose.

3.3.5.9. 5x pictures are taken by fluorescence microscopy, wavelength set to 620 to 750 nm. Fluorescent tumors are quantified by Image J software.
Table 1. Materials required for Colony formation assay.

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Table 2. Materials required for spheroid formation assay by the hanging drop technique.

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4. Results

In figure 1, chronic myeloid leukemia cell line K562 was grown in methylcellulose to form colonies after treatment with imatinib at the indicated concentrations. The results show that after 10 days of incubation, imatinib dose-dependently decreases the number of colonies compared to DMSO-treated controls.

In figure 2, U373 cells were grown to form tumor spheroids by the hanging drop technique. After 4 days of incubation, images of spheroids were taken. Quantification of the average size and volume of spheroids was determined using Image J and ReVisp software.

In figure 3, leukemia cell tumor formation was inhibited in the zebrafish yolk sac by compound treatment. Quantification of fluorescence intensity correlates to the inhibitory potential of the compound.
Figure 1. Inhibition of K562 colony formation by imatinib. (A) Images of a K562 colony formation experiment treated with indicated doses of imatinib. (B) Quantification of the number of colonies.
Figure 2. Formation of U373 tumor spheroids by the hanging drop technique. (A) Images of U373 tumor spheroids after 4 days. (B) Graph showing the average size of the spheroids. (C) Graph showing the volume of the spheroids.
Figure 3. Inhibition of tumor formation by a compound. (A) Pictures are showing the inhibitory effect of the drug. (B) Quantification of tumor formation.
5. Discussion

In the colony formation assay with methylcellulose, the rate of colony formation depends on the cell type. Usually, for non-adherent cells, the number of colonies is much higher compared to adherent cells. For example, for K562 cells, the number of colonies ranges from 180 to 200. However, for PC3 cells the number of colonies ranges from 80 to 100 [7]. The suggested seeding concentration may vary from 1 x 103 to 1.5 x 103. Colony formation assays using methylcellulose are a valuable technique routinely used for measuring tumorigenesis in various cancer cell lines, but it has some limitations. Often the recovery of viable colonies after the experiment is inconvenient, and cells do not grow well once isolated from methylcellulose and seeded in regular medium. Moreover, if the compound tested has a low bioavailability or stability, the treatment cannot be repeated, which limits the overall output of the experiment. Nevertheless, it is a well-accepted pre-clinical technique to better understand the mechanism of cancer progression in a 3D environment.

Spheroid formation assays are performed with adherent cells and gained wide popularity in cancer drug discovery as they exhibit physiological traits such as increased cell survival, tumor morphology and a hypoxic core which are more representative of an in vivo situation. The overall size, texture, and integrity of spheroids obtained by the hanging drop technique may vary between cell lines. For instance, the glioblastoma cell line U373 cells form spheroids which are uniform in size, have stable integrity and the outer boundary is very regular [8]. Prostate cancer cell line PC3 cells form spheroids whose size may vary, integrity
is looser, and the outer boundary is less regular [9]. Moreover, the starting concentration of cells used to form spheroids may also influence its overall development. To increase the throughput of compound screening such a 3D culture system, some companies are developing specific substrates, low attachment assay plates, and scaffolds improving the formation of 3D structures [10]. With further technical advances, development of the most representative 3D tumor model will allow a better understanding of the pharmacology of novel compounds for a given type of cancer.

Zebrafish xenografts are considered as the most cost-effective animal assays routinely used for anticancer drug development. We optimized the zebrafish in vivo system with various solid and hematological cancer types as well as with patient-derived cells. Our established system confirms the in vitro drug effect in an animal model. Moreover, cell dissemination from the yolk sac to the tail region of the zebrafish is mimicking metastasis. Altogether, this technique can contribute to rapid screening of drugs in a preclinical setting.
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DISCLOSURES

The authors have nothing to disclose.
6. References


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폐암은 조직형에 따라 크게 소세포성 폐암과 비소세포성 폐암으로 구분되며, 비소세포성 폐암은 발생 부위에 따라 선암, 폐평상피세포암, 대세포암 등으로 구분된다. 최근에는 비흡연자에서도 폐암 유병률이 증가하고 있는 한편, 종격동 림프절, 간, 뼈, 부신 등의 전이율 또한 높아 사망률이 증가하는 추세이다. 미국 국립 보건원 (national Institute ofHealth, NIH)의 최근 통계에 따르면 폐암은 전체 암으로 인한 사망률 대비 약 35.1%로 가장 높은 사망률을 보이고 있으며, 이는 폐암의 경우 초기에는 각 증상이 없는 경우가 많아 조기발견이 쉽지 않으며, 신뢰할만한 바이오마커의 부족 등에서 기인한다.

폐암 치료에 있어서는 수술적 절제를 통한 완치가 가장 이상적인 경우이지만, 실제 임상 통계에 의하면 폐암환자의 약 25% 이하에서만 수술적 절제가 가능하며, 이외에는 항암화학치료나 방사선 치료 등의 병용을 통해 양성주의 사망률을 유도한 후 수술적 치료를 고려하는 경우가 대부분이다. 따라서 폐암의 유전학적 세포신호전달 경로에 대한 심도 있는 이해와 이를 통한 적절한 항암제 개발을 통해 사망률감소 및 치료 효율성의 증가를 기대할 수 있을 것이며, 이에 본 연구는 천연물을 기반으로 한 항암제 발굴에 매우 유용한 정보를 제공할 수 있을 것으로 사료된다.

최근에는 특정 유전적 변이를 타겟으로 하는 2세대 표적치료제를 통해 양성주의 더욱 선별적으로 적용시키고 있으며, 뿐만 아니라 면역항암제로 알려진 3세대 항암제에 대한 임상 연구도 활발히 진행되고 있다. 이는 환자의 장기적인 면역활성을 활성화 시킴으로써 양성주의 사망 및 정상세포의 활성화를 목적으로 하기 때문에 항암제에서 기인하는 부작용이 적고, 암의 변이 종류나 특정 표적에 관계없이 적응 가능하며 새로운 항암제의 패러다임으로 각광받고 있다.

하지만 임상적으로 상당수에서 치료 효과가 크지 않아 치료 대상이 명확하게 분류되지 못했으며 자가면역계 질환 등의 면역계....
부작용이 뚜렷하게 규명되지 않았다. 이에 부작용을 줄이고 약리 효과를 항상시키고자 천연물에서 유래한 면역항암제에 대한 연구가 활발하게 진행되고 있으나 아직 임상적으로 적용 가능한 천연물 유래 면역 항암제는 전무하다.

이에 본 연구에서는 다양한 약리효과로 오랫동안 학계의 주목을 받고 있는 Coumarin 화합물을 대상으로 폐의 상피세포에서 유래한 신암을 타겟으로 하는 표적항암제 후보 물질을 제시하고자 분자 유전학적인 세포 신호전달 분석 및 면역반응을 통한 표적 항암기전에 관한 연구를 진행하였다.

쿠마린 (Coumarin) 은 hetero ring 계열의 유기화합물로서 통카빈, 카시아 계피, 라벤더 등 약 800여가지 이상의 약용 식물에서 분리되는 천연물로 알려져 있으며, 항산화, 항염증, 항혈전 등의 다양한 생리활성 기능을 가진 것으로 보고되고 있다. 또한 생물학적 중요성과 구조적 특징으로 인해 효과적인 약물 합성법 개발을 위한 많은 시도가 되어 왔으나, 아직까지 주목할만한 거시적인 성과는 전무하기에 쿠마린을 대상으로 한 항암제 연구는 항암제 개발에 있어 중요한 자료가 될 수 있을 것으로 사료된다.

본 연구에서는 쿠마린에 분자수식치환을 통하여 강력한 항암효과를 지닌 OT52 합성물을 생성하였고, OT52가 암을 촉진시키는 STAT3와 같은 주요한 세포신호전달 경로를 차단함으로써 암의 성장 및 진행을 억제할 수 있음을 규명하였다. 특히 OT52는 세포독성의 없는 무해한 농도에서 G1 단계 세포주기조절을 통해 효과적으로 암세포의 성장 및 전이를 선택적으로 억제하는 것을 확인하였다. 따라서 본 화합물을 통해 항암제로 인해 발생할 수 있는 부작용을 최소화 할 수 있을 것으로 사료된다. 또한 OT52는 세포소기관인 ER, Golgi, 미토콘드리아 등에 복합적인 경로를 통하여 세포 스타트레스를 유발하여 활성산소를 증가시키고 비정상적인 세포조직체를 형성함으로써 암세포의 성장을 저해시키는 기전을 규명하였다.

또한 OT52와 현재 임상시험단계에 있는 BH-3 mimetics 와의 병용요법을 통해 유의미한 상승효과가 있음을 확인하였고, 이는
OT52에 의한 세포 스트레스가 BH-3 mimetics 에 의해 직접적으로 면역세포 사멸을 유도함으로써 이는 3세대 항암제로서의 개발 가능성을 시사한다.

본 연구에서는 동물모델로서 제브라피지를 이용하여 OT52 와 BH-3 mimetics 의 생체 내 항암 효과를 입증하였다. 제브라피지는 사육 및 관리가 용이하고, 시험비용절감효과가 탁월하며 한번에 약 200여개 이상의 알을 수정하므로 개체수로 인한 실험 데이터 변동의 폭이 크지 않아 데이터의 신뢰도를 향상시킬 수 있다는 장점이 있다. 뿐만 아니라 본 연구에서 실험 대상으로 한 7일 이하의 치여에서는 면역계 시스템이 발달 되지 않았기 때문에 항암 효과를 입증할 때에 동물 자체에서 오는 면역 반응을 배제할 수 있고, 따라서 OT52 와 BH-3 mimetics 화합물 본연의 항암효과를 검증할 수 있다는 점에서 항암제 스케린에 있어 적합한 동물모델 개발에 기여할 수 있을 것으로 사료된다.

주요어: non-small cell lung cancer, Coumarin, OT52, ER/Golgi, ROS, ATP, Spheroid, Apoptosis, Immunogenic cell death, Zebrafish