SAPIENZA
UNIVERSITÀ DI ROMA

Department of Physiology and Pharmacology

"V. Erspamer"

Dissertation in fulfillment of requirements for the

Degree of Doctor of Philosophy in Pharmacology

XXXV Cycle

Study of expression and role of Metabotropic Glutamate Receptors in Human Acute Myeloid Leukemia cell lines and involvement of L1-CAM in migration of mGluR1^{+} mouse melanoma cell lines

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Abstract: English

mGluRs are a family of G-coupled protein receptors with widespread expression in the central nervous system (CNS), some normal tissues and in various cancers. The predominant functions of mGluRs in the CNS are modulating presynaptic neurotransmission but in cancers, mGluRs are involved in regulating cell proliferation.

Acute Myeloid Leukemia (AML) is a clonal, malignant disease of hematopoietic tissue and is the most common type of acute leukemia in adults and the elderlies. Despite a wide number of drugs available to treat this disease, there are many unmet needs in AML therapy; relapsed or refractory patients or those unable to receive intensive chemotherapy display a poor prognosis.

Skin cancer is described as the abnormal multiplication of skin cells, which is frequently detected in sun-exposed regions. Basal cell carcinoma, squamous cell carcinoma, merkel cell carcinoma, cutaneous lymphoma, kaposi sarcoma, and melanoma are the six primary forms. Melanoma is the most severe form of skin cancer and is caused by melanocyte transformation. In the United States, invasive melanoma is expected to account for about 100,000 new cases and over 7,000 fatalities from skin cancer in 2022.

In this study, there are two distinct studies, in one, we evaluated metabotropic glutamate receptors (mGluRs) to assess if one of more of mGluRs may be a putative therapeutic target in AML. We have found a novel, previously unknown role of mGluRs in AML. The second study is to use mGluR1^{+} mouse melanoma cell lines to determine if the presence of L1-NCAM (CD171), a neural cell adhesion molecule 1 is involved in cell migration as shown previously in glioblastoma cells.

Abstract: Italiano

I recettori metabotropici del glutammato (mGluR) sono una famiglia di recettori accoppiati a proteina G con espressione diffusa nel sistema nervoso centrale (SNC), in alcuni tessuti normali e in vari tipi di cancro. Le funzioni predominanti degli mGluR nel SNC sono la modulazione della neurotrasmissione presinaptica, invece nei tumori gli mGluR sono coinvolti nella regolazione della proliferazione cellulare.

La leucemia mieloide acuta (AML) è una malattia clonale maligna del tessuto ematopoietico ed è il tipo più comune di leucemia acuta negli adulti e negli anziani. Nonostante l'ampio numero di farmaci disponibili per il trattamento di questa malattia, ci sono molte esigenze non soddisfatte nella terapia dell'AML; i pazienti recidivanti o refrattari o quelli che non sono in grado di ricevere una chemioterapia intensiva presentano una prognosi sfavorevole. Il cancro della pelle è descritto come una moltiplicazione anormale delle cellule cutanee, che si riscontra frequentemente nelle regioni esposte al sole. Il carcinoma basocellulare, il carcinoma a cellule squamose, il carcinoma a cellule di Merkel, il linfoma cutaneo, il sarcoma di Kaposi e il melanoma sono le sei forme primarie. Il melanoma è la forma più grave di cancro della pelle ed è causato dalla trasformazione dei melanociti. Negli Stati Uniti, si prevede che il melanoma invasivo presenterà circa 100.000 nuovi casi e oltre 7.000 decessi nel 2022.

In questo lavoro sono stati condotti due studi distinti: in uno abbiamo valutato i recettori metabotropici del glutammato (mGluRs) per valutare se uno o più mGluRs possano essere un putativo bersaglio terapeutico nell'AML. Abbiamo trovato un ruolo inedito, precedentemente sconosciuto, degli mGluR nell'AML. Il secondo studio prevede l'utilizzo di linee cellulari di melanoma di topo mGluR1^{+} per determinare se la presenza di L1-NCAM (CD171), la molecola di adesione cellulare neurale 1, sia coinvolta nella migrazione cellulare, come dimostrato in precedenza nelle cellule di glioblastoma.

Background: leukemia

Rudolf Virchow, who detected an unusually large quantity of white blood cells in a patient's blood sample, identified the origin of leukemia in 1845. Virchow gave the term for the illness, "leukämie," which is derived from the Greek words "leukos," which means "white," and "aima," which means "blood"; literally, "white blood."[1].

The first scientific report of a case of leukemia in medical literature was made in 1827 by the French physician Alfred-Armand-Louis-Marie Velpeau. He described the case of a 63-year-old man who had developed a disease marked by fever, weakness, urinary stones, and significant enlargement of the liver and spleen; at the time, he had also noted that the patient's blood had the consistency of gruel and suggested that this was caused by white blood cells [1].

Based on extensive studies previous by others, we now know that mutations in the early phases of cell differentiation in myeloid or lymphoid hematopoietic cell lines yielded these two kinds of leukemia, and that leukemia is a complicated illness that can strike at any age.

Hematopoiesis

The term hematopoiesis refers to the development and maturation of blood components, or the process of producing blood cells. During the transition from intrauterine to extrauterine life, the red marrow of the bones and lymphoid tissue are primarily in charge of this function. Particular mesenchymal cells of the arterial walls supply in the initial stages of embryonic development, long before the creation of the liver. A second time, the liver is involved, and its hematopoietic activity reduces during the second month of intrauterine life, when bone marrow progressively takes over. Medullary hematopoiesis produces constituents of the red series, granulocytes, monocytes, and platelets [2].

Hematopoiesis is a biological process that occurs when self-renewing stem progenitor cells develop into mature blood cells that perform certain biologic roles. *In vivo* homeostasis of the whole hematopoietic system necessitates careful regulation of the systems that govern proliferation, cell destiny, cell death, differentiation, cell-cell contact, and migration [2].

Hematopoiesis stem cells give rise to cells of all blood lineages, including T and B cells in the lymphoid lineage and neutrophils, eosinophils, basophils, monocytes, macrophages, megakaryocytes, platelets, and erythrocytes in the myeloid lineage [3]. In this perspective, many transcription factors are necessary for the formation of a hematopoietic lineage, such as GATA-1 for the erythroid and megakaryocytic lineages and PU.1 for myeloid development [4]. The retinoic acid receptor (RAR), which is essential for neither lineage, definitely modulates blood cell formation; therefore, controlled expression of lineage-specific genes is essential for hematopoiesis proliferation and differentiation signals [4].

Hematopoietic stem cells (HSCs) constantly create uncommitted progenitor cells that proliferate and develop into fully functioning blood and immune cells of diverse lineages [5]. Because the HSC pool creates progenitors continually, the quantity of HSCs must be adequately maintained by coordinating dormancy, self-renewal, and lineage differentiation. These are largely adult blood cells with limited lives, such as erythrocytes, granulocytes, and platelets [5-7]. Hematopoiesis is, therefore, an organized and hierarchical process at the apex of which we find hematopoietic stem cells (totipotent cells) from which all the cellular components of the blood will originate. Multipotent progenitors (MPPs) are stem cells that give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs); they are the ancestors of the myeloid and lymphoid lineages, respectively. CMPs will give rise to two different cell lines: the progenitors of granulocytes and macrophages (GMPs) and the erythroid and megakaryocyte progenitors (MEPs), from which erythrocytes and platelets are derived. CLPs, on the other hand, is the source of immune system cells such as lymphocytes T and B, as well as natural killer (NK) cells.

Malignant Hematopoiesis

AML research has revealed that, like regular hematopoiesis, the leukemic clone has a hierarchy that matches the differentiation hierarchy of typical hematopoiesis. The leukemic clones have stem cell features as well as more developed cells that are unable to self-renew. These stem-like cells are known as leukemia stem cells (LSCs) [5]. LSCs have an infinite ability for self-renewal and constantly create immature blood cells. Undifferentiated leukemic "blasts" accumulate because leukemias commonly have mutations that block

hematopoietic maturation mechanisms; one of the distinguishing features of AML is this differentiation block [5]. The two possible ways for HSCs to transform into LSCs are: the HSCs can acquire infinite proliferative potential, or a committed progenitor can acquire self-renewal capabilities [5]. Because the phenotype of LSC reflects the consequences of successive and cumulative mutations, it is critical to realize that leukemias, particularly AML, are genetically different [5,8,9]. LSCs have been assumed to share many other biological properties with normal HSCs, such as a $CD34^{+}/CD38^{-}$ immunophenotype and being predominantly in the quiescent, G0 phase of the cell cycle [10]. However, given the wide variety of driver mutations in AML, the biological features (immunophenotype, cell cycle activity, and LSC frequency) vary substantially amongst different leukemias.

Global Epidemiology of Leukemia

Leukemias can appear at any age, from newborn to elderly, but distinct kinds have highly varied age distributions, with acute lymphoblastic leukemia (ALL, 1-10 years old) being most prevalent in young childhood and scarce in adults, whereas acute myeloid leukemia (AML, 65-84 years old) is less common in children but becoming more common in older individuals [11]. Leukemia was the 15th most often diagnosed disease and the 11th major cause of cancer death globally in 2018, according to GLOBOCAN, accounting for 437.033 incident cancer cases and 309.000 cancer fatalities [12]. As of now, the figures are different; according to the GLOBOCAN, in 2020 (Fig. 1), there would be a total of 474.519 (both sex) instances of leukemia, while 311.594 people would die from it worldwide.

Males experience a greater worldwide burden of the leukemia illness than females do [12].

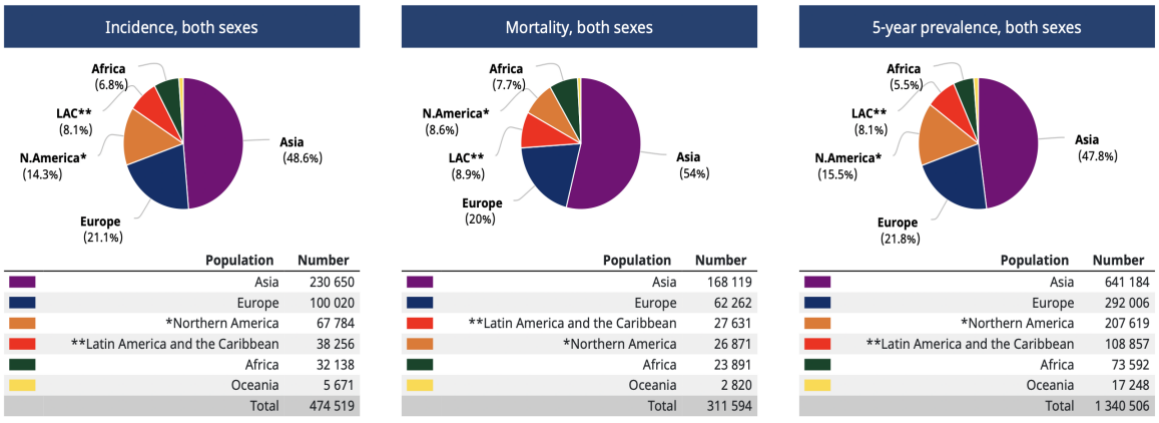


Fig. 1: https://gco.iarc.fr/today/data/factsheets/cancers/36-Leukaemia-fact-sheet.pdf WHO, GLOBOCAN The Global Cancer Observatory, 2020

In addition, the incidence of leukemia increases significantly in nations with high to very high human development index (HDI) when countries in the GLOBOCAN database are compared. Likewise, death rates are greater in nations with high HDIs (in both sex).

Risk factors for Leukemia

Radiation (therapeutic, occupational), chemotherapy, family history, genetic disorders and abnormalities, chemical exposures, and lifestyle variables including smoking are among those exposures that have been repeatedly identified as risk factors for leukemia. The most significant risk factors affect numerous subtypes of leukemia, even if specific ones have been linked to particular leukemias [12].

AML can result from treatment with a DNA-damaging modality for a prior malignant illness or as a side effect of a previously identified hematologic malignancy [11]. About 8% of adult AML patients had a condition linked to their treatment, and 12% experienced myeloproliferative neoplasia in the past [13]. Therapy-related illness, which is more prevalent in older patients, is mostly driven by chemotherapy, including alkylating drugs and topoisomerase II-inhibitors, and radiation used to treat breast cancer and lymphoma [11].

Physical and chemical risk factors

Ionizing radiation exposure has been associated with AML [12,14]. Furthermore, therapeutic radiation has been linked with an increase in the incidence of subsequent AML [11,12]. Chronic exposure to some substances increases the likelihood of AML development. Benzene is the most extensively researched and commonly utilized, possibly leukemogenic substance [14].

Drugs such as Pipobroman are also among the risk factors; other risk factors include cigarette smoke, herbicides, pesticides, embalming fluids, and ethylene oxides [14].

Genetic risk factors

Genetic risk factors with a greater association with AML include Down syndrome, Klinefelter syndrome, Patau syndrome, Ataxia telangiectasia, Shwachman syndrome, Kostman syndrome, Neurofibromatosis, Fanconi anemia, and Li-Fraumeni syndrome [14]. Furthermore, loss or deletion of chromosomes 5, 7, Y, and 9, translocations such as

t(8;21)(q22;q22); t(15;17)(q22;q11), trisomy 8 and 21, and other disorders affecting chromosomes 16, 9, and 11 [14]. A "2-hit-hypothesis" model for the AML phenotype has been proposed. It describes the cooperativity of FLT3 (Fms-like tyrosine kinase 3) activating mutations and gene rearrangements implicating hematopoietic transcription factors [14]. The AML phenotype may come from the expression of both groups. FLT3 mutations have been linked to all subtypes of AML as well as most of the known genetic alterations in AML [11,14].

Less than 1% of *de novo* AML patients has the Philadelphia chromosome (BCR/ABL1 fusion gene); AML with BCR/ABL1 translocation is thought to have a poor prognosis, even when normal intensive treatments are supplemented with a tyrosine kinase inhibitors (such as Imatinib) [11].

Leukemia Classification

On the basis of cell lineage (myeloid or lymphoid) and stage of maturation arrest, leukemias are often divided into subgroups: chronic or acute [12]. Leukemia is divided into a number of large groups and the first division is between its acute and chronic forms (Table1):

Acute form: the amount of immature blood cells increases quickly, and as a result of these cells clumping together, the bone marrow is no longer able to produce healthy blood cells.

Chronic form: it is distinguished by an aberrant concentration of mature or almost mature white blood cells that are morphologically or genetically defective. The process normally takes months or years, and because the cells are generated more quickly than usual, the bloodstream contains a large number of aberrant white blood cells. Additionally, leukemias are classified into lymphoblastic or lymphocytic and myelocytic or myeloid leukemias based on the kind of blood cell that is afflicted.

In lymphoblastic or lymphocytic leukemias, a kind of bone marrow cell that ordinarily gives rise to lymphocytes becomes malignant. A particular subtype of lymphocyte, such as B- or T-lymphocytes, is involved in the majority of lymphocytic leukemias.

In myeloid leukemias, the marrow cell type that usually produces red blood cells, as well as other kinds that produce white blood cells or platelets, has a malignant alteration. There are a total of four major categories when these two groupings are combined. There are generally

multiple sub-categories inside each of these four primary groups. Some rarer types are usually considered outside this classification scheme.

Table 1: Leukemia classification, Hematology Basic Principles and Practice, 6th Ed. , Elsevier

Cell Type	Acute	Chronic
Lymphocytic Leukemia	Acute Lymphocytic Leukemia (LLA)	Chronic Lymphocytic Leukemia (LLC)
Myelocytic Leukemia	Acute Myeloid Leukemia (AML)	Chronic Myeloid Leukemia (CML)

The French-American-British classification (FAB, Figure 2a), which is based on the morphological variations found during bone marrow aspirate or peripheral blood tests, is the first type of classification currently in use. Despite being the oldest, the FAB is still a very important tool in the diagnosis of leukemia.

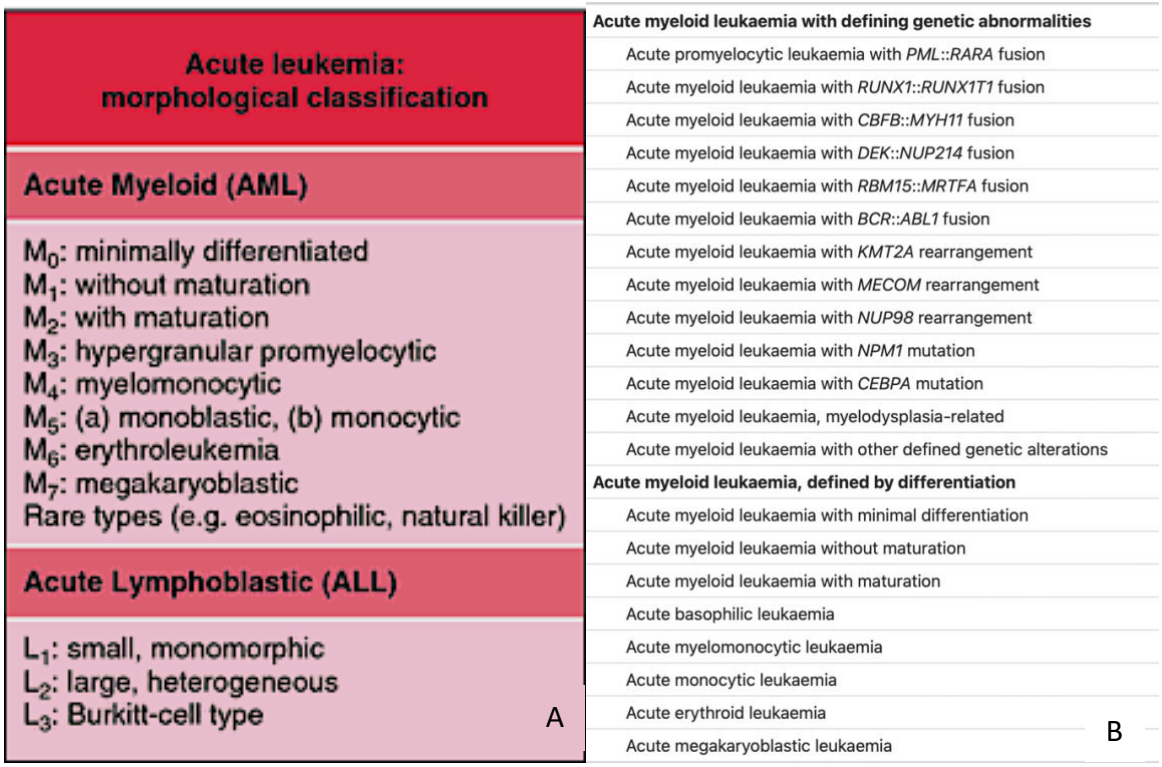


Figure 2a- b: a) FAB classification for Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL), based on cells morphology. b) WHO 2022 new classification based on morphology, molecular and cytogenetic determinants

The new 2022 classification (Figure 2b), proposed by the World Health Organization, considers not only the cell morphology but also the cytogenetic and molecular determinants, all in an attempt to develop a universally applicable and prognostically valid classification [15].

Acute Myeloid Leukemia

Acute Myeloid Leukemia (AML) is a blood cancer that develops clonally from blasts of the myeloid lineage that have not been adequately differentiated. As a result of this proliferation of immature myeloid cells, the buildup of immature progenitors (blasts) with disruption of normal hemopoiesis causes severe infections, anemia, and bleeding. In some circumstances, extramedullary disease such as central nervous system (CNS) involvement may also be present [16]. The most prevalent acute type of leukemia in adults and the elderly, acute myeloid leukemia is on the rise, primarily as a result of a rise in the prevalence of therapy-related acute myeloid leukemia as more people with cancer treated with cytotoxic chemotherapy recover from their underlying illness [16].

The presence of >20% blasts in peripheral blood or bone marrow, or the presence of specific genetic aberrations detected in bone marrow regardless of blast count [t(8;21), inv(16), or t(15;17)], is used to make the diagnosis [17].

Genetically, there are mutations that we know are common in AML or less rare than others. To name a few, the Nucleophosmin 1 (NPM1 or nucleolar phosphoprotein B23 or numatrin) protein has a strong correlation with concurrent receptor-type tyrosine-protein kinase 3 (FLT3), isocitrate dehydrogenase 1 and 2 (IDH1/2), and DNA methyltransferase 3 alfa (DNMT3A) mutations and can be used to monitor for minimal residual disease (MRD). Recurrent mutations in acute myeloid leukemia affect the biology and phenotype of the disease, responsiveness to therapy, and the likelihood of relapse in the future [18,19]; ASXL Transcriptional Regulator 1 (ASXL1) mutation has increased incidence in older adults with Clonal Hematopoiesis of Indeterminate Potential (CHIP) mutation [20] associated with secondary acute myeloid leukemia (AML) that has progressed from antecedent hematologic malignancy [21]. Tumor Protein 53 (TP53 or p53) associated with complex karyotype and secondary AML from antecedent hematological malignancy or therapy related [16].

Standard treatment in AML and targeted therapy

In AML, the two most prevalent induction therapy are cytotoxic chemotherapy with or without targeted drugs and hypomethylating drugs [17].

The backbone of therapy has not altered in 50 years for all individuals with AML who are medically fit to undergo chemotherapy. The addition of numerous targeted drugs to the standard 7+3 induction chemotherapy enhanced the outcomes [17]. The first therapy consists of a seven-day continuous infusion of Cytarabine with the addition of an anthracycline, usually Daunorubicin, administered daily for the first three days; this therapy is colloquially called the "7+3 therapy" [17]. In individuals with FLT3 mutations, adding Midostaurin to conventional chemotherapy increased survival from 25 to 74 months [17,22]. An unmet need in the treatment of acute myeloid leukemia is adverse risk illness. The results of standard 7+3 chemotherapy are still disappointing. The complete remission rate is only approximately 40%, and the median overall survival is 12–18 months. Patients are frequently referred to clinical trials if one is available due to the low survival rate [17].

Two targeted agents, CPX351 and Venetoclax (a highly specific inhibitor of the anti-apoptotic protein BCL-2), when combined with hypomethylating therapies, outperformed standard 7+3 treatment in patients with adverse risk disease [17,23,24].

Since 2017, a large number of new drugs are available to treat AML, the therapy landscape for AML has shifted dramatically. Venetoclax, which targets B-cell lymphoma 2 (BCL-2), Midostaurin and Gilteritinib, which target FLT3, and Ivosidenib and Enasidenib, which target mutant isocitrate dehydrogenase 1 and 2, respectively, have all emerged as new targeted treatments. Reapproval of Gemtuzumab Ozogomycin to target CD33, Glasdegib to target the Hedgehog pathway, and a liposomal version of Daunorubicin and Cytarabine (CPX-351) are among the other additions [25,26]. Nevertheless, there are still many unmet needs in the AML therapy, only 5 to 15% of patients older than 60 years could be cured [16,27]; moreover, the outcome in relapsed/refractory patients or in which ones who are unable to receive intensive chemotherapy treatment is particularly poor, with a median survival of only 5 to 10 months [16,27].

Glutamate

Glutamate is the most important excitatory neurotransmitter in nervous system, where it performs different actions through ionotropic and metabotropic glutamate receptors [28], both in physiological and pathological conditions. Glutamate receptors are the target of

several compounds, both approved or under clinical trial (like Riluzole, Memantine, Foliglurax). Beyond the nervous system, where glutamate receptors expression and functions are well defined, glutamate receptors also have a widespread distribution in non-neural tissues, where their mode of action and function are not completely understood. Glutamate mediates its activities via two main types of receptors; the first group it's composed by receptors with inotropic activity: AMPA receptor (AMPAR), NMDA receptor (NMDAR) and Kainate receptors (KARs). The second type has a metabotropic activity and the receptors are accordingly called metabotropic glutamate receptors (mGluRs), a family of eight subtypes which are classified into three groups based on amino-acid sequence, transduction mechanisms and pharmacological profile.

Glutamate mediates many actions at a physiological and pathological level. For example, an excess of glutamate production and release causes a massive cell death in the CNS through a mechanism called "excitotoxicity", which is the main cause of numerous neurological diseases [29].

Glutamate is a polar amino acid and a chiral molecule. The L-enantiomer is one of the 20 ordinary amino acids. Its lateral group has a carboxyl which gives it acid behavior, and in humans it is not an essential amino acid. Glutamate is involved in most aspects of normal brain functions: cognition, memory, learning, and during the CNS developing cell migration, differentiation, and cell death [30].

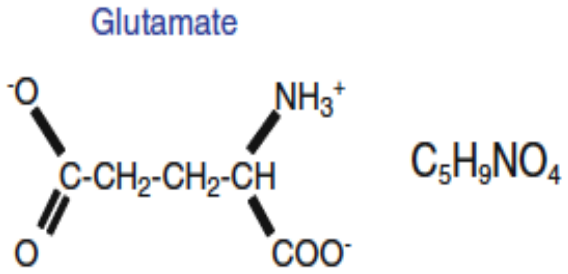


Figure 3: Glutamate, structural and chemical formula

Glutamate is important for hormone regulation, heart rhythm, blood pressure, circulation, and reproduction in peripheral organs and tissues, including the kidney, heart, intestine, lungs, muscles, liver, ovary, testis, bone, and pancreas [31]; and in the hormone production system and reproduction [31-33].

G-protein-coupled-receptors (GPCR)

The GPCR superfamily is made up of receptors with similar general structures and accounts for almost 4% of human protein-coding DNA sequences [34]. Their physiological responsibilities span from central nervous system (CNS) function to vision, hearing, taste, and immune system function [35]. GPCRs are classified into six groups based on sequence homology, function, ligand, and structural features. The six receptor classes include rhodopsin-like receptors (class A), secretin receptors (class B), metabotropic glutamate receptors (mGluRs) (class C), fungal mating pheromone receptors (class D), cyclic adenosine monophosphate (cAMP) receptors (class E), and frizzled/smoothened receptors (class F) [36]. The rhodopsin-like receptors are the most abundant classes of GPCRs, account for 85% of all known GPCRs [37]. Despite their diversity, GPCRs share structural units. They all feature a seven-transmembrane domain that is made up of hydrophobic residues that connect the N-terminal extracellular domain to the C-terminal intracellular domain [38]. The seven-transmembrane region has three intracellular and extracellular loops that link the transmembrane domain's alpha-helical components [39]. The receptor structure is stabilized by highly conserved disulfide connections in the N-terminal domain [40].

GPCRs can be activated by the binding of numerous ligands, which include neurotransmitters, lipids, ions, hormones, amines, nucleotides, and odorant compounds. When activated, the extracellular domain undergoes a conformational shift, prompting the intracellular G-protein component G_{α} to exchange guanine diphosphate (GDP) for guanine triphosphate (GTP), resulting in $G_{\beta\gamma}$ dissociation [41]. The activation of downstream signaling cascades is linked with adenylyl cyclase or phospholipase C (PLC) and others is directed by G_{α}-bound GTP [41].

mGluRs (mGlu=protein, GRM=gene for human, Grm= gene for mouse) are members of the class C GPCR family and are activated by glutamate, the most abundant neurotransmitter in the central nervous system (CNS) [42]. mGluRs are classified into three classes based on their structures, phylogenetics, signal transduction, and pharmacology (Fig. 4) [43]. Group I includes mGluR1 and mGluR5; group II includes mGluR2 and mGluR3; and group III includes mGluR4, mGluR6, mGluR7, and mGluR8 [43]. mGluRs have comparable structures

to other members of the GPCR family, but they include a unique characteristic known as the Venus-flytrap domain that is not seen in other GPCRs. They feature a cysteine-rich extracellular N-terminal domain that is coupled to a seven transmembrane domain that links to the cell's C-terminal domain [43,44]. This region is where glutamate binds and activates the receptor. The canonical role of mGluRs is to regulate neuronal signaling in the CNS [43]. mGluRs are engaged in a number of processes in the central and peripheral nervous systems, including learning, memory, anxiety, and pain perception [45]. Pre- and postsynaptic neurons in synapses in the hippocampus, cerebellum, cerebral cortex, and peripheral tissues contain mGluRs [46,47].

Group I mGluRs are found at synapses and play an important role in neuronal excitability by activating the $G_{\alpha q}/G_{\alpha/11}$ subunits, whereby stimulate PLC signaling [43,48]. PLC transmits Group I mGluR signals [43]. $G_{\alpha q}$ / $G_{\alpha/11}$ are activated when glutamate binds to group I mGluRs by exchanging GDP for GTP, which causes G_{α} to separate from the $G_{\beta\gamma}$ heterodimer [49]. As a result of GTP-bound $G_{\alpha q}/G_{\alpha/11}$ activating PLC, phosphatidylinositol-4,5-diphosphate (PIP2) is broken down into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) [49]. While IP3 diffuses into the cytoplasm and interacts with IP3 receptors on the endoplasmic reticulum to release calcium (Ca^{2+}) into the cytosol, DAG is still attached to the membrane [49,50]. Protein kinase C (PKC) is stimulated by increased Ca^{2+} and DAG, which activates downstream signaling pathways such MAPK and PI3K/AKT [49].

Group II/III mGluRs are activated via $G_{i/o}$ subunits, whose transduce signals by blocking the adenylyl cyclase pathway and may be located at both pre- and post-synapse, reducing neuronal excitability [43].

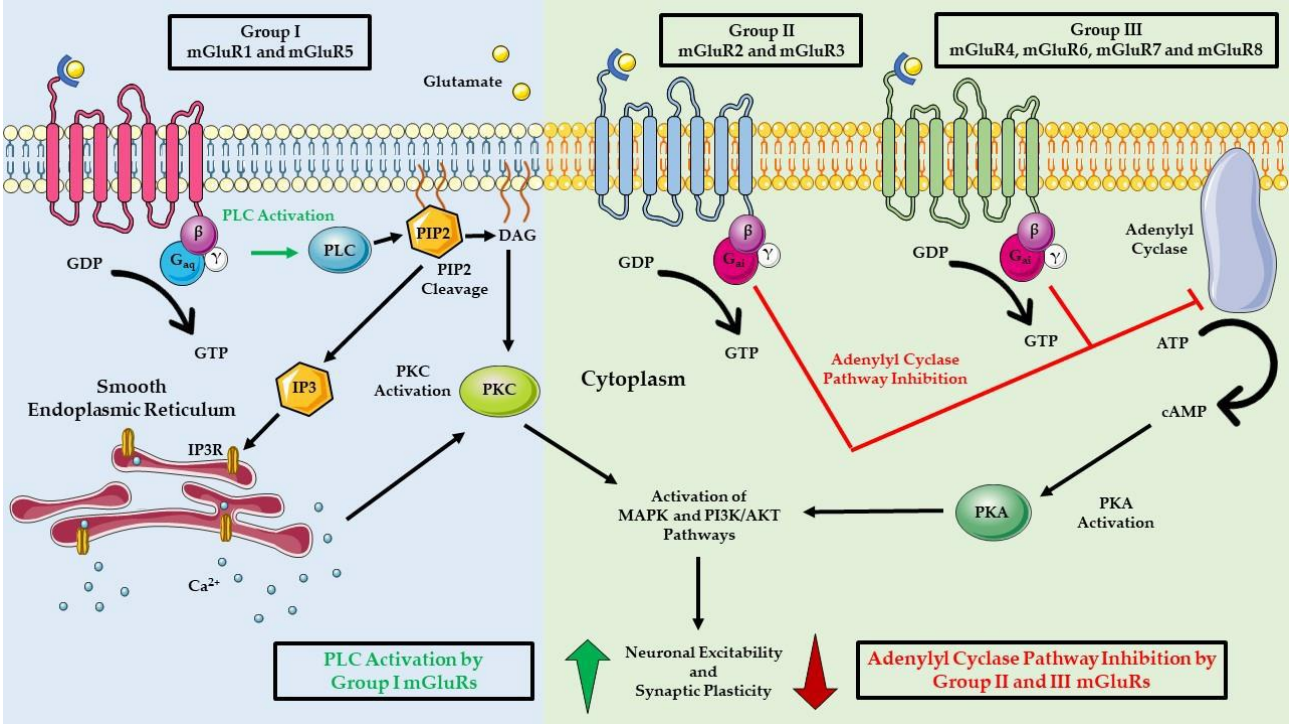


*Figure 4: Mechanisms of action of mGluRs. The known functions of Group I, II, and III mGluRs upon activation by the natural ligand, glutamate, with the downstream PLC, and adenylyl cyclase signaling pathways. Parts of the figure were created by using pictures from Servier Medical Art (Picture take from "**Implications of a Neuronal Receptor Family, Metabotropic Glutamate Receptors, in Cancer Development and Progression" Eddy et al., 2022;** with authors permission).[51]*

Metabotropic Glutamate Receptors in cancers

In recent years more and more studies have demonstrated the presence and role of metabotropic glutamate receptors in the development and maintenance of cancer, not only within the central nervous system (natural site of these receptors) as in the case of glioma but also in ectopic sites, outside the nervous system [42]. In fact, the importance of their role has also emerged in cancers such as melanoma, colorectal cancer, breast cancer, prostate cancer, gastrointestinal cancer, ovarian cancer, kidney cancer, osteosarcoma, in oral squamous cell carcinoma and upper aerodigestive tract cancer, where they are involved in proliferation, apoptosis, migration and immune evasion [48,52-72]. It has been shown that these receptors also play a role in healthy and leukemic T-cell biology and this activity correlates with glutamate levels outside the cell [47].

In human breast cancer, mGluR1 has been found to play a function in carcinogenesis and progression. Grm1 cDNA was introduced into immortalized Mouse Mammary Epithelial Cells (iMMECs), resulting in cell transformation *in vitro* and cancer *in vivo*, with increased angiogenesis [73]. mGluR1-expressing iMMECs had higher extracellular glutamate levels,

and Riluzole, a glutamate release inhibitor, inhibited cell proliferation *in vitro* and tumor development in MCF7 xenografts *in vivo* [73].

Speyer and colleagues, showed that in the presence of Riluzole, *in vivo*, tumor cells exhibited reduced tumor growth and reduced angiogenesis; furthermore, it appears that mGluR1 is able to up regulate the expression of pro-inflammatory cytokines (such as CXCL1, IL-6 and IL-8) during acute inflammation in Triple Negative Breast Cancer (TNBC). This implies that mGluR1-expressing cells could regulate cytokine production to regulate immune surveillance and infiltration of immune cells into the tumor microenvironment thereby allowing the tumor to evade the surveillance by the immune system and/or prevent immune cells from initiating an anti-tumor response [74,75]. Instead, GRM8, has been demonstrated to function as an onco-gene in breast cancer and has been associated to a worse overall survival rate. The overexpression of this receptor in breast cancer cells promoted tumor growth, migration, invasion, carcinogenesis, and inhibited cell death signaling. GRM8 has been shown to be adversely regulated by miR-33a-5p in breast cancer [76]. Furthermore, others have connected GRM4 overexpression to a decrease in cell proliferation, migration, and invasion in MDA-MB-231, a human breast cancer cell line. GRM4 knockdown, on the other hand, enhanced these activities [77].

The function of glutamatergic signaling in glioma formation and progression is well recognized. Glioma cells are a type of non-neuronal cell that regulates neuronal cell activity and metabolism. Ependymal cells, oligodendrocytes, and astrocytes are all components of glial cells [51]. Many studies have connected mGluR1, mGluR2, or mGluR3 signaling to enhanced tumorigenesis and metastatic spread of gliomas via activation of the MAPK and PI3K/AKT pathways. There is a significant quantity of circulating glutamate in glioblastoma, which stimulates mGluRs and promotes tumor development. Furthermore, Riluzole therapy lowers glioma cell aggressiveness [51]. The suppression of mGluR1 by RNA silencing was demonstrated to diminish the vitality, invasiveness, and migratory activities of human glioma cells (U87) *in vitro*, as well as the growth of U87 tumors *in vivo* [51].

REST limits neuronal gene expression by interacting with two different co-repressors, SIN3A and RCOR1, which attract histone deacetylase to REST-regulated gene promoters. It

mediates repression by recruiting the BHC complex (lysine-specific demethylase 1) to RE1/NRSE sites and works as a chromatin modification by deacetylating and demethylating certain histone sites. The recruitment of histone methyltransferase EHMT2 by REST-CDYL may be significant in inhibiting cell transformation [78,79]. It inhibits the expression of SRRM4 (Serine/Arginine Repetitive Matrix Protein 4) a splicing factor essential for neural cell development in non-neural cells in order to prevent the activation of neural-specific splicing events and the creation of REST isoform 3.

Repressor activity can be reduced by building heterodimers with isoform 3, which prevents binding to NRSE or binding to co-repressors, resulting in target gene de-repression. It also keeps neuronal genes repressed in neural stem cells and allows transcription and differentiation into neurons by dissociating from target genes RE1/NRSE sites. As a result, it plays a role in keeping adult neural stem cells dormant and avoiding premature differentiation into mature neurons. It acts as a "switch" in development by changing the composition of the synaptic NMDA receptor throughout postnatal development, inhibiting GRIN2B expression and therefore changing the characteristics of the NMDA receptor from containing mostly GRIN2B as a subunit to primarily GRIN2A. It is a key repressor of gene expression in hypoxia: it represses genes during hypoxia by directly binding to a RE1/NRSE site on their promoter regions. It may also function in stress resistance in the brain during aging, possibly by regulating the expression of genes involved in cell death and stress response. It also acts as a repressor of gene expression in the hippocampus during ischemia by directly binding to RE1/NRSE sites and recruiting SIN3A and RCOR1 to target gene promoters, inducing chromatin alterations and ischemia-induced cell death. It may have a role in inhibiting miRNA-132 expression in hippocampus neurons following ischemia, leading to neuronal cell death. It also suppresses the expression of SRRM3 in breast cancer cell lines [78-85]. At this point, it is safe to assume that genes encoding for metabotropic glutamate receptors may be under partial control of RE1/REST and that, in the case of ectopic and aberrant expression of these receptors, the problem is probably attributable to repressor malfunction.

Members of the mGluR family have been demonstrated to be important participants in glioma pathogenesis, as evidenced by the targeting of group II mGluRs (mGluRs 2/3) with LY341495, a group II antagonist, or Riluzole, which lowered the aggressiveness of gliomas [51]. Furthermore, Khan and colleagues reported that dosing mGluR3-expressing U87 glioma cells with Riluzole increases DNA damage and glioma cell cytotoxicity both *in vitro* and *in vivo*; additionally, Riluzole treatment sensitizes U87 cells to γ-radiation [51,86].

When colorectal cancer cells are compared to normal colon cells, mGluR4 result overexpressed [72,87]. mGluR4 signaling increased cancer cell proliferation and infiltration, according to functional experiments [72]. In human colorectal adenocarcinomas it was shown that 68% overexpressed mGluR4, which was linked to poor prognosis and disease-free survival, implying mGluR4's role as an oncogene [72]. Elevated expression of mGluR4 has been demonstrated to contribute to 5-fluorouracil (a chemotherapeutic drug) resistance when compared with non-resistant parental cells [72,87]. Defective drug absorption, changed anabolic and/or catabolic enzyme activity, and numerous pathways revolving within thymidylate synthase in terms of substrate binding, gene amplification, and mutations have all been hypothesized as reasons for resistance to 5-fluorouracil [51].

Another example, is the role of mGluR4 and mGluR5 in osteosarcoma. Osteosarcoma is a type of malignant bone cancer that primarily affects children and adolescents [51]. Overexpression of mGluR4 in osteosarcoma results in reduced cell proliferation, migration, and invasion [51]. Liao and colleagues demonstrated the formation of autocrine loops in mGluR5-expressing osteosarcoma cells, SaOS-LM7, where glutamate is released into the tumor microenvironment, activating the mGluR5 receptor on these cells and promoting tumor development [88]. Blocking mGluR5 signaling using pharmacological inhibitors (Riluzole or Fenobam, a negative allosteric modulator of mGluR5) or genetic approaches resulted in decreased osteosarcoma cell proliferation, tumor cell motility, and increased apoptosis [88].

mGlu Receptors and Leukemia

Many cells of the immune system (IS) express glutamate receptors including T lymphocytes. By its nature, glutamate activates T-lymphocytes and stimulates transcription, cellular

adhesion, chemotactic migration, production and secretion of different cytokines [89]. Furthermore, glutamate has a strong mitogenic effect on T-activated lymphocytes. T-lymphocytes produce and secrete glutamate that has at least two effects: first, it has an autocrine effect and, second, it has a paracrine effect on other cell types [89]. An interesting finding by Long and colleagues demonstrated the involvement of glutamate in promoting lymphocytes T-reg proliferation (a subpopulation of lymphocytes), their activation, suppressive action, and stimulation in the mGluR1 receptor expression on cells surface [90]. Through immunohistochemistry and reverse transcription and PCR (RT-PCR), Pacheco and colleagues provided strong evidence for the involvement of group I mGluRs in glutamate connection in human lymphocytes [91]. Specifically, they found that the expression of mGluR1 and mGluR5 in human lymphoid cells and in resting and activated lymphocytes from human peripheral blood. They also detected high expression of both receptors in the Jurkat T cell lines, but mGluR5 is expressed only in the human B cell lines. Interestingly, mGluR5 is constitutively expressed in blood lymphocytes and may contribute to the maintenance of the resting status, but mGluR1 is expressed only upon activation via the T cell receptor-CD3 complex [91]. Another study by Ciocchetti and her group, investigating the influence of glutamate on T cell activation-induced cell death (AICD), a mechanism that causes the death of lymphocytes if they are stimulated two times by TCR, a process that the body needs to preserve the peripheral tolerance of the immune system [92]. This study showed that micromolar concentrations of glutamate inhibit AICD and that the effect was mediated by group I mGluR activation with consequent downregulation of FasL (Fas Ligand) expression. Indeed, the direct activation of these receptors' expression on T lymphocytes and the nearby cells (macrophages and monocytes), protects the cells from AICD [92]. mGluRs activation does not modulate cell proliferation or responses to other apoptotic stimuli at low concentration. Instead, millimolar concentrations of glutamate established in damaged tissues during acute and chronic inflammation had a metabolic consequence (i.e., modifications of intracellular thiol compounds). These modifications result in a modulation of the immune system response with diminished cell proliferation and increased levels of INF-γ and IL-10 [92]. The high expression level of mGluRs was found

on T cells and dendritic cells (DC), and the DC mediated the protective effect of mGluR4 signaling, as elucidated through mGluR4's inhibitory effect on intracellular cAMP activation [93]. This failure to activate cAMP prevents the production of IL-6 and IL-23 and causes the dendritic cells to shift the production of cytokines in such a way as to induce the differentiation of T lymphocytes into regulatory T lymphocytes (T-reg), which are responsible for protecting mice from "Experimental Autoimmune Encephalomyelitis"; through mGluR4 [93].

The influence of glutamate on T-cells is dependent on the individual mGluR expression on T cell subtypes, whether T-cells are resting or active, and the availability or lack of other contemporaneous stimuli [47]. Glutamate has been found to be involved in a variety of T-cell activities, such as T-cell activation, survival, adhesion, migration, proliferation, and the prevention of antigen-induced apoptosis [47]. It has been postulated that tumor immune evasion in T-cell cancers is mediated by high glutamate levels in mGluR-expressing cells, which enhance tumor growth via hyperactivation of mGluRs on tumor cells, as well as mGluR activation on normal T-cells, which reduces cytotoxic T-cell expansion within the tumor environment [47]. Human T-cell leukemia (Jurkat, FRO, and SUP-T1) and T-cell lymphoma (HUT-78, and H9) cell lines exhibit expression of group I (mGluR1/5), group II (mGluR2/3), and group III (mGluR4/6/7/8) mGluRs [47,58,94].

When glutamate concentrations are low and normal, glutamate typically activates or elevates numerous T cell characteristics and activities. When glutamate concentrations are high, as in various pathological circumstances, glutamate usually does the reverse and suppresses T cell activity. Thus, glutamate-induced effects are determined by a set of elements including the environment [47]. The concentration of glutamate appears to be the most crucial factor regulating the functions and the interactions between glutamate and T cells [47]. Therefore, distinct immunological reactions are induced by glutamate at nanomolar, micromolar, and millimolar levels, at such concentrations, it truly represent glutamate levels *in vivo* under normal and healthy physiological settings, as opposed to excess glutamate in a variety of pathological situations [47]. Based on the reviews of Ganor and Levite (2014), we can divide the properties of glutamate on T cells according to its

concentration. Furthermore, it has been shown that mGluR1, 2, 3, 5, and 8 are all present on healthy T cells [47]. Under different conditions, glutamate, through metabotropic receptors, induces different responses in activated or resting T lymphocytes: I) Glutamate, at physiological mid-micromolar concentrations, protects activated T lymphocytes against Apoptotic Activation-Induced Cell Death (AICD) via group I mGluRs; II) Glutamate suppresses the proliferation of activated T lymphocytes via group I mGluRs at pathologically high-millimolar concentrations; III) Glutamate modulates T cell cytokine production via group I mGluRs at pathologically high-millimolar concentrations [47].

In pathological conditions, we know that Group I (mGluR1/5), group II (mGluR2/3), and group III (mGluR4/6/7/8) mGluRs are expressed in human T-cell leukemia (Jurkat, FRO, and SUP-T1), and T-cell lymphoma (HUT-78, and H9) cell lines [47,58,94].

Taken together, there are fewer differences between the nervous, immune, and endocrine systems, and we can investigate the consequences of perturbing one of them on the others [95]. It will be of great interest (especially in blood malignancies) if we could manipulate the activating/resting status of lymphocytes through the mGluRs, but addition studies are necessary to perform first.

Melanoma history

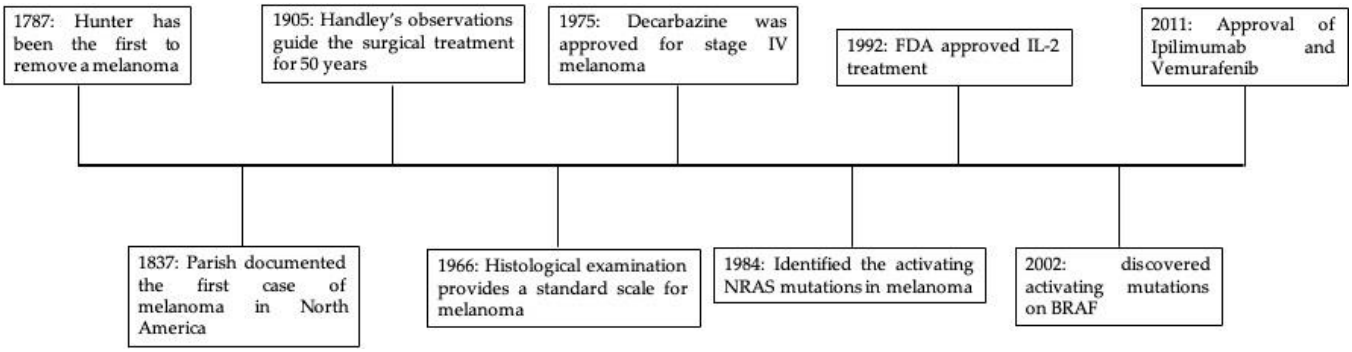
Skin cancer is described as the abnormal multiplication of skin cells, which is frequently detected in sun-exposed regions. Basal cell carcinoma, squamous cell carcinoma, Merkel cell carcinoma, cutaneous lymphoma, Kaposi sarcoma, and melanoma are the six primary forms. Melanoma is the most severe form of skin cancer and is caused by melanocyte transformation. In the United States, invasive melanoma is expected to account for about 100,000 new cases and over 7,000 fatalities from skin cancer in 2022 [96].

Melanoma has been known for a long time, below I will summarize the most significant milestones. In 1787, John Hunter from St. George's Hospital Medical School in London operated on the jaw of a 35-year-old man and he was able to effectively remove a recurring melanoma. Hunter described the tumor as a "cancerous fungous excrescence," while Everard Home described it as "soft and black" in his 1805 book Observations on Cancer [97,98]. In 1837, Isaac Parish recorded the first melanoma case in North America. His patient,

a widowed 43-year-old woman with a toe "fungal tumor" [97,98]. In 1905, William Handley recommended aggressive lymph node removal combined with the excision of around 5 cm of subcutaneous tissue down to the level of muscle fascia based on one case of metastatic melanoma, and for over 50 years it served as a reference for surgical melanoma therapy [97]. Melanoma is highly dangerous because it spreads to the lymph nodes, brain, liver, and lungs. It is classified into two types: cutaneous and non-cutaneous. Cutaneous melanoma forms on sun-exposed regions of the body, such as the skin. Non-cutaneous melanoma occurs in sun-protected tissues such as the mucosa, uvea, and acral tissue. Melanoma progresses over several phases. A melanocytic neoplasm develops from melanocytes during the initial hyperplasia stage, and is a benign lesion known as a melanocytic naevus. The naevi advance to a more aggressive stage called dysplastic naevi, which develops into melanoma *in situ* and subsequently invasive melanoma. Straume and colleagues [99] discovered that metastatic melanoma has the highest proliferative index of all melanoma stages. Melanoma metastases, like many solid tumors, typically occur first in the lymph nodes draining the main tumor, with distant metastases affecting visceral locations appearing later [100]. Commonly reported melanoma-inducing variables include cumulative Ultra Violet Rays (UVR) exposure, host age, mutational load, and the kind of neoplastic change [101]. UVR has long been linked to melanoma development. It is believed that UVR exposure alone accounts for 60-70% of all instances of cutaneous melanoma [102] and, UVR exposure is thought to be the most common external cause of all cutaneous melanoma cases [103]. Among the most commonly observed driver somatic mutations in chronic sun-induced (CSID) are key proliferation governing genes (BRAF, NF1, and NRAS), growth and metabolism genes (PTEN and KIT), cell identity genes [AT-rich interaction domain 2 (ARID2)], apoptosis resistance genes (TP53), cell cycle control (cyclin-dependent kinase inhibitor 2A (CDKN2A), and replicative lifespan associated genes (telomerase reverse transcriptase, TERT). Each melanoma case's variance is explained by the order of mutation and accumulation of these genes [104,105].

From 1975 to the present time, progress and new discoveries in melanoma therapy have been very rapid with the approval in 1975 of Decarbazine for the treatment of stage IV

melanoma; the identification of NRAS-activating mutations in 1984; FDA approval for the use of IL-2 in 1992; the discovery of mutations in BRAF in 2002; and the approval of immunotherapy with Ipilimumab and targeted therapy with Vemurafenib in 2011 [97,98].



Metabotropic glutamate receptor 1 (mGluR1/Grm1-mice gene) ectopic expression in mice melanocytes was sufficient to cause melanoma growth *in vivo* with 100% penetrance. Chen's group also discovered that mGluR1 was expressed in around 60% of human melanoma biopsies and cell lines but not in benign nevi or normal human melanocytes, indicating that GRM1 is involved in melanomagenesis. [106].

mGluR1 is predominantly expressed in neurons it's expression in neuronal cells is controlled by the binding of Neuron-Restrictive-Silencer-Factor (NRSF, also named RE1-Silencing Transcription Factor) to a Neuron-Restrictive-Silencer-Element (NRSE), NRSF is a transcriptional repressor that binds to the "neuron-restrictive silencer element" (NRSE) and inhibits the transcription of neuronal genes in non-neuronal cells [107]. In melanoma cells Lee et al., showed that NRSF/NRSE indeed are involved in the regulation of mGluR1 expression in melanocytes but additional regulatory elements are also involved, not all have been identified [106].

First-line of treatment in melanoma

In this section, we will mainly discuss inoperable stage III and IV melanoma and the different drugs that are currently used in therapy. We will also briefly discuss the mutations affecting the Rapidly Accelerated Fibrosarcoma (RAF) family of proteins, specifically BRAF, found in 50% of melanocytes and melanoma patients.

Currently, the main criterion for deciding the treatment of an advanced patient with Vemurafenib, targeted therapy is the presence or absence of the most common V600E BRAF gene mutation. This BRAF mutation, which is found in approximately 50% of melanocytes and whether this population of melanocytes progress to melanoma is not known. Mutated V600E BRAF is also detected in 50% of melanoma patients, resulting in hyperactivation of MEK, a kinase in the Mitogen-Activated Protein Kinase (MAPK) pathway that phosphorylates the Extracellular Signal-regulated Kinase (ERK). Hyperactivation of ERK promotes cell proliferation and growth and inhibits apoptosis [108-110]. Melanomas that form on sun-exposed skin are more likely to have BRAF mutations [110-112]. Based on the signaling pathway data, drugs termed "molecular targeting" have been developed; combining a BRAF inhibitor with a MEK inhibitor has also been shown to improve tolerance and efficacy of therapy but also increased in toxicity.

Currently the first-line of treatment is immunotherapy with antibodies targeting immune check point, Nivolumab and Pembrolizumab: they target patients diagnosed with advanced or metastatic melanoma (inoperable III or IV) [113,114]. Nivolumab and Pembrolizumab are antibodies that target the PD-1 (Programmed Death 1 receptor) receptor on immune cells. When triggered by attaching to particular components, the PD-1 receptor silences the immune response by sending inhibitory signals. The drug's interaction with PD-1 inhibits immune response suppression and boosts antitumor response.

Ipilimumab is a monoclonal anti-CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) antibody that targets freshly activated $CD4^+$ and $CD8^+$ T cells. The drug's binding to the receptor inhibits CTLA-4 function, restoring a primary immune response towards cancer cell [115].

Both BRAF-mutated and non-BRAF mutant melanoma patients benefit from immunotherapeutic treatments.

For patients carrying the BRAF mutation, two different drug therapies are available, both based on a BRAF inhibitor and a MEK inhibitor; thus, these therapies consist of Dabrafenib and Tametinib or Vemutafenib and Cobimetinib. In two trials, COMBI-d and COMBI-v, Dabrafenib and Trametinib outperformed Dabrafenib in monotherapy and Vemurafenib in monotherapy, respectively. In terms of survival, the findings of both studies were very

similar, with 44–45% of patients living after three years of monitoring compared to 31-32% for monotherapy. In another study, coBRIM, Vemurafenib and Cobimetinib indicated a benefit above Vemurafenib alone. In this case, the target combination resulted in 48% of patients being alive at 2 years compared to 38% [116-118].

L1CAM (CD171) as possible target in melanoma invasiveness

Much studies have been performed to identify molecule(s) involved in tumor cell migration, the first step in invasiveness and metastasis of tumor cells. L1, also known as L1-NCAM or CD171, is a transmembrane protein expressed by the L1-NCAM gene, that belongs to the L1 protein family. This 200-220 kDa protein is a neuronal cell adhesion molecule that plays an important role in cell migration, adhesion, neurite outgrowth, myelination, and neuronal differentiation [119]. L1 serves a static role as a cell adhesion molecule that joins various cells. It is involved in the adhesion of neurons as well as the development and connection of neurites, a process known as neurite fasciculation [120]. Some studies have shown that L1 has a role in tumor development, tumor cell invasion, and metastasis of melanoma, ovarian, and colon cancer owing to overexpression of the protein L1, which promotes malignant cell mobility [121]. L1CAM has a conserved cytoplasmic portion, five fibronectin type III repeats, and six immunoglobulin-like domains (Fig.5) [122].

L1 protein is linked to the activation of multiple cancer related pathways such as extracellular signal-regulated kinase (ERK), focal adhesion kinase (FAK), and p21-activated kinase (PAK) [122-124].

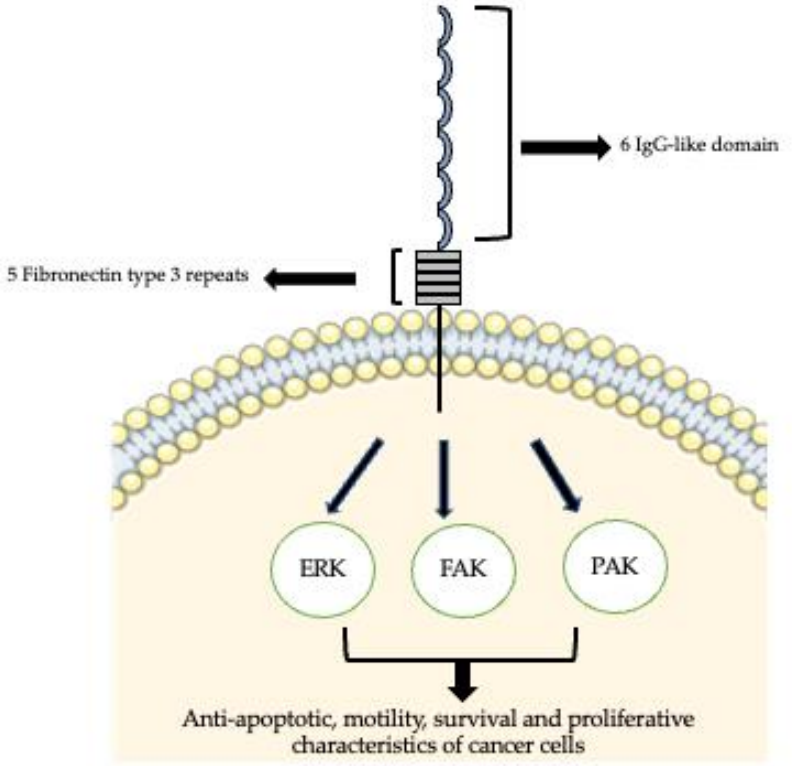


Figure 5: Structure and general pathway related with L1CAM in cancer. We can see the six Ig-like domains and the five Fibronectin III domains. ERK = Extracellular-related Kinase; FAK = Focal Adhesion Kinase; PAK = p21-activated Kinase. In cancer these pathways are overactivated and cause acquiring of anti-apoptotic, motility, survival and proliferative characteristics.

L1 was found to be associated with the activation of the ERK pathway in melanoma, upregulating ERK-dependent, motility and invasion products such as α-v-β3 integrin. L1 expression is linked with B3 integrin expression, although it promotes invasive melanoma development without an increase in B3 integrin expression, according to one of the three primary findings in Meier's studies. They also discovered that L1 overexpression transforms the Radiant Growth Phase (RGP) melanoma into the Vertical Growth Phase (VGP) melanoma in the absence of B3 integrin [125]. Their recent report revealed that suppressing L1 considerably decreased melanoma migration and invasion but did not entirely stop growth. According to their findings, L1 does not enhance melanoma formation but does help in migration and progression from RGP to VGP with invasive and metastatic capability [125].

Based on previous work by Nagaraj and colleagues, where they tested several mimetic antagonist drugs that block the action of L1CAM, we decided to use two antagonist drugs

to LICAM, anagrelide and 2-hydroxy-5-fluoropyrimidine to evaluate their *in vivo* and *in vitro* efficacy in melanoma cell migration *in vivo* in allograft studies [126].

Anagrelide is a drug that reduces the number of platelets to treat thrombocythemia. It inhibits megakaryocyte development in the bone marrow by lowering platelet numbers without interacting with other progenitor cell lines [126]. Anagrelide inhibits the maturation of megakaryocytes, resulting in lower levels of transcription factors GATA-1, FLI-1, and NF-E2 [126]. It also reduces platelet numbers by preventing pro-platelet production. Pro-platelets are mature megakaryocyte extrusions that extend into the sinusoidal lumen of the bone marrow and give rise to platelets at their protrusions' terminals [126].

Hepatic aldehyde oxidase converts 2-hydroxy-5-fluoropyrimidine to 5-fluorouracil; 5-fluorouracil inhibits metabolic activities in cells and is more abundantly integrated into the RNA of tumor cells than non-tumor cells because tumor cells upregulate protein production more than non-tumor cells. 5-fluorouracil incorporation into RNA renders RNA non-functional, limiting key cell processes [126].

Aims of the studies

Part I:

The first objective of this study was to demonstrate the expression of metabotropic glutamate receptors in human acute myeloid leukemia cell lines; the second objective was to evaluate the efficacy, if any, of specific drugs that can modulate the activity of these receptors for each subtype. Finally, if it is possible to modulate the activity of these receptors, to evaluate at the biological level what consequences were apparent with respect to proliferation, resistance to chemotherapeutics, and modulation of signal transduction pathways.

Part II:

In mGluR1 driven mouse melanoma cell lines, we are evaluating if two mimetic antagonist drugs, anagrelide and 2-hydroxy-5-fluoropyrimidine, against L1-NCAM protein could reduce these melanoma cells motility *in vitro* and *in vivo*.

Matherials and Methods

Drugs table

Complete Drugs Name	Abbreviation
(1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid	LY379268 (group II mGluR agonist)
(RS)-α-Cyclopropyl-4-phosphonophenylglycine	CPPG (group II/III mGluR antagonist)
(S)-3,5-Dihydroxyphenylglycine	DHPG (group I mGluR agonist)
3,4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone	JNJ16259685 (non-competitive mGluR1 antagonist)
7-Hydroxy-3-(4-iodophenoxy)-4H-1-benzopyran-4-one	XAP-044 (mGluR7 antagonist)
4-(4-Fluorophenyl)-5-[(1-methyl-1H-pyrazol-3-yl)methoxy]-2-pyridinecarboxamide	VU6001966 (negative allosteric modulator of mGluR2)
(1S,2S,4S,5R,6S)-2-amino-4-[(3-methoxybenzene-1-carbonyl)amino]bicyclo[3.1.0]hexane-2,6-dicarboxylic acid	LY2794193 (mGluR3 receptor agonist)
(1S,2S,4R,5R,6S)-2-amino-4-methylbicyclo[3.1.0]hexane-2,6-dicarboxylic acid	LY541850 (orthosteric mGluR2 agonist and mGluR3 antagonist)
N-(4-(trifluoromethyl)oxazol-2-yl)-9H-xanthene-9-carboxamide	RO0711401 (positive allosteric modulator of mGluR1)
GDC-0973	Cobimetinib (MEK inhibitor)
ABT-199	Venetoclax (antagonist of the BCL-2 anti-apoptotic protein)
N-(3-Chloro-4-fluorophenyl)-1H-pyrazolo[4,3-b]pyridin-3-amine	VU0418506 (Positive Allosteric Modulator of mGluR4)
3-(2,3-Difluoro-4-methoxyphenyl)-2,5-dimethyl-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine	VU6005649 (Positive Allosteric Modultator of mGluR 7/8)
Forskolin	
3-isobutyl-1-methylxanthine	IBMX
Anagrelide Hidroxychloride	
2-hydroxy-5-fluoropyrimidine	2H5F

LY379268, CPPG, DHPG, JNJ16259685, XAP-044, VU6001966 were purchased from Tocris Biosciences (Bristol, UK); LY2794193 and LY541850 were purchased from Eli Lilly Italia

S.p.A. RO0711401 was kindly provided by La Roche Ltd, Pharmaceutical division (Basilea, Switzerland). Cobimetinib and Venetoclax were kindly provided by Professor A. Tafuri (Department of Clinical and Molecular Medicine, Hematology, Sant'Andrea University Hospital, Sapienza University, Roma, Italy). VU0418506 and VU6005649 were kindly provided by Professor Jeffrey Conn. CPPG, LY2794193, LY379268 and LY541850 were dissolved in distilled water and NaOH 5N; DHPG was dissolved in distilled water. All others compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma Life Science®).

Cell cultures

Human leukemia cell lines: Three different hematological human cancer cell lines were used for *in vitro* tests: OCI AML-3, OCI AML-2 and U937. Jurkat (acute T-cell leukemia), MOLM13 (acute myeloid leukemia), HL60 and HL60/MX2 (acute promyelocytic leukemia) were used only for qRT-PCR screening for mGluRs expression; cell lines were kindly provided by Professor A. Tafuri. OCI AML-3 (Ontario Cancer Institute/ Acute Myelogenous Leukemia-3) has NPM1 and DNMT3A mutated and OCI AML-2 (Ontario Cancer Institute/ Acute Myeloid Leukemia-2) has NPM1 wild-type and DNMT3A mutated these cells are human leukemia cells; U937 (*ATCC® CRL-1593.2™),* this cell line was derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma. All cell lines were cultured with RPMI-1640 (*Gibco® Life Technologies™*) supplemented with 10% Fetal Bovine Serum Heat Inactivated (*Gibco® Life Technologies™*), 1% L-Glutamine (*Gibco® Life Technologies™*) and 1% Streptomycin / Penicillin (*Gibco® Life Technologies™*); cells were kept at 37°C and 5% CO_2 in a humid environment. These cells grow in suspension and 25 cm^2 (*Corning 430639, 2* μm vent cap) and 75 cm^2 flasks were used (*Corning 31464U,* 2 μm vent cap). Medium was changed twice a week and the cells were used during the exponential growth. Viable cells were counted with Trypan Blue 0.4% solution (*Sigma Life Science®*) and then transferred in Burker counting chamber.

Mouse melanoma cell lines: Mass 3 cell line were cultured with RPMI-1640 (*Gibco® Life Technologies™*) supplemented with 10% Fetal Bovine Serum Heat Inactivated (*Gibco® Life Technologies™*), and 1% Streptomycin / Penicillin (*Gibco® Life Technologies™*); cells were kept at 37°C and 5% CO_2 in a humid environment.

Mass 3 infection and clones selection: Cell lines were infected using a lentivirus: the lentivirus contained the puromycin resistance gene, the gene for Green Fluorescent Protein (GFP) and the luciferase gene. Infections were performed by diluting the lentivirus 1:1 in RPMI supplemented with 10% FBS (Fetal Bovine Serum) and 1% penicillin/streptomycin and then adding Polybrene (7.5 μg/ml), an adjuvant that enhances the uptake and integration of lentivirus into cells. After 24h, the lentivirus was removed and replaced with complete RPMI 1640. Then cells were plated into fresh 60 mm petri-dishes and puromycin was added for selection of resistant clones (0.8 mg/ml for puromycin).

Luminescence assay

The luminescence assays were used to observe luciferase in cells infected with lentivirus. We used "Dual-Luciferase® Reporter Assay System" purchase from Promega (*Promega Corporation, Madison, USA*).

qRT-PCR Analysis

Total RNA was extracted from OCI AML-3, OCI AML-2, U937, Jurkat, Molm13, HL60 and HL-60/MX2 with Trizol reagent according to manufacturer's protocol for mGlu receptors screening. The RNA was further treated with DNAse (Quiagen, Hilden, Germany) and single strand cDNA was synthesized from 2 μg of total RNA using SuperScript III (Invitrogen, Carlsbad, CA) and random hexamers. Real Time PCR was performed on 20 ng of cDNA by using specific primers and Ssoadvanced Universal SYBR Green on an Applied Biosystem Step-One instrument. Thermal cycler conditions were as follows: 10 min at 95°C, 40 cycles of denaturation (15s at 95°C) and combined annealing/extension (1 min at 60°C).

Primers used are as follow:

GRM1	Forward: TTGGAAGTGATGGATGGGCA Reverse: TGGAACCGATGTTGCCAGAA
GRM2	Forward: CCAGGAGCTGGGTCCCTT Reverse: AAGTCTCCCTCCAGGGTCAG
GRM3	Forward: CGCTTTGCACAAAATGCAGC Reverse: AACACGTTGTATCGCCCCAT
GRM4	Forward: CAACTTCTCAGGCATCGCAG Reverse: ACTGTCTTCTTCCGCTCACC
GRM5	Forward: GCCAGATCAAGGTGATCCGAA Reverse: TAACAAACAGGGTGGCCAGG
GRM7	Forward: AGGCTCTTCCAGATCCCCC Reverse: TGGGCAATGCAGAGTCCAC
GRM8	Forward: AGAAATGCACAGGGCTGGAG Reverse: TCATTCGTGGACAAAGGCCA
GAPDH	Forward: TTGCCATCAATGACCCCTTCA Reverse: CGCCCCACTTGATTTTGGA

mRNAs copy number of each gene analyzed was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized against GAPDH copy number.

Immunoblotting Protein Extraction

Cultured cells were washed three times with ice-cold phosphate-buffered-saline (PBS W/O $CaCl_2$ and $MgCl_2$), NaF 10 mM, EDTA 0,5 M pH= 8, Sodium Pyrophosphate, Na_3VO_4 1M and NaN_3 10%) and lysed with lysis buffer (PBS W/O $MgCl_2$ and Cl_2Ca), TritonX-100, Iodoacetamide 0,5 M, NaF 10 mM, Na_3VO_4 1M, NaCl 1,5 M, $MgCl_2$ 1M, $CaCl_2$ 1M, NaN_3 10%, Protease and Phosphatase inhibitors (tablets, LaRoche). Proteins were resuspended in SDS-bromophenol blue reducing buffer containing 5% 2-mercaptoethanol and separated by electrophoresis on 8% SDS polyacrylamide gels running at 100 Volts for 1.30 hour. Samples

were never boiled for mGlu receptors before loading. The proteins were transferred into nitrocellulose membranes using Trans-Blot Turbo® Transfer System (BioRad) and thereafter, membranes were blocked for unspecific binding with 5% non-fat dry milk in TBST (TBS containing 0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated with the following primary antibodies: Human primary Anti-mGluR1α (Abcam ab82211 Rabbit polyclonal) overnight at 4°C (in 5% milk TBST); mouse primary anti-GAPDH (Abcam) 1:1000, 1 hour at Room Temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore) using horseradish peroxidase-conjugated secondary antibodies.

Immunoblotting for L1CAM:

Due to the pigmentation in *in vivo* excised tumor samples, protein concentration was not performed, 30 µl of proteins extracts (from cells and from tissues samples) were loaded in a 7.5% SDS-page gels and then separated by electrophoresis running at 100 volts for 2 hours. Proteins were transferred into nitrocellulose membranes preparing the "sandwich", and the transfer ran at 160 mm Volts for 4 hours. Membranes were blocked for unspecific binding with 5% non-fat dry milk in TBST for 1 hour at room temperature. Membranes were incubated with the following primary and secondary antibody prepared in 0.25% non-fat dry milk in TBST.

For L1 (CD171) protein the primary antibody was purchased from Sigma-Aldrich anti-CD171 rabbit polyclonal (used 1: 1.000, *Cat. #SAB4501674*); tubulin primary antibody was used 1:10.000 (monoclonal Anti-α-Tubulin produced in mouse, *Sigma-Aldrich, Cat. # T6074*). Secondary anti-rabbit antibody for L1 (*EMD Millipore, AP182P, USA*) and anti-mouse secondary antibody for tubulin (*Sigma-Aldrich, anti-mouse IgG, Cat. #A4416*) were used 1: 5.000.

Proteins extraction of mouse melanoma cell lines:

Cells were allowed to grow in a 60-mm Petri dish (*Corning*) in complete medium to a confluence of about 85 percent. The plate was then washed twice with cold PBS 1X and Laemmli Sample Buffer (*Bio-Rad Laboratories Inc. Cat. # 1610747*) was then used; the buffer consisted of PBS 1X, Laemmli buffer and 5% β-mercaptoethanol. Using a scraper, the cells

submerged in the lysis buffer were detached (100 µl per 60 mm plate). The protein extract was then heated at 99°C for 10 minutes and centrifuge at 14.000 rpm for 10 minutes; the samples were then stored at -80°C for future use.

Primary tumor protein extraction:

Lysis buffer was prepared with 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 5% Glycerol and 1% Igepal at pH=7.75. DTT 1mM, cocktail phosphatase 3 inhibitors (Sigma-Aldrich, P0044), cocktail phosphatase 2 inhibitors (*Sigma-Aldrich, P5726*), and 25X proteinase inhibitors (*Complete Mini-EDTA-free, 11836170001*) were added to the lysis buffer. Keeping the samples on ice, mortars and pestles with liquid nitrogen were used. The pestles and mortars were pre-cooled by pouring nitrogen and then the samples were added. Each sample was crushed and reduced to powder using the pestle and adding liquid nitrogen from time to time. Once a fairly fine powder was obtained it was resuspended in liquid nitrogen and poured into a test tube kept on ice while waiting for the nitrogen to evaporate. Next, 250 µl of lysis buffer was added to the test tube and, through the use of a mechanical sonicator (*Polytron, OMNI-TH International*), the suspension was further grinded. Once the sample became quite liquid and somewhat sticky, it was incubated for 2 hours at 4°C on a shaker. The sample was then transferred to a fresh tube and centrifuged at 14.000 rpm at 4°C for 20 min. The supernatant was recovered and placed in a new tube and placed in the -80°C.

Polyphosphoinositide hydrolysis

The cell lines OCI AML-3, OCI AML-2 and U937 was counted with Trypan Blue solution 0,4% and the cell suspension was centrifuged at 1200 rcf for 5 minutes (*Megafuge 1.0 R, Heraeus Instruments*) and the pellet was resuspended in Krebs-Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.18 mM, KH_2PO_4 1.18 mM, $NaHCO_3$ 24.8 mM, $CaCl_2$ 1.2 mM, D-Glucose 10 mM) pre-gassed with 95% O_2 and 5% CO_2 at pH=7.4 in order to obtain a final concentration of $1.5x10^6$ cells in 0.5 ml. The cellular suspension was labelled with 1 µCi diluted 1:2 of Myo-[^{3}H] inositol for 40 minutes oxygenating the suspension and then challenged with DHPG 10 µM alone, JNJ16259685 10 µM alone, RO0711401 10 µM alone and JNJ16259685 plus RO0711401, 10 µM respectively. The cells were incubated with

treatments for 30 minutes and then added LiCl 10 μM and incubated for 40 minutes. The incubation was stopped by the addition of 300 μl dH_2O, 800 μl chloroform and 800 μl methanol; samples were centrifuged at low speed to facilitate phase separation. The [^{3}H] inositolmonophosphate (InsP) present in the supernatant was separated by anion exchange chromatography in 10 ml columns containing 1.5 ml of Dowex 1-X-8 resin (*formate form, 100-200 mesh, Bio-Rad, Milan, Italy*). Columns were washed twice with distilled water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate, and the [^{3}H] InsP was eluted with 6.5 ml of 0.2 M ammonium formate and 0.1 M of formic acid.

Annexin V and Propidium Iodide staining

Cells were seeded at 6 x 10^5 cells in a 6-wells plate in RPMI-1640 complete medium. Treatments with different reagents were performed either alone or in combination in incubator at 37°C and 5% CO_2 in a humid environment for 72 hrs. Afterwards, the cell suspension was recovered and centrifuged at 1200 rcf for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 μl of sterile PBS 1X (*Gibco® Life Technologies™*). Then the cells were transferred in a V-shaped 96-wells plate and washed again. Discarded the supernatant and resuspended the pellet in 50 μl of Annexin V and Propidium Iodide Staining Solution 1X (Staining Solution 1X, Annexin V and Propidium Iodide, *BD Pharmingen*) and incubated for 15 minutes in the dark. Centrifuge at 1200 rcf for 5 minutes and discard the supernatant and add 200 μl of Annexin Binding Buffer 1X (*BD Pharmingen*), resuspended the cells and transferred to FACS tubes.

Measurements of cAMP formation in leukemia cell lines

Measurements of cAMP were performed in all cell lines for the mGluR2/3 and mGluR7/8 receptors. Cells were expanded in their medium, centrifuged and resuspended in Krebs-Henseleit buffer (equilibrated with 95% O_2/ 5% CO_2, pH 7.4 and previously incubated for 35-45 min at 37°C under constant oxygenation to allow metabolic recovery). Then 1x10^6 cells were transferred to polyethylene tubes and incubated for 45 min before the addition of the specific drugs. Cells were incubated with 0.5 mM IBMX for 15 min, randomly distributed and then were challenged with VU6001966 (10 μM) or vehicle followed by LY541850 (1 μM) or LY379268 (10 μM) or XAP044 (10 μM) or VU6005649 (10 μM) or in combination and 2

minutes later by forskolin (10 μM) or its vehicle. The incubation was continued for additional 20 min. The reaction was stopped with 0.4 N perchloric acid (PCA). Samples were sonicated for 25 s, added K_2CO_3 2N, and centrifuged at 600 *g*. cAMP levels were measured in the supernatant using an enzyme-linked immunoassay (ELISA) kit (*Tema Ricerca, Castenaso, BO, Italy*).

Analysis of cell proliferation (MTT viability Assay)

OCI AML-3 and U-937 cell lines were seeded in a 6-wells plates and incubated for 72 hours with JNJ16259685 (10 μM), RO0711401 (10 μM), Cobimetinib (0,1 and 1 μM) and Venetoclax (1 and 2 μM) alone or in combination. After 72 hours, 100 μL of cells were put in 96 wells plate and added 10% of MTT (5 mg/ml in PBS 1X), then the plate was incubated for 3 hours at 37°C in a humidified atmosphere with 5% CO_2; after 3 hours DMSO was added and the plate was incubated for another 15 minutes, detected the formazan developed with ELISA reader at a wavelength of 565 nm. For mouse melanoma cells, the cells were seeded in a 96-well plate at a concentration of $1x10^4$ in 50 μl in complete RPMI-1640 and incubated 24 hr. After 24 hours, anagrelide and 2H5F were added at a concentration of 100 μM in 50 μl, and readings were taken at 24, 48, 72 and 96 hours using a plate reader at a wavelength of 565 nm and a reference wavelength of 750 nm.

Migration of mouse melanoma cells using scratch assay

Mouse melanoma cells at 2×10^5 cells were seeded in a 6-wells plate (*Corning*) and let them growth for 24 hours in complete media; the next day with a 200 μl pipette tip a scratch was made along the entire length of the well. The wells were then washed twice with sterile PBS 1X and the medium with treatments was added, changing it after 48 hours. Images were acquired every 24 hours at the same time and location. Treatments were added as follows: No treatment (NT), Vehicle (Veh), Anagrelide 1 μM, Anagrelide 10 μM and Anagrelide 100 μM; for 2H5F the scheme used was the same.

Measurement of tumor volume with vernier caliper *in vivo*

Measurements of tumor volumes by digital caliper were performed twice a week, and the tumor volume, in mm^3, was calculated as $(AxB^2)/2$. The caliper used was a Fowler (*Tools and Instruments, ultra-cal IV 6"/150 mm*).

In vivo imaging using IVIS system

The mouse melanoma cell lines carried the luciferase reporter gene. To visualize the luciferase reporter, we prepared 100 mg of Luciferine Patassium Salt (*Regis, Technologies Inc., Code. 1-360223-200*) in 6.6 ml of sterile 1x PBS to obtain a concentration of 15 mg/ml and 200 µl of Luciferine Potassium Salt are used, via IP injection for each mouse at least 15 min before the images were acquired from the small animal imaging system, IVIS. We used the intensities of the emitted bioluminescence to monitor the tumor growth and metastasis development through luciferase reporter.

In vivo allograft

For the *in vivo* studies, female and male BL6/SKH (hairless C57BL/6) mice at 8-10 weeks of age were used. The animals were housed in controlled environmental conditions: temperature 20 ± 2 °C, relative humidity 50 ± 10%, ventilation with 15 spare parts air hour, regular day / night cycle (12 light / 12 dark) and standard feed with complete feed for mice and water *ad libitum*. All the experiments were carried out in compliance with Rutgers IACUC. Mouse melanoma cell lines, Mass3 clone 1 cells were inoculated by subcutaneous injection in a volume of 100 µl (50 µl cell and 50 µl Matrigel) with a concentration of $1x10^6$ cells in one flank. When the tumor became palpable, the animals were divided into three groups, Vehicle, Anagrelide (20 mg/kg) and 2H5F (10 mg/kg) and treatment was carried on three times a week. The tumor was measured twice a week with a two-dimensional caliper. The animals were sacrificed at the end of the treatment, or at the first distress signal or when the tumor exceeded 700 mm^3 in volume.

Statistical analysis

Statistical analysis of data was performed using SigmaPlot software, version 12 and GraphPad Prims, software version 9. In all experiments, data are presented as means ± standard error of the mean (SEM) or simple SD, and $p \leq 0.05$ was considered significant for One-way ANOVA followed by Fisher's least significant difference (LSD). To quantify western blots we used ImageJ software version 1.52.

Results

1.Transcripts of different mGluRs subtypes are found in oncohematological cell lines

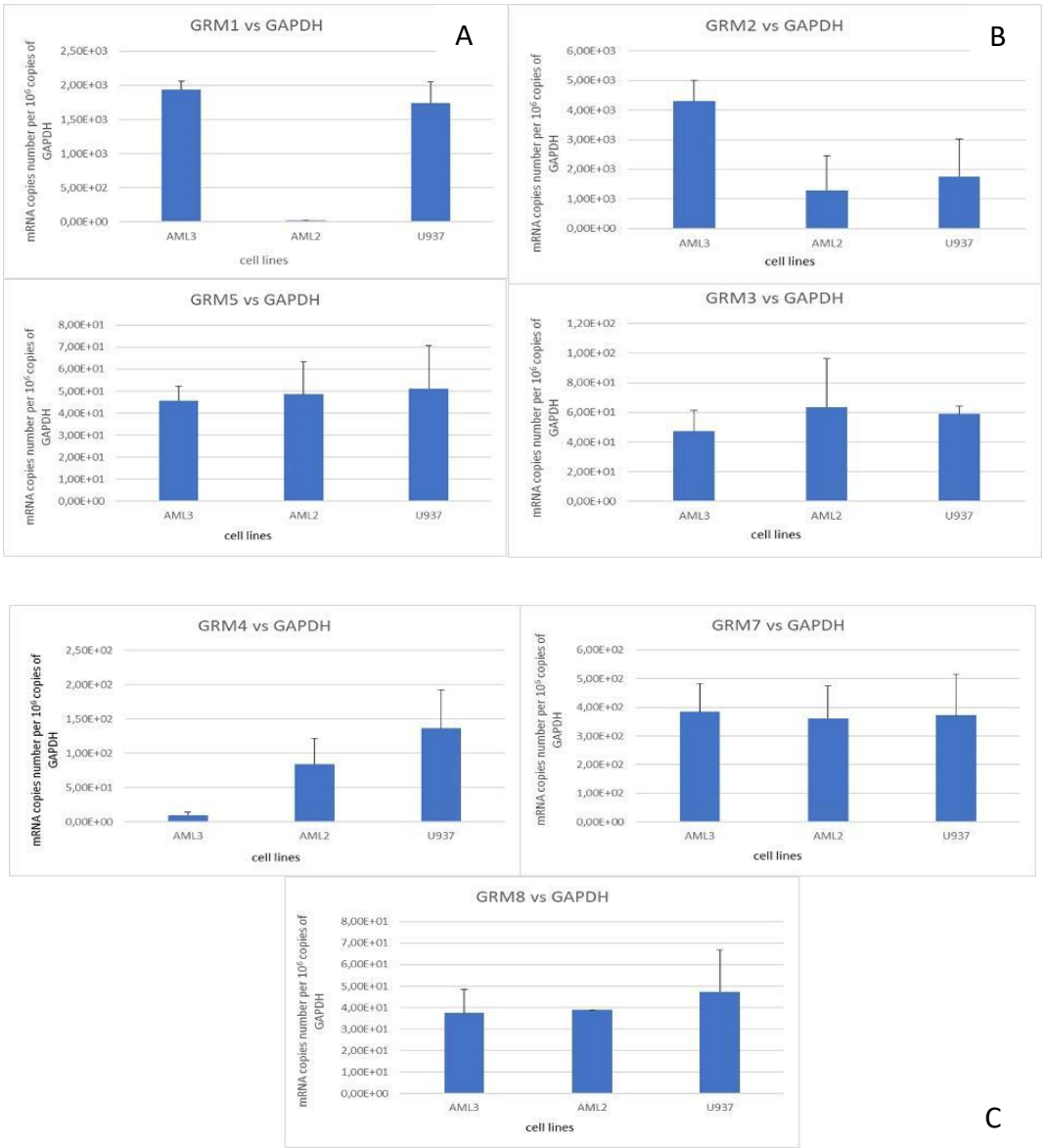


Figure 6: qPCR analysis of GRM1, GRM2, GRM3, GRM4, GRM7, GRM8 mRNAs in oncohematologic cell lines; OCI AML-3, OCI AML-2 and U937 cell lines; all the expression levels are normalized against GAPDH; a) qRT-PCR mGluRs group I; b) qRT-PCR mGluRs group II; c) qRT-PCR mGluRs group III; N=4 ± SEM

Expression of mGluRs genes (GRM1, 2, 3, 4, 5, 7, 8) (Supplementary data 1) were examined by quantitative RT-PCR (qRT-PCR) in several AML cell lines (HL60, HL60/MX2, OCI AML-2, OCI AML-3, U937) and as control group we examined also Acute Lymphoid Leukemia (ALL) cell lines (JURKAT and MOLM13), due to the better established roles of mGluRs in ALL [47]. We have not examined GRM6 expression because its expression and role outside the retina is poorly understood. We have detected expression of all mGluRs genes in both

AML and ALL cell lines (Supplementary data 1). GRM2, GRM3, GRM4, GRM5, GRM7, and GRM8 showed no difference in expression between AML and ALL cell lines; additionally, expression levels of these genes were quite low, with around 10 copies per 10^6 housekeeper copies (GAPDH). Also, GRM1 expression was detected in all ALL and AML cell lines. On the contrary to other genes, two AML cell lines (OCI AML-3 and U937) displayed GRM1 expression a hundred times higher compared to others. We have described above that GRM1 is considered an oncogene and its overexpression seems to play a fundamental role in the development and maintenance of carcinogenesis, so we concentrated preliminarily in analyzing mGluR1 in AML cell lines and follow by the other mGluR sub-family. We selected OCI AML-3, OCI AML-2, and U937 for further studies (Fig. 6a, 6b, 6c). From the results shown in Figure 6, it is apparent that the highest levels are those of mGluR1 and mGluR2 (Fig.6a and 6b), with the exception of the cell line AML-2, where the expression levels of mGluR1 are near zero. Furthermore, the expression levels of mGluR5 and mGluR8 are relatively low in all three cell lines, while the levels of mGluR4, mGluR7, and mGluR3 are average. Among the different expression levels, it is noted that those of mGluR2 are higher than those of mGluR1, even if they are on the same order of magnitude.

2. mGluR1 protein is expressed in AML cell lines

Once we detected GRM1 mRNA expression in AML cell lines, we investigated whether the transcript was translated into protein. We performed Western Blot analysis on OCI AML-3 and U937, the AML cell lines that display the highest GRM 1 transcript levels. As a positive control, we have used human brain lysate and as a negative control, we have chosen OCI AML-2, which has displayed the lowest levels of GRM 1 expression (1.16 copies on 10^6 copies of GAPDH). The expression of mGluR1 protein on OCI AML-3 and U937 was detected, and the protein's signal was also detected on the positive control. As expected, no signal was detected in the negative control.

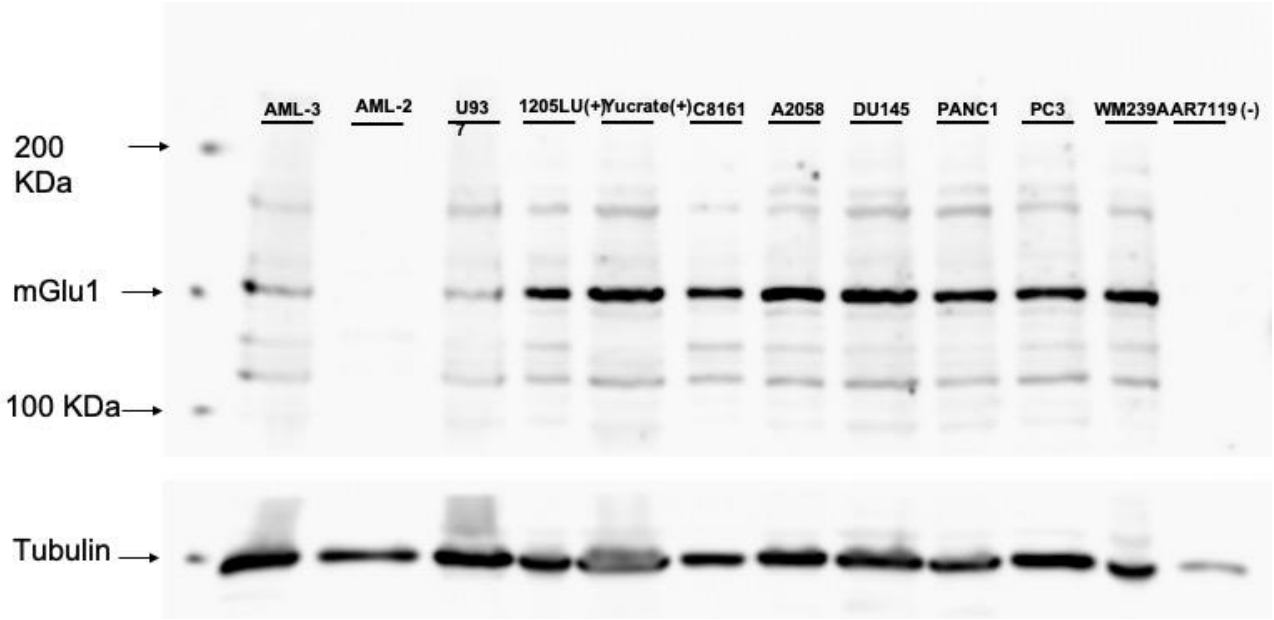


Figure 7: Western blot analysis of mGlu1α receptor in U937, AML3, and AML2 cells. Immunolabeling of mGlu1 receptor in other human cancer cell lines is also shown. Western Blot confirm mGlu1 receptor expression in AML3 and U937 cell lines but not in AML2 cell line.

3. mGluR1 is functional in OCI AML-3 but not in AML-2 and U937 cell lines

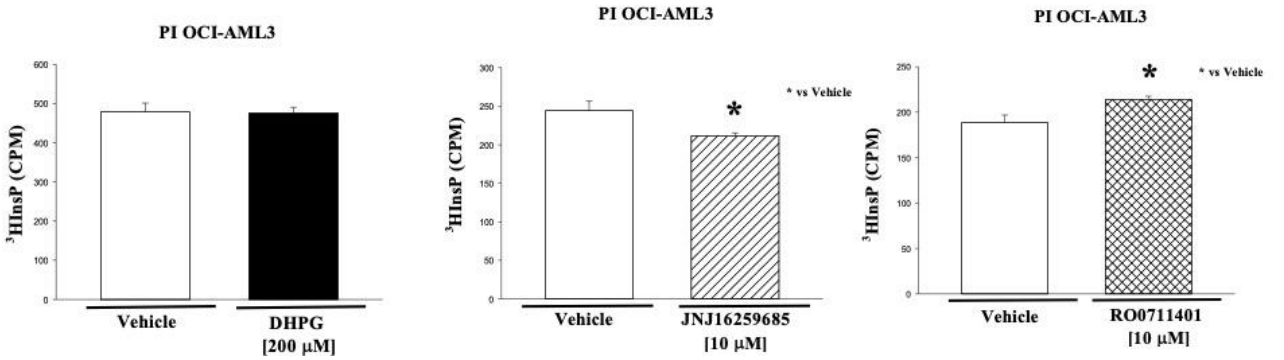


*Figure 8: [^{3}H]Polyphosphoinositide hydrolysis assay in AML-3 cells induced by DHPG 200 µM, no effects are observed n=6, One WayAnova ± SEM; PI hydrolysis assay on AML-3 cell line treated with JNJ16259685 (NAM) and RO0711401 (PAM) both at 10µM; note that NAM and PAM block and stimulate, respectively, the PI hydrolysis with statistical significance. Values are means ± SEM (n=6), * $p \leq 0,05$*

Up to this point, we have demonstrated that mGluR1 is expressed in some AML cell lines, so the next step was to demonstrate the functionality of this receptor in these cells. We have performed a [^{3}H]polyphosphoinositide ([^{3}H] PI) hydrolysis assay on OCI AML3, U937, and OCI AML2; with this assay, we wanted to study the consequences of mGluR1 modulation in the [^{3}H]PI hydrolysis pathway. To our knowledge, this is the first time that this technique, usually employed in neuroscience [127] is used in oncohematological cell lines. First, we analyzed the effect of DHPG (Fig.8), an orthosteric agonist of mGluR1 and mGluR5. We have detected no effect of DHPG on PI hydrolysis (Fig.8). However, DHPG is an orthosteric

agent, so we have postulated that the lack of effect could be linked to a high amount of glutamate in the medium, which could either maximally activate the receptor or induce an acute desensitization of it [128]. Subsequently, we have decided to modulate mGluR1 activity with an allosteric modulator; this allosteric modulator could increase (positive allosteric modulator, PAM) or decrease (negative allosteric modulator, NAM) receptor affinity and/or efficacy in a glutamate-independent path. We have performed a [^{3}H] PI assay where cell lines were treated with mGluR1 NAM (JNJ16259685) and PAM (RO0711401) (Fig.8).

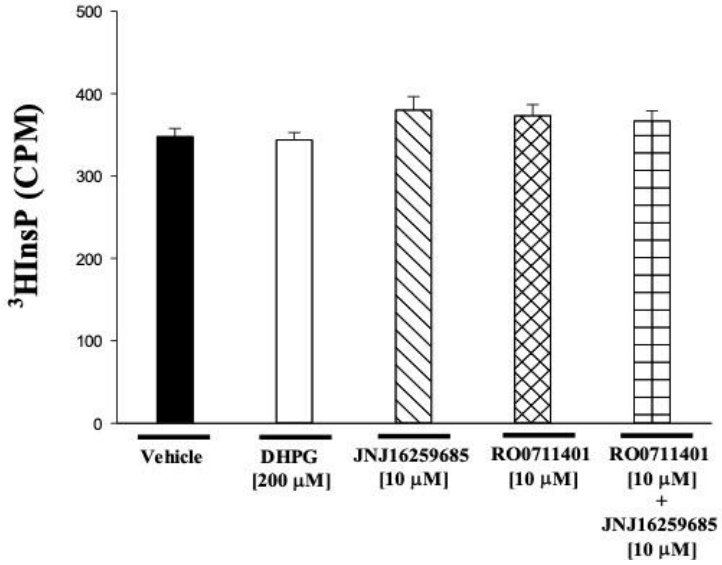


Figure 9: PI hydrolysis assay on AML-2 (n=4) cell lines treated with JNJ16259685, RO0711401 and DHPG; no effects were observed, One Way Anova ± SEM

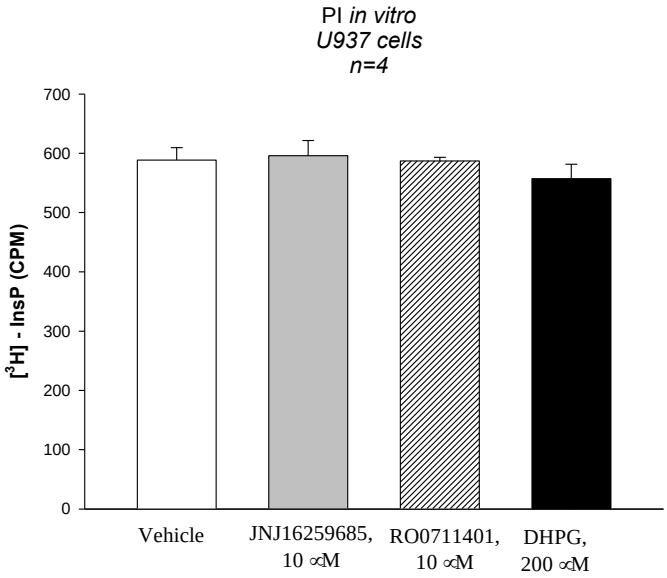


Figure 10: PI hydrolysis assay on U937 (n=4) cell lines treated with JNJ16259685, RO0711401 and DHPG; no effects were observed, One Way Anova ± SEM

We only observed statistical significance where mGluR1 PAM and NAM respectively enhanced or inhibited PI in OCI AML-3 cell line. Additionally, no effects were observed on U937 cells even though they expressed mGluR1 (Fig. 10). On OCI AML-2, where no mGluR1 protein or transcript were detected, it was not surprising that no effect of NAM or PAM was observed (Fig. 9). Taken together, we concluded that mGluR1 is expressed and functional in some AML cell lines, expression alone did not translate to functionality.

4. mGluR1 modulation did not alter AML-3 cell proliferation or viability

Once we demonstrated the presence and functionality of mGluR1 in AML cell lines, we assessed the cytotoxic effects of mGluR1 modulation as a putative target in AML. First, we performed an Annexin V and Propidium Iodide staining assay. We did not detect any changes in the percentage of apoptotic cells in OCI AML-3 cell lines treated with NAM or PAM versus the spontaneous apoptosis rate of OCI AML-3 as a result of mGluR1 blockade or activation (Fig. 11).

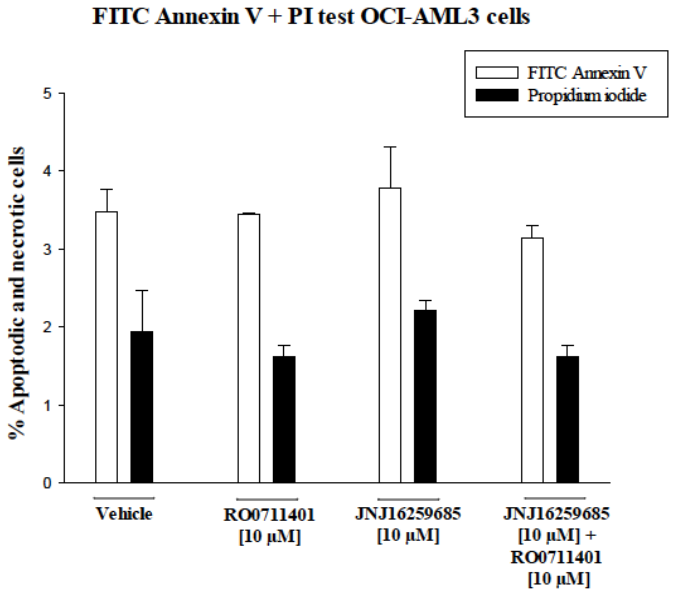


Figure 11: no effect of mGluR1 receptor blockade or activation on cells AML-3 apoptosis (n=3; One Way Anova ± SEM)

The AML-2 and U937 cell lines were not tested because AML-2 does not express mGluR1 receptor and the mGluR1 receptors in U937 were likely non-functional (data not shown).

5. MTT assay: cells viability evaluation

In order to investigate a possible "priming" of mGluR1 PAM or NAM, we have combined these agents with targeted therapy drugs: Cobimetinib (a MEK inhibitor) or Venetoclax (a

Bcl-2 inhibitor). We treated the OCI AML-3 and U937 cell lines to determine if mGluR1 was implicated in chemoresistance or chemosensitivity. No changes in cell proliferation were observed using mGluR1 PAM or NAM alone or in combination with Cobimetinib or Venetoclax (Fig. 12-13).

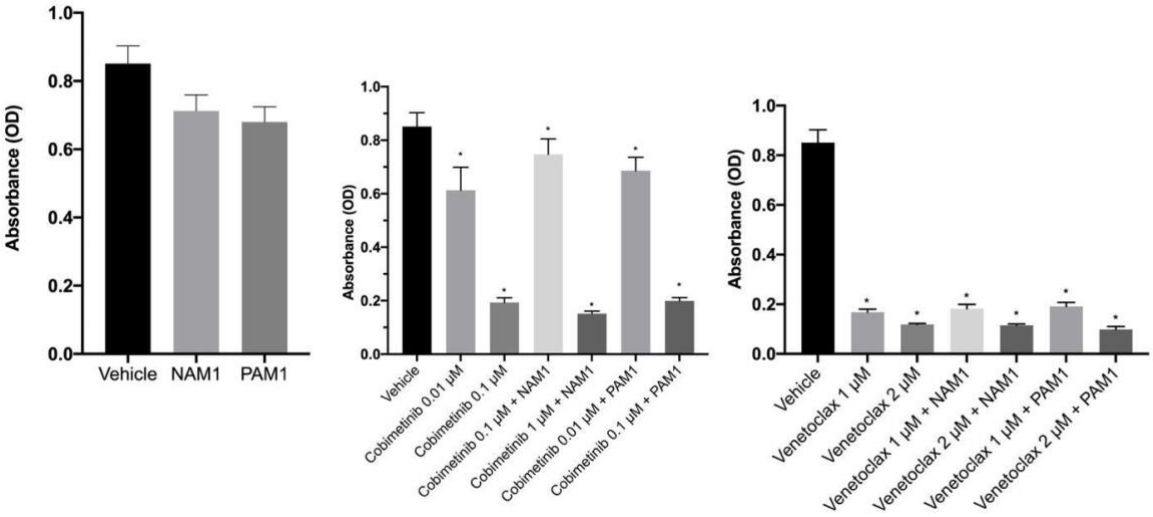


*Figure 12: Proliferation assay on AML-3 cell line treated with NAM and PAM alone and in combination with Cobimetinib and Venetoclax. OneWay ANOVA test ± SEM n=3, * p ≤ 0.05*

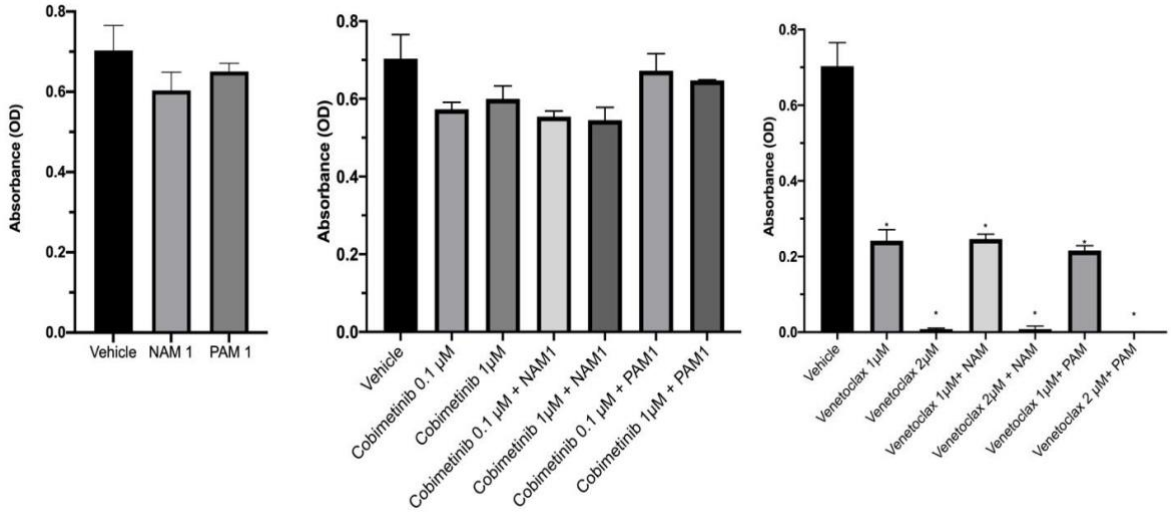


*Figure 13:Proliferation assay on U937 cell line treated with NAM and PAM alone and in combination with Cobimetinib and Venetoclax. OneWay ANOVA test ± SEM n=3, * p ≤ 0.05*

6. mGluR2/3 modulation influences cAMP levels in leukemia cell lines in a non-predictable pathway

We have evaluated the cAMP formation in leukemia cell lines after stimulation with LY379268 (selective group II mGlu receptor agonist), LY541850 (selective orthosteric mGluR2 agonist and mGluR3 antagonist, 10 µM), VU6001966 (NAM mGluR2, 10 µM). As

shown in Figure 14, furthermore, there is no difference when the two compounds are administered concurrently (Fig. 14). Administration of a selective orthosteric mGluR2 agonist and mGluR3 antagonist induces a decrease in cAMP level, which is amplified by concurrent administration of mGluR2 inhibitors. All of the data presented above show that, while mGluR2/3 modulation has an effect on cAMP levels, it occurs via a non-canonical pathway, and the interpretation of these findings remains unclear.

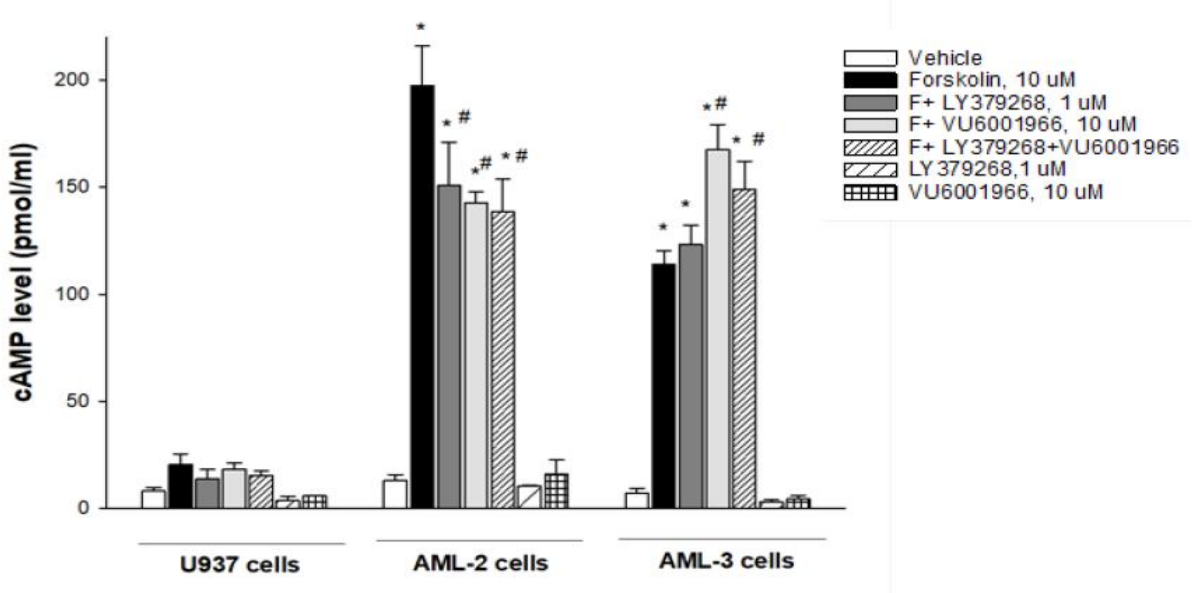
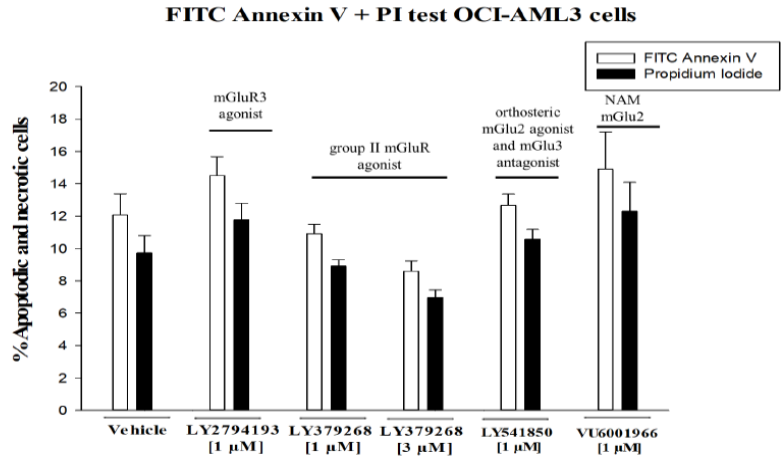


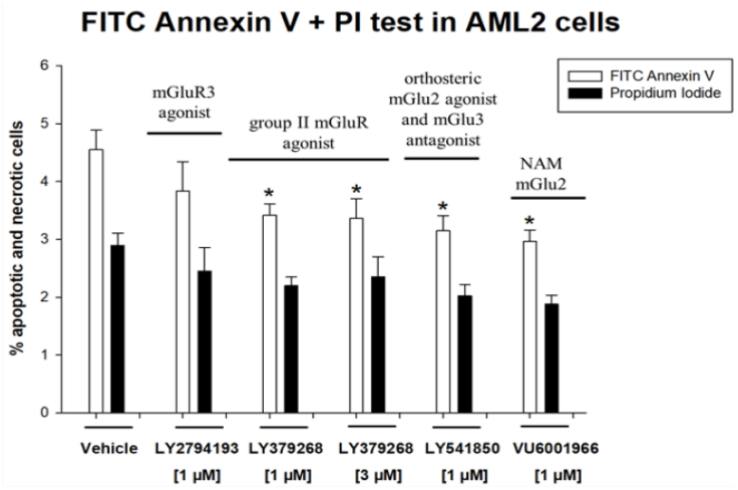
Figure 14: mGluR2-3 modulation displays no effect in the absence of forskolin, a compound that directly activates the adenylate cyclase; in U937 no significant effect is shown by the modulation of mGluR2-3; in OCI AML-2 and OCI AML-3 the modulation of mGluR2-3 induces a significant change in cAMP levels; however, these changes happen in a non-predictable way. One Way ANOVA ± SEM n=3

7. mGluR2/3 modulation does not induce a biologically significant increase in leukemia cell lines apoptosis

Despite the unclear mechanism of action of mGluR2/3 in leukemia cell lines, we aimed to assess cytotoxic effects of mGluR2/3 modulation. We have performed Annexin V and Propidium Iodide staining assay (Fig. 15). We have challenge cell lines with several compounds active on mGluR2/3 but, even if a statistically differences has emerged, no significant biological effect has been detected (percentage of apoptotic and necrotic cells was less than 2%).



mGlu2-3, Vehicle n=8, Treated n=6



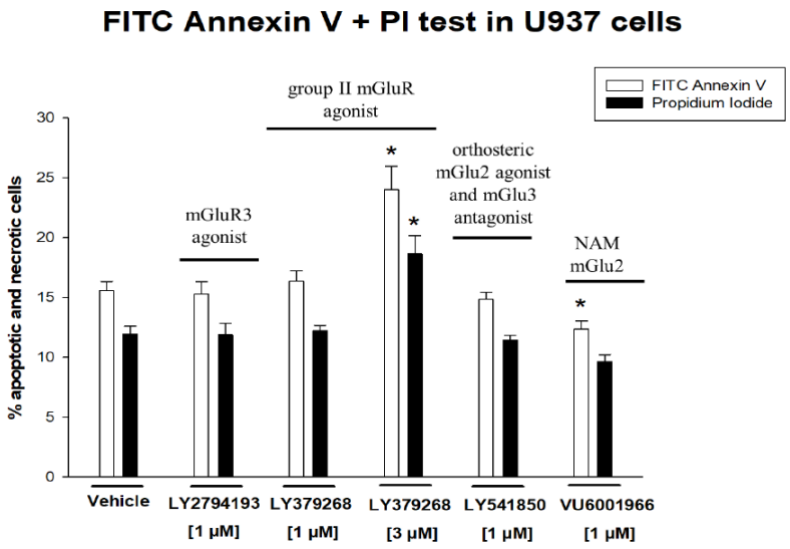


Figure 15: no significant biological effect of mGluR2/3 receptors blockade or activation on OCI AML-2, OCI AML-3 and U937 apoptosis. n=6, OneWay ANOVA ± SEM

8. mGluR7 activation induced a strong and receptor specific pro-apoptotic responses in U937 and AML-3 cell lines and differences in cAMP levels

Group III represents the largest and least known of the mGluR family. Several studies in the literature, suggested that these receptors may have anti-tumor properties. Therefore, we have challenged U937 and OCI AML-3 with several compounds against group III mGluRs: an mGluR4 PAM (VU0418506), an mGluR7 antagonist (XAP044), a mGluR7-8 PAM (VU 6005649) and a potent group II/III mGlu receptor antagonist (CPPG). mGluR4 PAM significantly decreased the apoptotic rate in OCI AML2 but did not affect apoptotic rate in OCI AML3 and U937 cell lines (Fig. 16). mGluR7 antagonist alone led a slight but significant decrease in apoptotic rate in OCI AML2, but not in OCI AML3 or U937 cell lines (Fig. 16). Interestingly, mGluR7-8 activation (mediated by a PAM, VU6005649) displayed a strong pro-apoptotic response in all three AML cell lines (Fig 16). These different responses are probably mediated by different mechanism(s) of cAMP modulation; moreover, as seen in Figure 17, no significant cAMP modifications are induced by mGluR7-8 PAM. In order to investigate the receptor-specificity of this observation, we incubated AML cell lines both with mGluR7-8 PAM and with the mGluR7 antagonist. In U937 cell lines, we observed an intermediate result, with a statistically significant increase in apoptotic rate but less robust compared to cell lines treated with mGluR7-8 PAM alone. These data demonstrated that mGluR7 activation induced strong and receptor-specific pro-apoptotic responses in U937 cells. In OCI AML-2, mGluR7-8 activation still induced a statistically significant pro-apoptotic effect, but after a challenge with both mGluR7-8 PAM and mGluR7 antagonist, the apoptotic responses increased. In summary, these findings suggest that mGluRs group III play important roles in AML biology; but more research is needed to investigate the role of mGluR7-8 in AML that may shed light on a possible therapeutic role for mGLuR7-8 in AML.

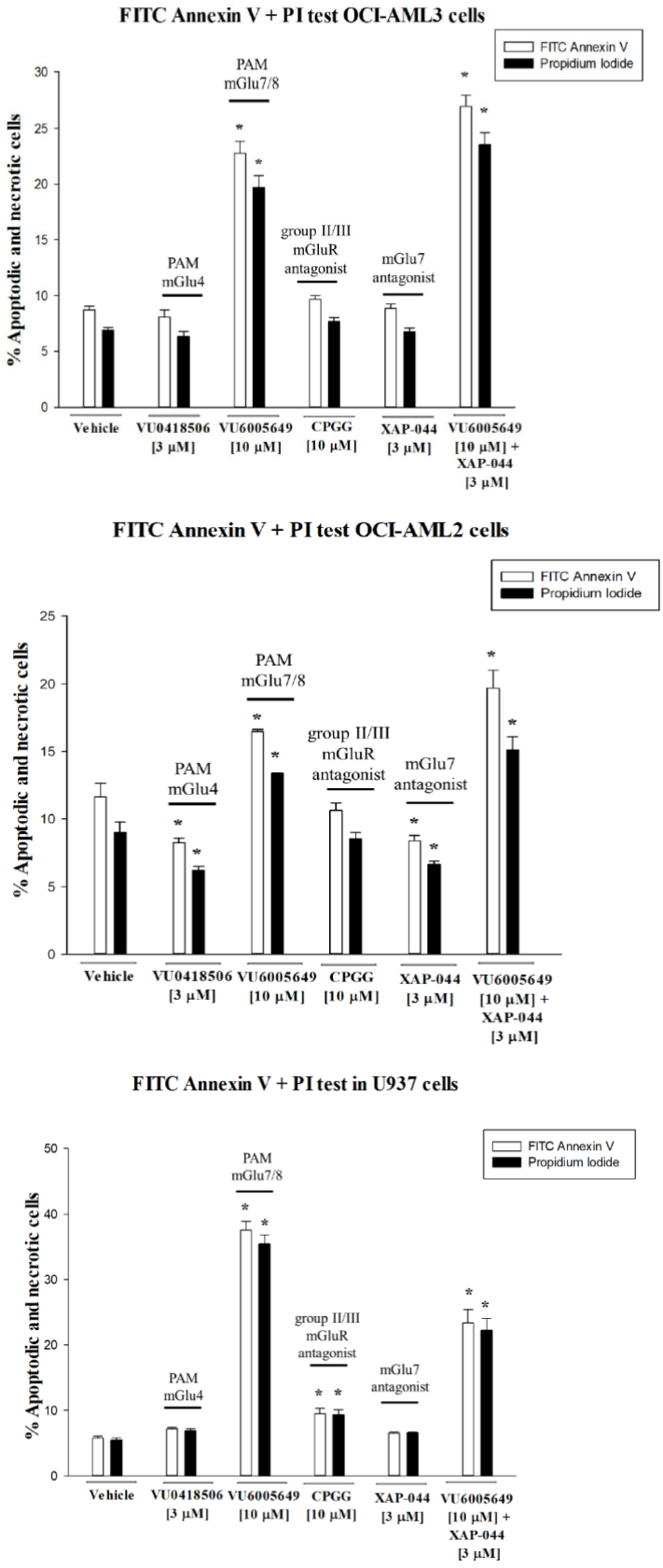


*Figure 16: OCI AML-3, OCI AML-2 and U937 cell lines were treated with VU418506, XAP-044, VU6005649, CPPG and a combination of VU6005649 and XAP-044. One Way Anova ± SEM: * $p \leq 0.05$ Treated VS Vehicle, # $p \leq 0.05$ VU6005649 VS VU6005649 + XAP-044; Cells were treated for 72 hours; n=4*

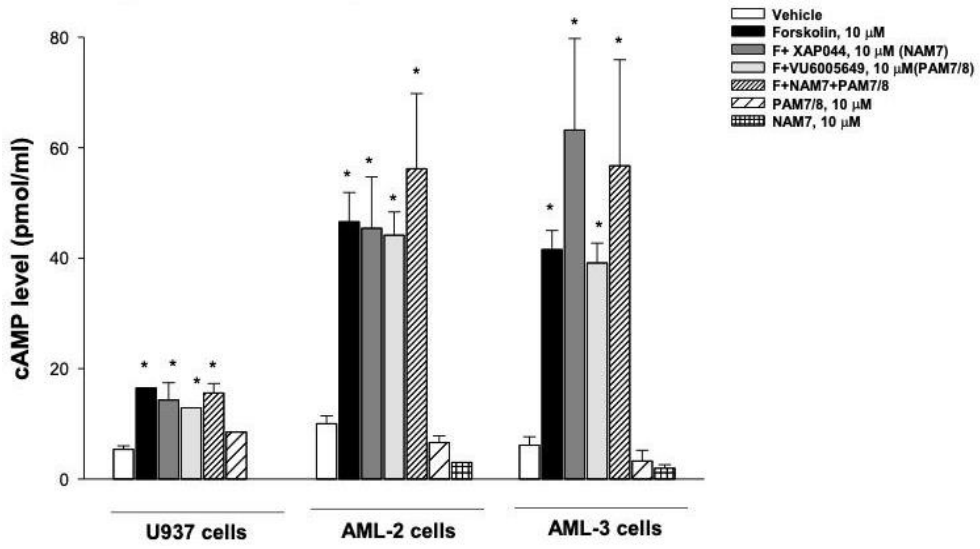


*Figure 17: cAMP levels in OCI AML-3, OCI AML-2 and U937 cell lines treated with NAM and PAM of mGluR7/8. We can observe a variation in cAMP levels especially in OCI AML-3 and OCI AML-2 cell lines. n=3, OneWay ANOVA ± SEM, * $p \leq 0.05$.*

After evaluating the effects of different drugs on the biology of leukemic cell lines, we also investigated the molecular pathway of AKT, which is involved in cancer cell survival. We considered both total AKT and p-AKT (pSer-473), and by western blot, both forms were quantified and assessed. As show in figure 18, in OCI AML-3 cell line treated for 20 minutes with PAM for mGluR7/8 in both complete medium and starvation medium, no significant changes in AKT and p-AKT levels were found (Fig.18). Similarly, total AKT and p-AKT levels were measured for the U937 cells treated with the group II agonist (LY379268) under both normal and starvation conditions (Fig.19). We observed significant differences in p-AKT/AKT levels only in cells treated under starvation conditions. Thus, we propose that the presence and amount of glutamate in the complete culture medium may interfere with receptor modulation by the agonist for group II.

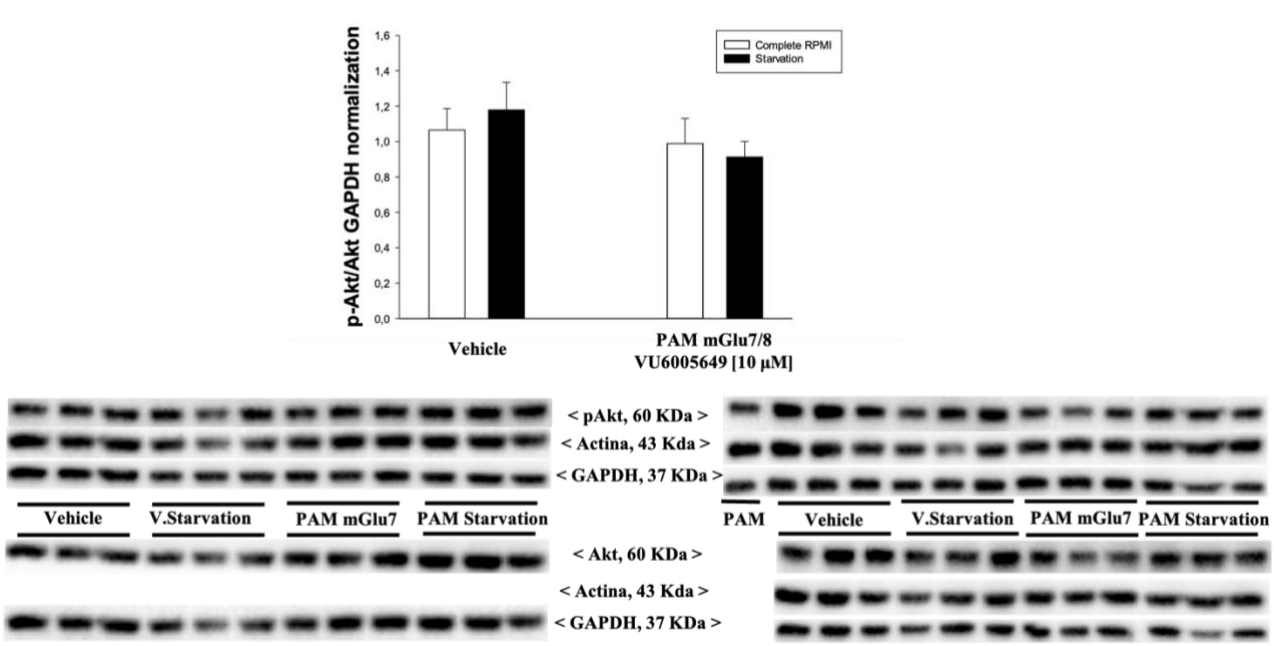


Figure 18: Western Blot and quantification levels for AKT and p-AKT after 20 minutes of treatments in complete medium and starvation medium on OCI AML-3 with mGluR7/8 PAM.

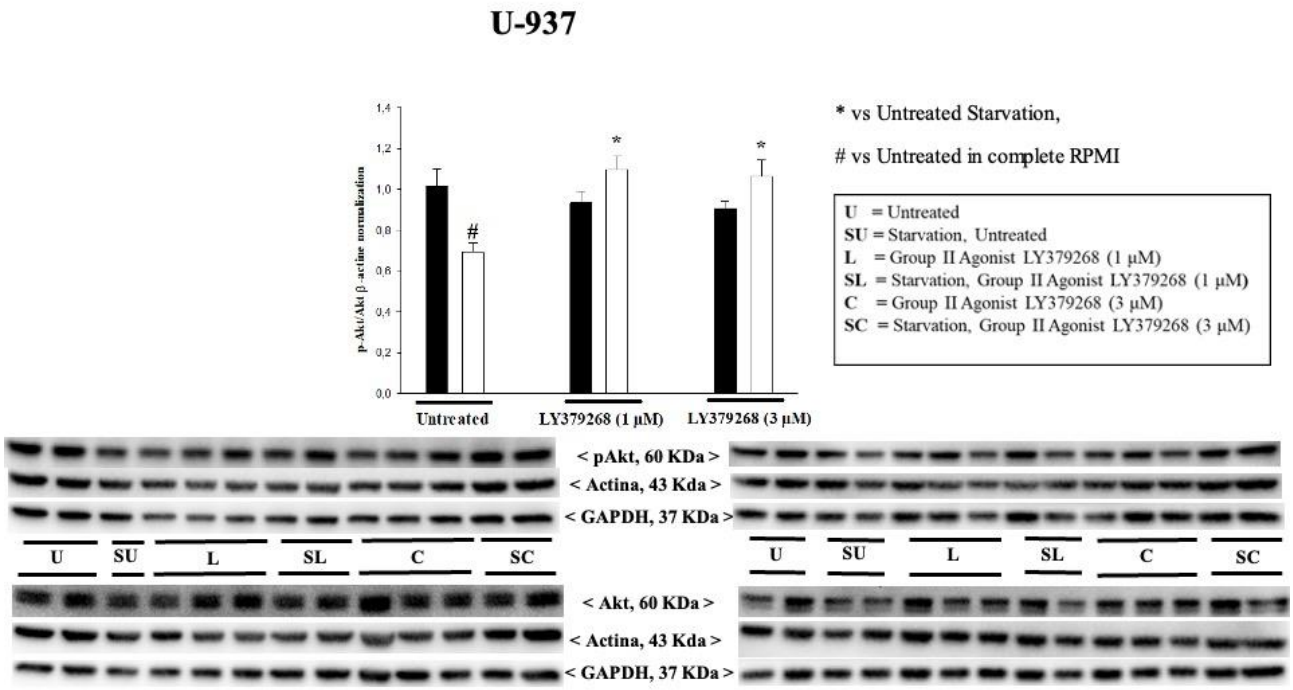


Figure 19: Western Blot and quantification levels for AKT and p-AKT after 20 minutes of treatment with mGluR2/3 agonist in complete and starvation medium on U937 cell line. Black columns=complete medium, white columns=starvation medium.,# $p \leq 0.05$*

Results Part II

10. Mass 3 clone 1 Scratch assay

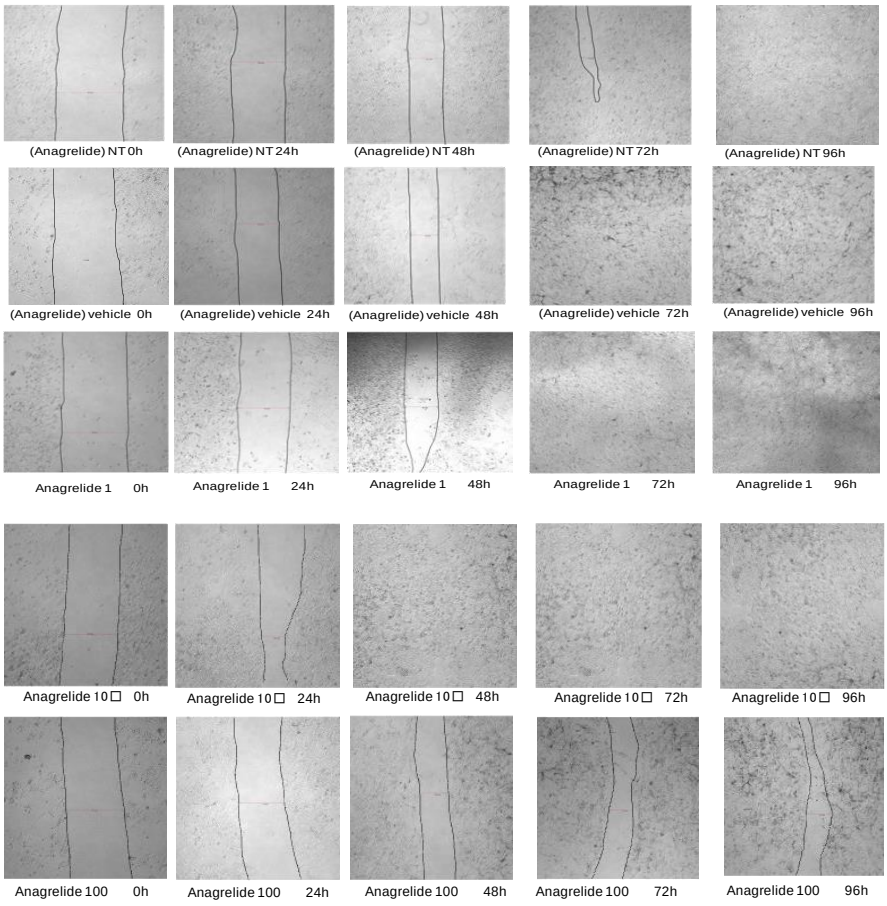


Figure 20: Scratch assay, at 5 different acquisition time and three different anagrelide concentration; anagrelide inhibits migration of Mass3 clone1 cell. After 24 h, monolayers were scratched, immediately imaged, and then treated with different concentrations of anagrelide (1, 10 and 100 μM). Cells were imaged every 24 h up to 96 h.

Using the scratch assays, we evaluated the time for the mouse melanoma cells to migrate and fill the space using three different concentrations (1, 10 and 100 μM) of anagrelide and 2H5F (Fig.20). As shown in Figure 20, anagrelide at 100 μM yielded the greatest limitation on cell motility, perhaps mediated through a blockade on L1, a protein important for melanoma mobility and invasiveness.

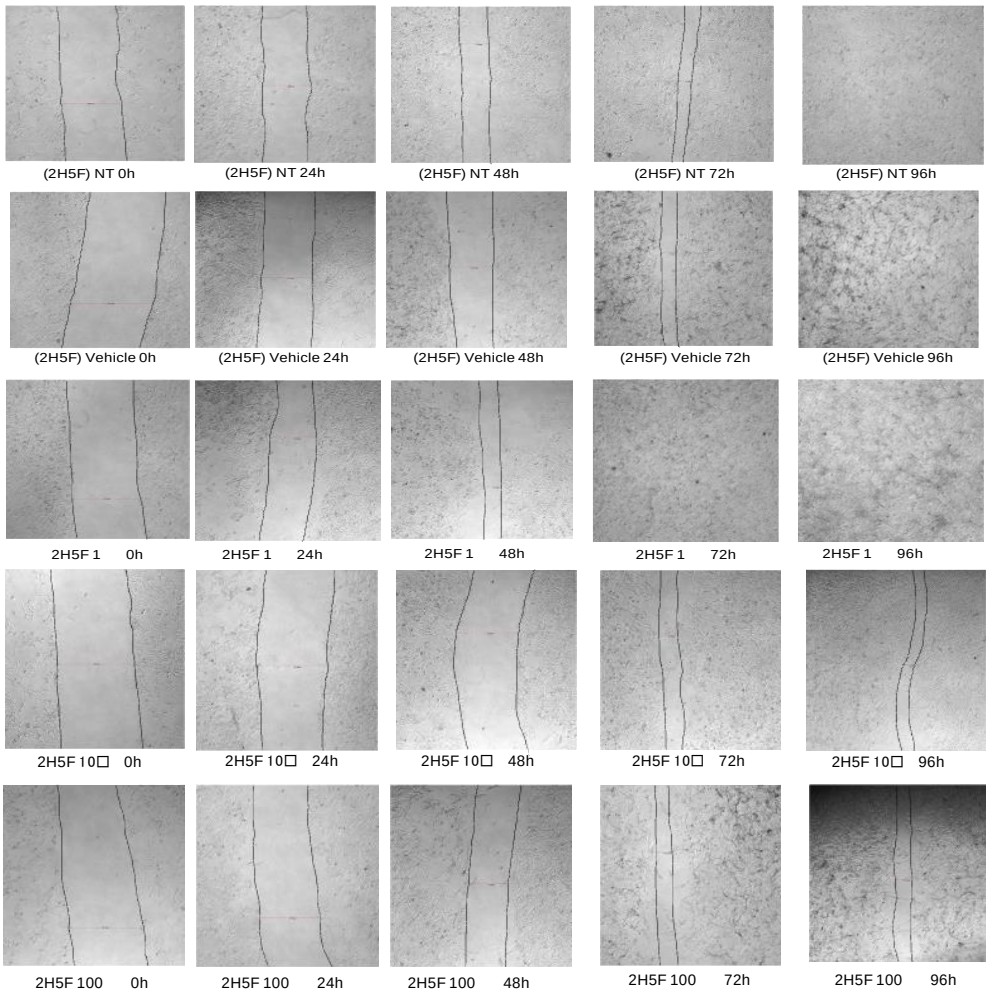
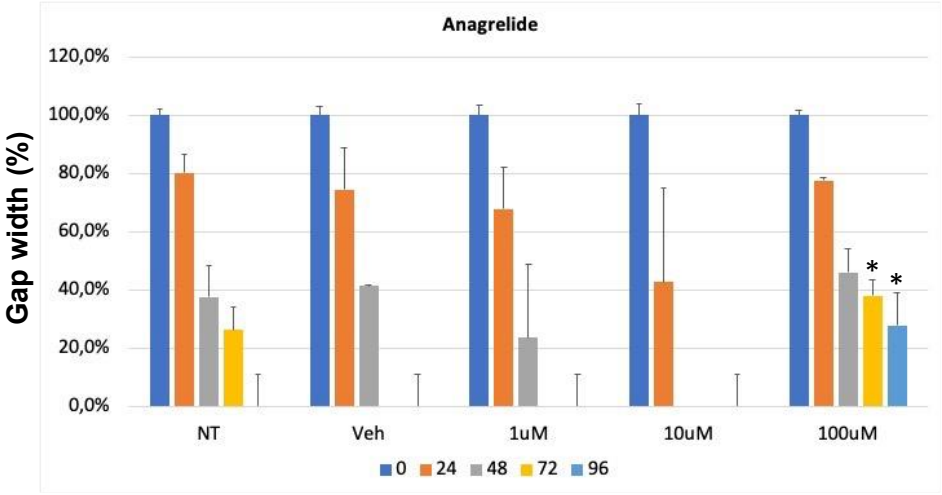


Figure 21:Scratch assay, at 5 different acquisition time and three different 2H5F concentration. 2-Hydroxy-5-fluoropyrimidineinhibits migration in Mass3 clone 1 cell. After 24 h, monolayers were scratched, immediately imaged, and then treated with different concentrations of 2-Hydroxy-5-fluoropyrimidine (2H5F) (1, 10 and 100 μM). Cells were imaged every 24 h up to 96 h.

With 2H5F, at concentration of 10 μM at both 72 and 96 hours, noticeable space was present compared to untreated (NT) or vehicle (Fig.21). Taken together, either antagonist mimetic of L1 was able to reduce cell migration at different concentrations.



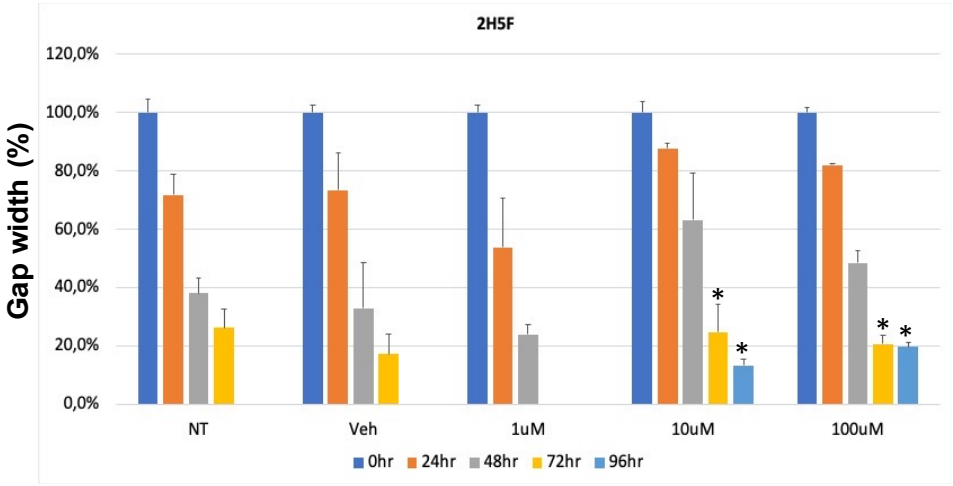


*Figure 22: Graphs represents the gap percentage (%) of untreated cells, vehicle-treated cells and cells treated with 1, 10, 100 µM anagrelide or 2H5F. Three images were taken per well and values for each condition were averaged. ANOVA ± SD, *p ≤ 0,05*

11. Proliferation Assay

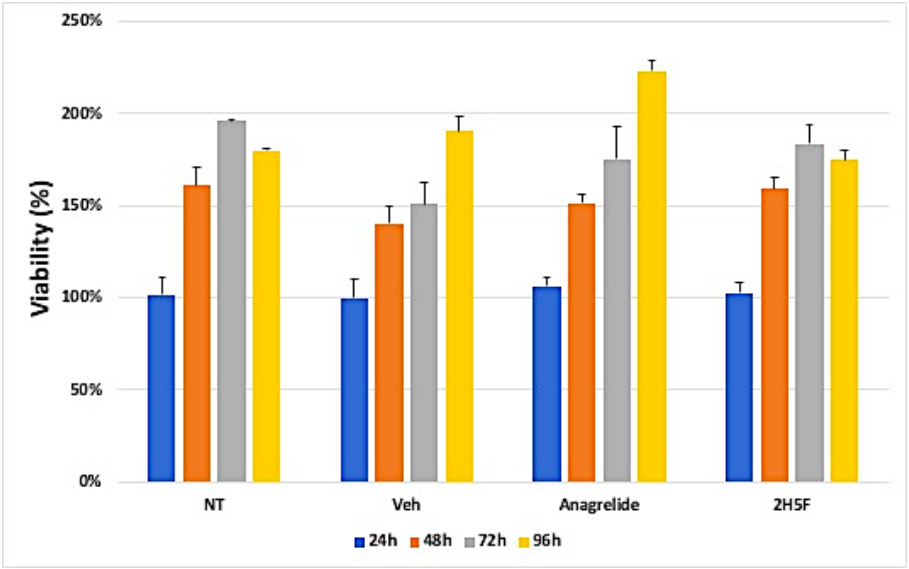


Figure 23: MTT Assay on Mass3 Clone 1 cell line at different time points treated with 100 µM Anagrelide and 2H5F. n=3 TwoWay ANOVA Test ± SD

We then performed cell proliferation/cell viability assays to examine if one or both antagonist mimetics of L1 may influence mouse melanoma cells in cell proliferation and/or viability (Fig.23). No effect is observed after a 96 hours treatment, these results suggest that Anagrelide and 2H5F compounds not affect cell proliferation. Next, we perform *in vivo* assays to determine if one or both antagonist mimetics of L1 may possess same inhibitory activity *in vivo*. In order for us to monitor the growth and migration of the inoculated mouse melanoma cells, we took advantage of the luciferase reporter gene.

12. Luciferase reporter and luminometer assay

After infecting cells with lentivirus carrying the luciferase reporter gene and the gene for puromycin resistance, the cells were allowed to proliferate and emerging of putative resistant clones were selected and tested for luminescence (Fig.24). We isolated 30 puromycin resistant clones, those with the highest luminescence levels were selected, namely clone 1, 7 and 8 for further studies. Then, Mass3 clone 1 was chosen to be used in the *in vivo* allograft study.

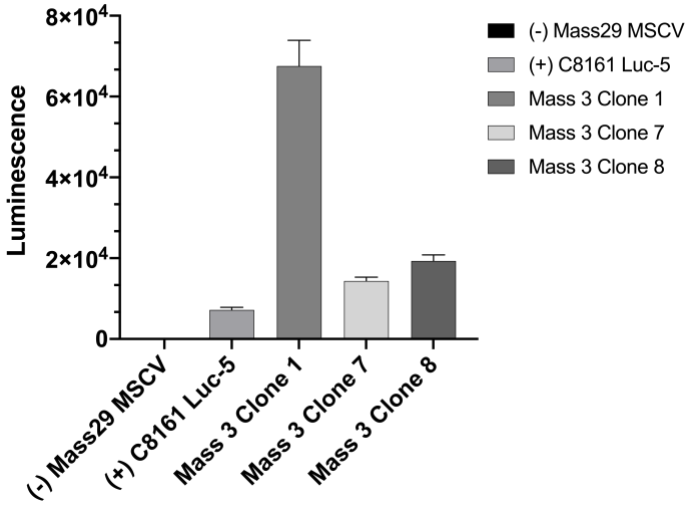


Figure 24: Luminometer assay results on Mass3 clone 1, 7 and 8 showed the higher readings for luminescence, so we choose them to work with, other clone's tests are not graphed

13. Immunoblotting for L1 (CD171) in melanoma cell line at different time points

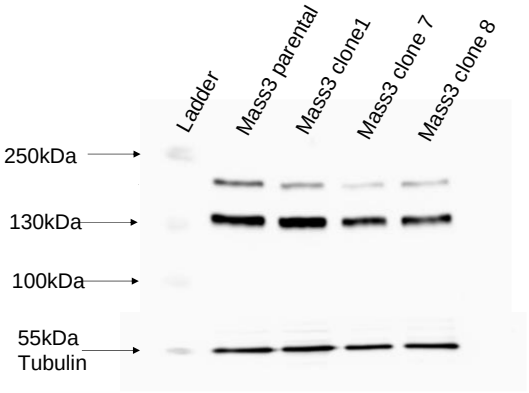


Figure 25: Immunoblotting assay on Mass3 cell line, parental and clones 1,7 and 8. 20 μl of extract are loaded in SDS-gel. The molecular weight for L1 in mouse cell line is ~150 kDa for the truncated form as showed by immunoblotting and 200-220 kDa for the full lenght; tubulin was used as internal control

We knew from the published reports by others that L1 protein is expressed in melanoma cells, but to be certain that the mouse melanoma cells we use for the studies also express L1, immunoblottings were performed (Fig. 25) on protein extracts obtained from the parental Mass 3 and three different Mass 3-derived clones that had a very high level of active luciferase compared with controls, based on the luminometer assay (see below). As expected, we detected L1 protein in all our samples used. We then performed westerns on protein lysates prepared from the cell proliferation/viability assays at 4 different times, L1 expression was detected in all samples (Fig. 26A and 26B).

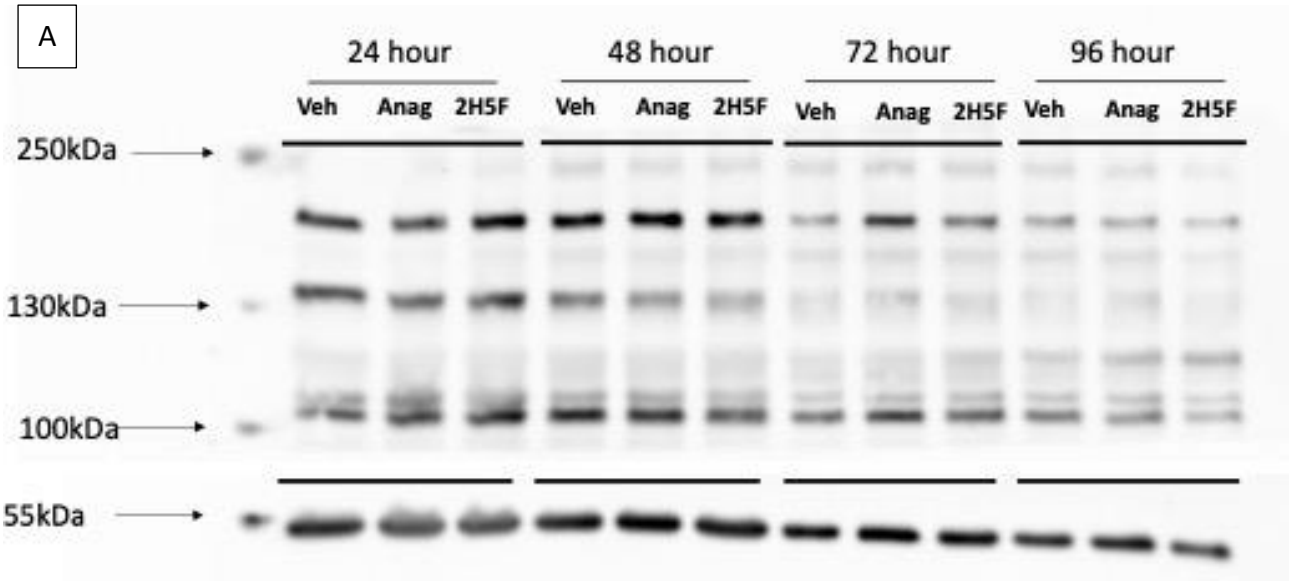


Figure26A: Western blot of Mass3 Clone 1 treated at 24, 48, 72, and 96 hours with Anagrelide 100 μM and 2H5F 100 μM. Molecular weight: 200-220 kDa, full length protein. 20 μl/each sample.

B

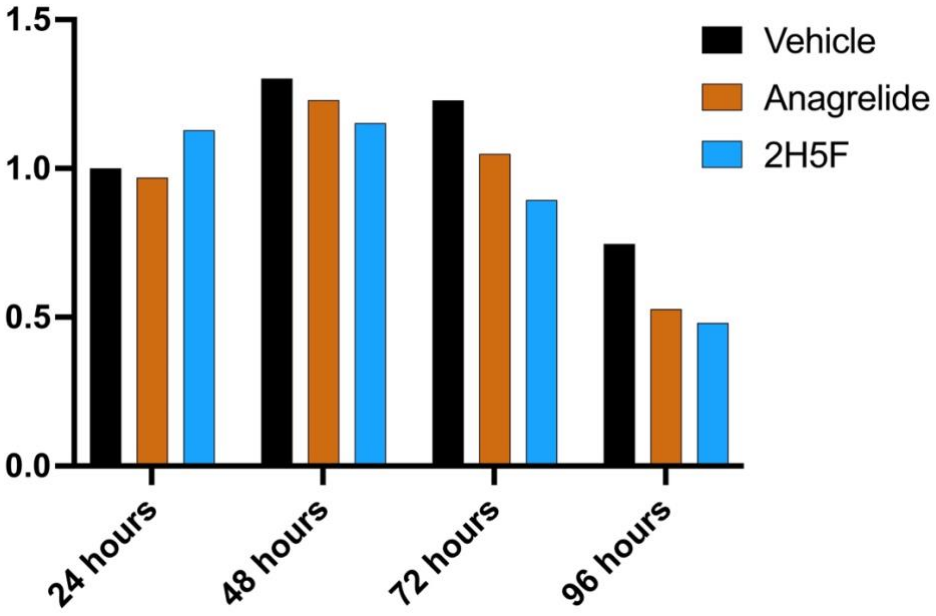
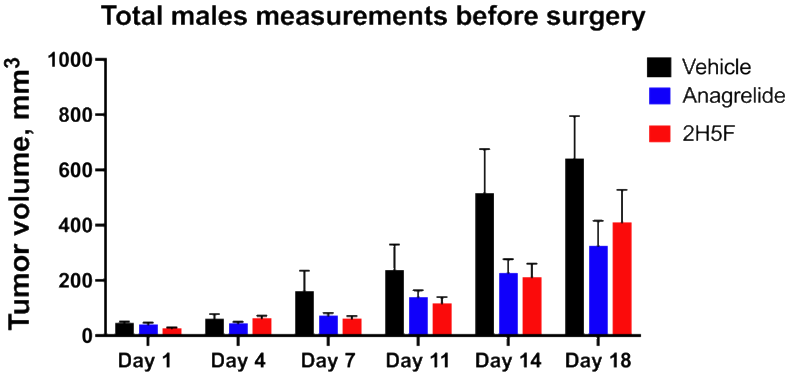


Figure26B: Quantification of western shown in Figure 26A.

15. *In vivo* imaging and caliper tumor measurements



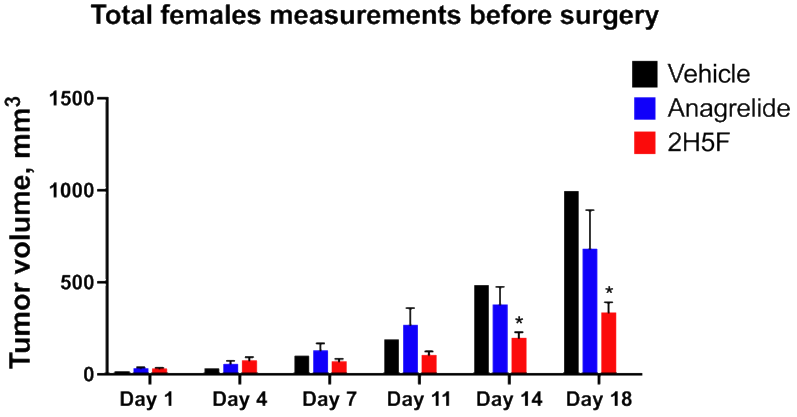


*Figure 28: Caliper measurements, volume unit in mm³; treatments VS vehicle, Student T test *p≤0.05*

Caliper measurements were performed twice a week for about 18 days. Based on these measurements, it appears that in female group treated with 2H5F was the most effective in reducing tumor growth significantly (Fig.28). At the same time, luminescence images were also acquired by IVIS of all three animal groups for the purpose of monitoring tumor development and the possible appearance of metastasis (Fig. 29).

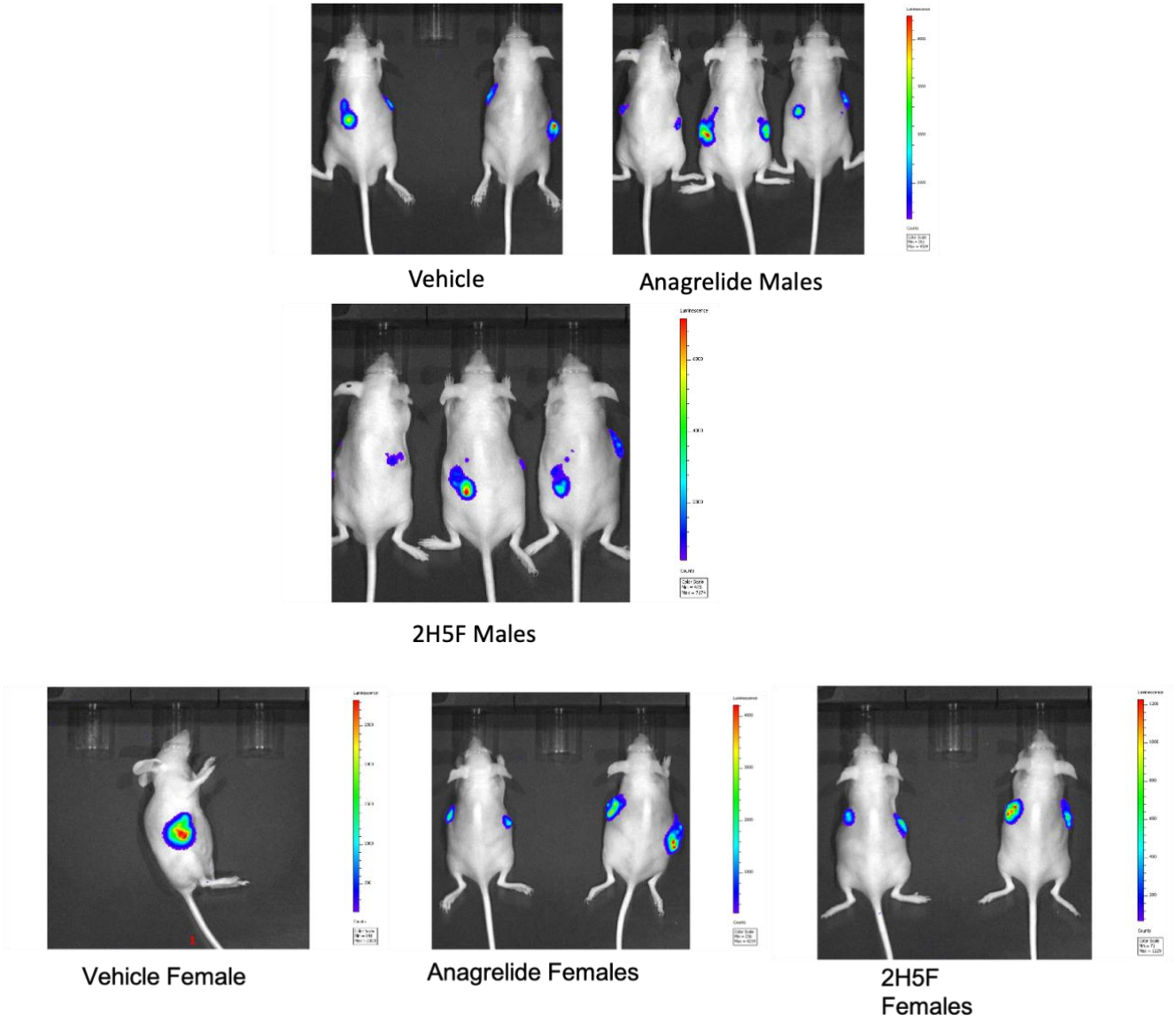


Figure 29: Luminescence images from representative mice before primary tumor surgery, Males and Females groups Vehicle and Treatments

14. Expression of L1 (CD171) in excised primary tumors

After inoculation of one million MASS3 clone1 cells into the flank of immunocompetent hairless C57BL/6, palpable tumors appear after 10 days. The tumor volumes were measured twice a week and the small animal imaging system was used to observe the

growth/migration of the inoculated cells. When the primary tumors reach 600 mm^3, we performed survival surgeries to remove the tumors and continue to monitor the tumor progression. We performed westerns on some of the excised tumors for the L1 expression (Fig. 27) and between primary and relapses tumors (Fig. 28).

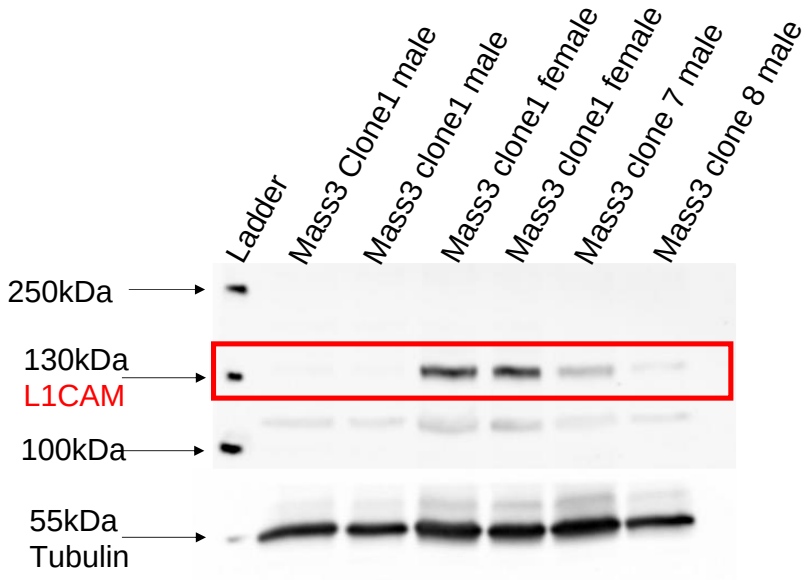


Figure 27: Immunoblotting assay on Mass3 clone 1, 7 and 8, from proteins extracts derived from tumor tissues after tumor removal surgery on mice. 30 µl of extracts were loaded in SDS-gel, 7.5%. Tubulin was used as internal control

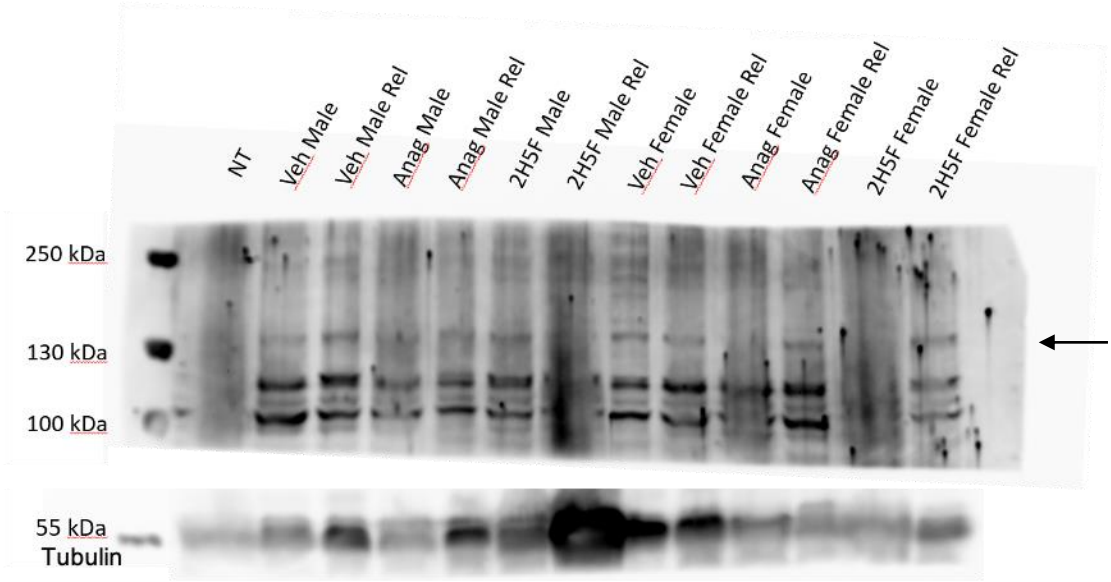


Figure 28: Immunoblotting assay on Mass3 clone 1 (primary and relapses) from proteins extracts derived from tumor tissues after tumor removal surgery on mice. 30 µl of extracts were loaded in SDS-gel, 7.5%. Tubulin was used as internal control

Discussion

Since they were discovered in the 1980s, metabotropic glutamate receptors (mGlus) have attracted increasing interest, especially in their function within the central nervous system and their correlation with neurodegenerative diseases such as Parkinson's and Alzheimer's and nervous central system cancers. To date, we know that these receptors are important for both neural plasticity and long-term memory development. Most studies, therefore, are focused almost entirely on the pathophysiology of the central nervous system; however, after the discovery that these receptors are also expressed in ectopic sites and consequently led to onset of tumors. The interest has increased dramatically to study their expression and function in tissues outside the central nervous system. In the literature we try to understand the role of these receptors in tumor development going so far as to define their differential roles as oncogenes or tumor suppressors. In breast cancer, colorectal cancer, kidney cancer, oral squamous cell carcinoma, pancreatic cancer, osteosarcoma, and prostate cancer, the metabotropic glutamate receptors are involved in regulating cell proliferation, cell differentiation, resistance to chemotherapy and induce oncogenic transformation. Despite these studies, there was lack of the systematic analysis of metabotropic glutamate receptors in leukemia with regard to the receptor's expression, function, and activity. The objective of this work is to begin to uncover putative role(s) of these receptors in leukemia.

In this work, we evaluate mGlu Receptors as a putative therapeutic target in Acute myeloid leukemia (AML). AML is the most common acute type of leukemia in adults and elderlies; despite the high incidence of this disease and a large number of new drugs available, prognosis remains poor, especially in patients unable to receive intensive chemotherapy. Based on the data obtained from the first part of the current work on human acute myeloid leukemia cell lines, we conclude that all three groups of metabotropic glutamate receptors are expressed in the OCI AML-3, OCI AML-2 and U937 AML cell lines. The expression levels of these receptors are variable in the different lines but in general they are low for GRM3, GRM4, GRM5, GRM7 and GRM8 while they are higher for GRM1 and GRM2. Specifically, the results obtained by hydrolysis of radioactive-labeled polyphosphoinositides, where we showed that mGluR1 is not only expressed but that its

activity can also be modulated through the use of specific drugs in only one AML cell line, OCI AML-3, but not in U937 or third AML cell line, OCI AML-2, did not have detectable mGluR1 expression. Role(s) of mGluR1 function(s) in AML remain obscure; despite cellular responses alter with mGluR1 modulators. However, results obtained with the cell viability assays, MTT in the presence of different drugs specifically the targeted ones, Cobimetinib and Venetoclax, plus the labeling with annexin V and propidium iodide for cellular apoptosis and necrosis, suggested that mGluR1 is not involved in these cellular proliferation and/or survival activities rather some other biological functions.

Similar ambiguity exists for the roles of mGluR2/3 in AML. mGluR2/3 had high levels of expression assessed by RNA and protein also modulation by different drugs led to alterations of the second messenger, cAMP. However, the cytofluorimetry data did not show any biological consequences. Additional studies by genetic manipulations of mGluR2/3 may yield indications how one or both receptors mediate its activity in AML. Group III represents the largest and least known of the mGluR family. Several earlier studies suggested that these receptors may have anti-tumor properties. mGluR7 activation induced strong and receptor-specific pro-apoptotic responses in AML-3 and U937 cells. In another AML cell line, OCI AML-2, mGluR7/8 activation induced a statistically significant pro-apoptotic response, but after a challenge with both mGluR7/8 PAM and mGluR7 antagonist, the apoptotic responses increased. Therefore, it is not clear if there are some interactions between mGluR7 and 8. Taken together, these findings suggest that mGluRs group III play important roles in AML biology; but further investigations are warrant to elucidate the role(s) of mGluR7/8 in AML. In addition, *in vivo* studies are currently underway to determine the possible consequences in tumor progression of inoculated OCI AML-3 cells in immunodeficient mice in the presence of mGluR7/8 PAM, VU6005649.

Skin cancers have the highest cancer rate, about 1% of skin cancers is melanoma but it accounts for the majority of death from skin cancer. In the United States, about 100,000 new cases of invasive melanoma and over 7,000 fatalities are predicted in 2023. Much progress was made in the past 10-15 years with targeted therapies against mutated BRAF, the most common mutation in cutaneous melanoma, and immune-checkpoint blockade immunotherapies.

However, melanoma remains one of the most difficult cancer to treat with high frequencies of relapse and resistance. Ectopic expression of mGluR1 was shown to be able to induced *in vitro* cell transformation and *in vivo* tumorigenesis. Clinical trials with riluzole, an FDA approved drug for the treatment of amyotrophic lateral sclerosis (ALS) showed stable disease in about 40% of the patients, combining riluzole with immune-checkpoint inhibitor resulted in good responses but resistance emerged with time in a melanoma mouse model, suggesting that better understanding of the basis of melanoma biology is still needed

In this study, we explore the role of an adhesion molecule, L1CAM or CD171 in melanoma cell migration. Earlier studies by others demonstrated that L1CAM's involvement in tumor development, tumor cell invasion, and metastasis of melanoma, ovarian, and colon cancers by overexpression of L1CAM. LICAM was shown to promote malignant cell mobility and it is linked to the activation of multiple signaling pathways known to be critical in tumor cell proliferation and survival including extracellular signal-regulated kinase (ERK), focal adhesion kinase (FAK), and p21-activated kinase (PAK). Two mimetic antagonists of L1CAM, anagrelide and 2H2F were used in our studies, we confirmed these two antagonists reduced mouse melanoma cell migration. *In vivo* tumorigenicity assays also showed 2H5F is effective in reducing tumor volume significantly compared with control as early as after 14 days of treatment. Unfortunately, we did not observe any metastasis development but only relapses after surgically removal of primary tumors. These results clearly showed that there is a limiting effect on cell proliferation *in vitro* as well as *in vivo*.

Future perspective

Given the promising results, the *in vivo* study of mGluR7/8 in AML is still ongoing. However, it would be interesting to be able to produce knockout lines for mGluR1, 2 and 3 in AML cells and investigate the significances of these receptors in AML pathogenesis. For melanoma, we need to use a more aggressive cell line and examine if anagrelide or 2H5F may modulate tumor cell migration, invasion, and metastasis.

Aknowledgements

I would like to dedicate this space to those who, with dedication and patience, contributed to the realization of this project.

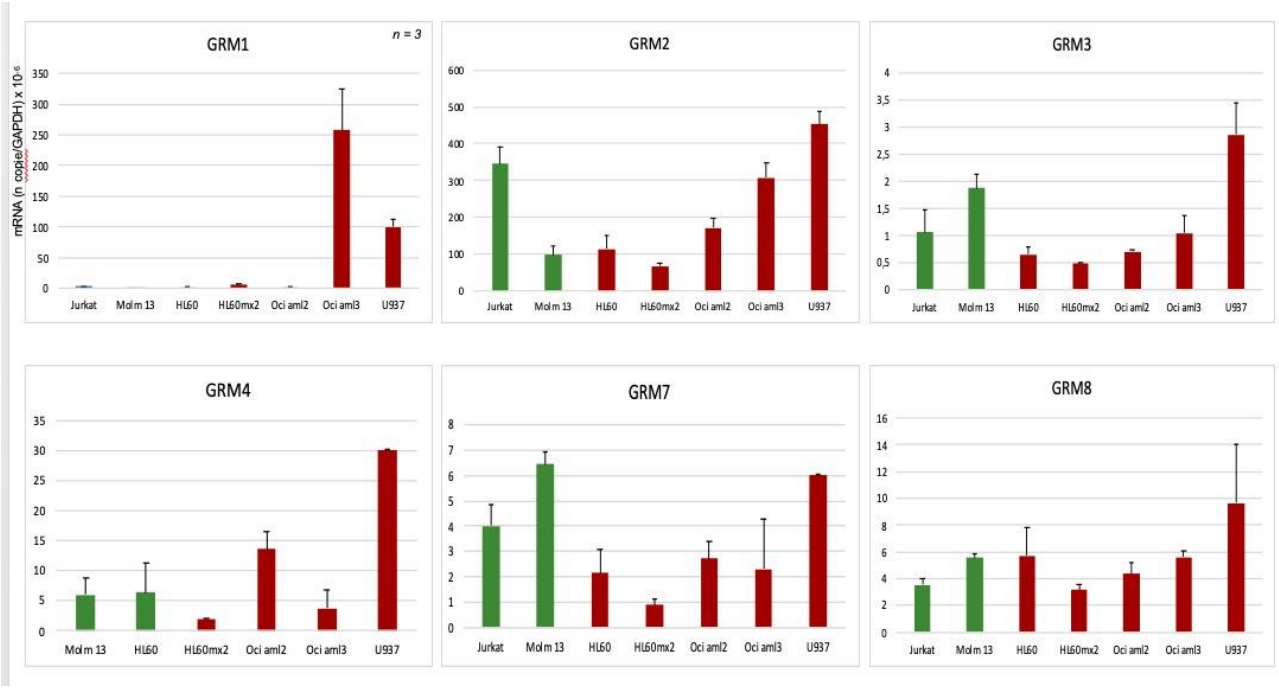
A special thanks goes to my supervisor Professor Ferdinando Nicoletti who has followed me, with his endless helpfulness, in every step of realization of this project, since the choice of the topic.

Thanks also to my co-advisor Professor Suzie Chen for her valuable advice and for promptly suggesting me the right changes to make to my thesis and for everything she taught me during our time together and especially for hosting me in her lab.

Thanks, are also due to all the institutions that have hosted me in different forms in the past years: the "Sapienza" University of Roma (Italy), Istituto di Ricovero e Cura a Carattere Scientifico (I.R.C.C.S) Neuromed (Italy) and Rutgers, The State University of New Jersey (USA).

Special thanks go to the Department of Clinical and Molecular Medicine, Hematology at Sant'Andrea where Dr.sa Ricciardi and Dr. Mirabilii taught me how to work with leukemia cells but especially to Professor Agostino Tafuri who kindly provided us with the cell lines used, without which this project would not have been born.

Supplementary Data



Supplementary Data 1: qRT-PCR of all mGluRs transcripts in lymphoid and myeloid cell lineages (Green: lymphoid; Red: myeloid); n= 3, all copies are normalized versus GAPDH copy numbers.

Publications

Serena Notartomaso, Nico Antenucci, Francesca Liberatore, Giada Mascio, Stefano Vito Boccadamo Pompili, Joan Font, Mariarosaria Scioli, Livio Luongo, Antonietta Arcella, Roberto Gradini, Amadeu Llebaria, Ferdinando Nicoletti **Light-Induced Activation of a Specific Type-5 Metabotropic Glutamate Receptor Antagonist in the Ventrobasal Thalamus Causes Analgesia in a Mouse Model of Breakthrough Cancer Pain.** *Int. J. Mol. Sci.,* **2022** Jul 20;23(14):8018. doi: 10.3390/ijms23148018.

Kevinn Eddy, Mohamad Naser Eddin, Anna Fateeva, Stefano Vito Boccadamo Pompili, Raj Shah, Saurav Doshi and Suzie Chen. **Implications of a Neuronal Receptor Family, Metabotropic Glutamate Receptors, in Cancer Development and Progression.** ***Cells*** **2022, *11*(18), 2857; https://doi.org/10.3390/cells11182857**

Bibliography

1. Kampen, K.R. The discovery and early understanding of leukemia. *Leukemia Research* **2012**, *36*, 6-13, doi:https://doi.org/10.1016/j.leukres.2011.09.028.
2. Ronald Hoffman, E.J.B.J., Leslie E. Silberstein, Helen Heslop, Jeffrey Weitz, John Anastasi. *Hematology-Basic principles & Practice 6th Ed.*, 6 ed.; Sunders, E., Ed.; Churchill Livingstone; 6th edition: 2013; p. 2384.
3. Zhu, J.; Emerson, S.G. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* **2002**, *21*, 3295-3313, doi:10.1038/sj.onc.1205318.
4. Rice, K.L.; Hormaeche, I.; Licht, J.D. Epigenetic regulation of normal and malignant hematopoiesis. *Oncogene* **2007**, *26*, 6697-6714, doi:10.1038/sj.onc.1210755.
5. Chopra, M.; Bohlander, S.K. The cell of origin and the leukemia stem cell in acute myeloid leukemia. *Genes Chromosomes Cancer* **2019**, *58*, 850-858, doi:10.1002/gcc.22805.
6. Eaves, C.J. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood* **2015**, *125*, 2605-2613, doi:10.1182/blood-2014-12-570200.
7. Kondo, M.; Wagers, A.J.; Manz, M.G.; Prohaska, S.S.; Scherer, D.C.; Beilhack, G.F.; Shizuru, J.A.; Weissman, I.L. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* **2003**, *21*, 759-806, doi:10.1146/annurev.immunol.21.120601.141007.
8. Schepers, K.; Campbell, T.B.; Passegué, E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell* **2015**, *16*, 254-267, doi:10.1016/j.stem.2015.02.014.
9. Tenen, D.G. Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer* **2003**, *3*, 89-101, doi:10.1038/nrc989.
10. Pollyea, D.A.; Jordan, C.T. Therapeutic targeting of acute myeloid leukemia stem cells. *Blood* **2017**, *129*, 1627-1635, doi:10.1182/blood-2016-10-696039.
11. Juliusson, G.; Hough, R. Leukemia. *Prog Tumor Res* **2016**, *43*, 87-100, doi:10.1159/000447076.
12. Bispo, J.A.B.; Pinheiro, P.S.; Kobetz, E.K. Epidemiology and Etiology of Leukemia and Lymphoma. *Cold Spring Harb Perspect Med* **2020**, *10*, doi:10.1101/cshperspect.a034819.
13. Hulegårdh, E.; Nilsson, C.; Lazarevic, V.; Garelius, H.; Antunovic, P.; Rangert Derolf, Å.; Möllgård, L.; Uggla, B.; Wennström, L.; Wahlin, A.; et al. Characterization and prognostic features of secondary acute myeloid leukemia in a population-based setting: a report from the Swedish Acute Leukemia Registry. *Am J Hematol* **2015**, *90*, 208-214, doi:10.1002/ajh.23908.
14. Deschler, B.; Lübbert, M. Acute myeloid leukemia: epidemiology and etiology. *Cancer* **2006**, *107*, 2099-2107, doi:10.1002/cncr.22233.
15. Arber, D.A.; Orazi, A.; Hasserjian, R.; Thiele, J.; Borowitz, M.J.; Le Beau, M.M.; Bloomfield, C.D.; Cazzola, M.; Vardiman, J.W. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **2016**, *127*, 2391-2405, doi:10.1182/blood-2016-03-643544.
16. Short, N.J.; Rytting, M.E.; Cortes, J.E. Acute myeloid leukaemia. *The Lancet* **2018**, *392*, 593-606, doi:https://doi.org/10.1016/S0140-6736(18)31041-9.
17. Pelcovits, A.; Niroula, R. Acute Myeloid Leukemia: A Review. *R I Med J (2013)* **2020**, *103*, 38-40.
18. Papaemmanuil, E.; Gerstung, M.; Bullinger, L.; Gaidzik, V.I.; Paschka, P.; Roberts, N.D.; Potter, N.E.; Heuser, M.; Thol, F.; Bolli, N.; et al. Genomic Classification and Prognosis in

Acute Myeloid Leukemia. *N Engl J Med* **2016**, *374*, 2209-2221, doi:10.1056/NEJMoa1516192.
19. Ivey, A.; Hills, R.K.; Simpson, M.A.; Jovanovic, J.V.; Gilkes, A.; Grech, A.; Patel, Y.; Bhudia, N.; Farah, H.; Mason, J.; et al. Assessment of Minimal Residual Disease in Standard-Risk AML. *N Engl J Med* **2016**, *374*, 422-433, doi:10.1056/NEJMoa1507471.
20. Jaiswal, S.; Fontanillas, P.; Flannick, J.; Manning, A.; Grauman, P.V.; Mar, B.G.; Lindsley, R.C.; Mermel, C.H.; Burtt, N.; Chavez, A.; et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* **2014**, *371*, 2488-2498, doi:10.1056/NEJMoa1408617.
21. Lindsley, R.C.; Mar, B.G.; Mazzola, E.; Grauman, P.V.; Shareef, S.; Allen, S.L.; Pigneux, A.; Wetzler, M.; Stuart, R.K.; Erba, H.P.; et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood* **2015**, *125*, 1367-1376, doi:10.1182/blood-2014-11-610543.
22. Stone, R.M.; Mandrekar, S.J.; Sanford, B.L.; Laumann, K.; Geyer, S.; Bloomfield, C.D.; Thiede, C.; Prior, T.W.; Döhner, K.; Marcucci, G.; et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med* **2017**, *377*, 454-464, doi:10.1056/NEJMoa1614359.
23. De Bellis, E.; Imbergamo, S.; Candoni, A.; Liço, A.; Tanasi, I.; Mauro, E.; Mosna, F.; Leoncin, M.; Stulle, M.; Griguolo, D.; et al. Venetoclax in combination with hypomethylating agents in previously untreated patients with acute myeloid leukemia ineligible for intensive treatment: a real-life multicenter experience. *Leuk Res* **2022**, *114*, 106803, doi:10.1016/j.leukres.2022.106803.
24. Lancet, J.E.; Uy, G.L.; Cortes, J.E.; Newell, L.F.; Lin, T.L.; Ritchie, E.K.; Stuart, R.K.; Strickland, S.A.; Hogge, D.; Solomon, S.R.; et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. *J Clin Oncol* **2018**, *36*, 2684-2692, doi:10.1200/jco.2017.77.6112.
25. DiNardo, C.D.; Wei, A.H. How I treat acute myeloid leukemia in the era of new drugs. *Blood* **2020**, *135*, 85-96, doi:10.1182/blood.2019001239.
26. Wei, A.H.; Tiong, I.S. Midostaurin, enasidenib, CPX-351, gemtuzumab ozogamicin, and venetoclax bring new hope to AML. *Blood* **2017**, *130*, 2469-2474, doi:10.1182/blood-2017-08-784066.
27. Döhner, H.; Weisdorf, D.J.; Bloomfield, C.D. Acute Myeloid Leukemia. *N Engl J Med* **2015**, *373*, 1136-1152, doi:10.1056/NEJMra1406184.
28. Köles, L.; Kató, E.; Hanuska, A.; Zádori, Z.S.; Al-Khrasani, M.; Zelles, T.; Rubini, P.; Illes, P. Modulation of excitatory neurotransmission by neuronal/glial signalling molecules: interplay between purinergic and glutamatergic systems. *Purinergic Signal* **2016**, *12*, 1-24, doi:10.1007/s11302-015-9480-5.
29. Iovino, L.; Tremblay, M.E.; Civiero, L. Glutamate-induced excitotoxicity in Parkinson's disease: The role of glial cells. *J Pharmacol Sci* **2020**, *144*, 151-164, doi:10.1016/j.jphs.2020.07.011.
30. Danbolt, N.C. Glutamate uptake. *Prog Neurobiol* **2001**, *65*, 1-105, doi:10.1016/s0301-0082(00)00067-8.
31. Gill, S.S.; Pulido, O.M. Glutamate receptors in peripheral tissues: current knowledge, future research, and implications for toxicology. *Toxicol Pathol* **2001**, *29*, 208-223, doi:10.1080/019262301317052486.
32. Nedergaard, M.; Takano, T.; Hansen, A.J. Beyond the role of glutamate as a neurotransmitter. *Nat Rev Neurosci* **2002**, *3*, 748-755, doi:10.1038/nrn916.

33. Hinoi, E.; Takarada, T.; Ueshima, T.; Tsuchihashi, Y.; Yoneda, Y. Glutamate signaling in peripheral tissues. *Eur J Biochem* **2004**, *271*, 1-13, doi:10.1046/j.1432-1033.2003.03907.x.
34. Klotz-Weigand, L.; Enz, R. Metabotropic Glutamate Receptors at Ribbon Synapses in the Retina and Cochlea. *Cells* **2022**, *11*, doi:10.3390/cells11071097.
35. Lämmermann, T.; Kastenmüller, W. Concepts of GPCR-controlled navigation in the immune system. *Immunol Rev* **2019**, *289*, 205-231, doi:10.1111/imr.12752.
36. Basith, S.; Cui, M.; Macalino, S.J.Y.; Park, J.; Clavio, N.A.B.; Kang, S.; Choi, S. Exploring G Protein-Coupled Receptors (GPCRs) Ligand Space via Cheminformatics Approaches: Impact on Rational Drug Design. *Front Pharmacol* **2018**, *9*, 128, doi:10.3389/fphar.2018.00128.
37. Zhou, Q.; Yang, D.; Wu, M.; Guo, Y.; Guo, W.; Zhong, L.; Cai, X.; Dai, A.; Jang, W.; Shakhnovich, E.I.; et al. Common activation mechanism of class A GPCRs. *Elife* **2019**, *8*, doi:10.7554/eLife.50279.
38. Tuteja, N. Signaling through G protein coupled receptors. *Plant Signal Behav* **2009**, *4*, 942-947, doi:10.4161/psb.4.10.9530.
39. Kobilka, B.K. G protein coupled receptor structure and activation. *Biochim Biophys Acta* **2007**, *1768*, 794-807, doi:10.1016/j.bbamem.2006.10.021.
40. Cook, J.V.; Eidne, K.A. An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation. *Endocrinology* **1997**, *138*, 2800-2806, doi:10.1210/endo.138.7.5233.
41. Ritter, S.L.; Hall, R.A. Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **2009**, *10*, 819-830, doi:10.1038/nrm2803.
42. Di Menna, L.; Joffe, M.E.; Iacovelli, L.; Orlando, R.; Lindsley, C.W.; Mairesse, J.; Gressèns, P.; Cannella, M.; Caraci, F.; Copani, A.; et al. Functional partnership between mGlu3 and mGlu5 metabotropic glutamate receptors in the central nervous system. *Neuropharmacology* **2018**, *128*, 301-313, doi:10.1016/j.neuropharm.2017.10.026.
43. Niswender, C.M.; Conn, P.J. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* **2010**, *50*, 295-322, doi:10.1146/annurev.pharmtox.011008.145533.
44. Cao, J.; Huang, S.; Qian, J.; Huang, J.; Jin, L.; Su, Z.; Yang, J.; Liu, J. Evolution of the class C GPCR Venus flytrap modules involved positive selected functional divergence. *BMC Evol Biol* **2009**, *9*, 67, doi:10.1186/1471-2148-9-67.
45. Ohashi, H.; Maruyama, T.; Higashi-Matsumoto, H.; Nomoto, T.; Nishimura, S.; Takeuchi, Y. A novel binding assay for metabotropic glutamate receptors using [3H] L-quisqualic acid and recombinant receptors. *Z Naturforsch C J Biosci* **2002**, *57*, 348-355, doi:10.1515/znc-2002-3-425.
46. Hinoi, E.; Ogita, K.; Takeuchi, Y.; Ohashi, H.; Maruyama, T.; Yoneda, Y. Characterization with [3H]quisqualate of group I metabotropic glutamate receptor subtype in rat central and peripheral excitable tissues. *Neurochem Int* **2001**, *38*, 277-285, doi:10.1016/s0197-0186(00)00075-9.
47. Ganor, Y.; Levite, M. The neurotransmitter glutamate and human T cells: glutamate receptors and glutamate-induced direct and potent effects on normal human T cells, cancerous human leukemia and lymphoma T cells, and autoimmune human T cells. *J Neural Transm (Vienna)* **2014**, *121*, 983-1006, doi:10.1007/s00702-014-1167-5.
48. Teh, J.L.; Chen, S. Glutamatergic signaling in cellular transformation. *Pigment Cell Melanoma Res* **2012**, *25*, 331-342, doi:10.1111/j.1755-148X.2012.00983.x.
49. Eddy, K.; Chen, S. Glutamatergic Signaling a Therapeutic Vulnerability in Melanoma. *Cancers (Basel)* **2021**, *13*, doi:10.3390/cancers13153874.

50. Marciel, M.P.; Khadka, V.S.; Deng, Y.; Kilicaslan, P.; Pham, A.; Bertino, P.; Lee, K.; Chen, S.; Glibetic, N.; Hoffmann, F.W.; et al. Selenoprotein K deficiency inhibits melanoma by reducing calcium flux required for tumor growth and metastasis. *Oncotarget* **2018**, *9*, 13407-13422, doi:10.18632/oncotarget.24388.
51. Eddy, K.; Eddin, M.N.; Fateeva, A.; Pompili, S.V.B.; Shah, R.; Doshi, S.; Chen, S. Implications of a Neuronal Receptor Family, Metabotropic Glutamate Receptors, in Cancer Development and Progression. *Cells* **2022**, *11*, 2857.
52. Kan, Z.; Jaiswal, B.S.; Stinson, J.; Janakiraman, V.; Bhatt, D.; Stern, H.M.; Yue, P.; Haverty, P.M.; Bourgon, R.; Zheng, J.; et al. Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature* **2010**, *466*, 869-873, doi:10.1038/nature09208.
53. Esseltine, J.L.; Willard, M.D.; Wulur, I.H.; Lajiness, M.E.; Barber, T.D.; Ferguson, S.S. Somatic mutations in GRM1 in cancer alter metabotropic glutamate receptor 1 intracellular localization and signaling. *Mol Pharmacol* **2013**, *83*, 770-780, doi:10.1124/mol.112.081695.
54. Sjöblom, T.; Jones, S.; Wood, L.D.; Parsons, D.W.; Lin, J.; Barber, T.D.; Mandelker, D.; Leary, R.J.; Ptak, J.; Silliman, N.; et al. The consensus coding sequences of human breast and colorectal cancers. *Science* **2006**, *314*, 268-274, doi:10.1126/science.1133427.
55. Durinck, S.; Ho, C.; Wang, N.J.; Liao, W.; Jakkula, L.R.; Collisson, E.A.; Pons, J.; Chan, S.W.; Lam, E.T.; Chu, C.; et al. Temporal dissection of tumorigenesis in primary cancers. *Cancer Discov* **2011**, *1*, 137-143, doi:10.1158/2159-8290.Cd-11-0028.
56. Stransky, N.; Egloff, A.M.; Tward, A.D.; Kostic, A.D.; Cibulskis, K.; Sivachenko, A.; Kryukov, G.V.; Lawrence, M.S.; Sougnez, C.; McKenna, A.; et al. The mutational landscape of head and neck squamous cell carcinoma. *Science* **2011**, *333*, 1157-1160, doi:10.1126/science.1208130.
57. Parsons, D.W.; Jones, S.; Zhang, X.; Lin, J.C.; Leary, R.J.; Angenendt, P.; Mankoo, P.; Carter, H.; Siu, I.M.; Gallia, G.L.; et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* **2008**, *321*, 1807-1812, doi:10.1126/science.1164382.
58. Stepulak, A.; Luksch, H.; Gebhardt, C.; Uckermann, O.; Marzahn, J.; Sifringer, M.; Rzeski, W.; Staufner, C.; Brocke, K.S.; Turski, L.; et al. Expression of glutamate receptor subunits in human cancers. *Histochem Cell Biol* **2009**, *132*, 435-445, doi:10.1007/s00418-009-0613-1.
59. Brocke, K.S.; Staufner, C.; Luksch, H.; Geiger, K.D.; Stepulak, A.; Marzahn, J.; Schackert, G.; Temme, A.; Ikonomidou, C. Glutamate receptors in pediatric tumors of the central nervous system. *Cancer Biol Ther* **2010**, *9*, 455-468, doi:10.4161/cbt.9.6.10898.
60. Zhang, C.; Yuan, X.R.; Li, H.Y.; Zhao, Z.J.; Liao, Y.W.; Wang, X.Y.; Su, J.; Sang, S.S.; Liu, Q. Anti-cancer effect of metabotropic glutamate receptor 1 inhibition in human glioma U87 cells: involvement of PI3K/Akt/mTOR pathway. *Cell Physiol Biochem* **2015**, *35*, 419-432, doi:10.1159/000369707.
61. Martino, J.J.; Wall, B.A.; Mastrantoni, E.; Wilimczyk, B.J.; La Cava, S.N.; Degenhardt, K.; White, E.; Chen, S. Metabotropic glutamate receptor 1 (Grm1) is an oncogene in epithelial cells. *Oncogene* **2013**, *32*, 4366-4376, doi:10.1038/onc.2012.471.
62. Kalariti, N.; Lembessis, P.; Papageorgiou, E.; Pissimissis, N.; Koutsilieris, M. Regulation of the mGluR5, EAAT1 and GS expression by glucocorticoids in MG-63 osteoblast-like osteosarcoma cells. *J Musculoskelet Neuronal Interact* **2007**, *7*, 113-118.
63. D'Onofrio, M.; Arcella, A.; Bruno, V.; Ngomba, R.T.; Battaglia, G.; Lombari, V.; Ragona, G.; Calogero, A.; Nicoletti, F. Pharmacological blockade of mGlu2/3 metabotropic glutamate receptors reduces cell proliferation in cultured human glioma cells. *J Neurochem* **2003**, *84*, 1288-1295, doi:10.1046/j.1471-4159.2003.01633.x.

64. Prickett, T.D.; Samuels, Y. Molecular pathways: dysregulated glutamatergic signaling pathways in cancer. *Clin Cancer Res* **2012**, *18*, 4240-4246, doi:10.1158/1078-0432.Ccr-11-1217.
65. Arcella, A.; Carpinelli, G.; Battaglia, G.; D'Onofrio, M.; Santoro, F.; Ngomba, R.T.; Bruno, V.; Casolini, P.; Giangaspero, F.; Nicoletti, F. Pharmacological blockade of group II metabotropic glutamate receptors reduces the growth of glioma cells in vivo. *Neuro Oncol* **2005**, *7*, 236-245, doi:10.1215/s1152851704000961.
66. Ferrigno, A.; Berardo, C.; Di Pasqua, L.G.; Siciliano, V.; Richelmi, P.; Vairetti, M. Localization and role of metabotropic glutamate receptors subtype 5 in the gastrointestinal tract. *World J Gastroenterol* **2017**, *23*, 4500-4507, doi:10.3748/wjg.v23.i25.4500.
67. Cancer Genome Atlas, N. Genomic Classification of Cutaneous Melanoma. *Cell* **2015**, *161*, 1681-1696, doi:10.1016/j.cell.2015.05.044.
68. Frati, C.; Marchese, C.; Fisichella, G.; Copani, A.; Nasca, M.R.; Storto, M.; Nicoletti, F. Expression of functional mGlu5 metabotropic glutamate receptors in human melanocytes. *J Cell Physiol* **2000**, *183*, 364-372, doi:10.1002/(sici)1097-4652(200006)183:3<364::Aid-jcp9>3.0.Co;2-x.
69. Koochekpour, S.; Majumdar, S.; Azabdaftari, G.; Attwood, K.; Scioneaux, R.; Subramani, D.; Manhardt, C.; Lorusso, G.D.; Willard, S.S.; Thompson, H.; et al. Serum glutamate levels correlate with Gleason score and glutamate blockade decreases proliferation, migration, and invasion and induces apoptosis in prostate cancer cells. *Clin Cancer Res* **2012**, *18*, 5888-5901, doi:10.1158/1078-0432.Ccr-12-1308.
70. Park, S.Y.; Lee, S.A.; Han, I.H.; Yoo, B.C.; Lee, S.H.; Park, J.Y.; Cha, I.H.; Kim, J.; Choi, S.W. Clinical significance of metabotropic glutamate receptor 5 expression in oral squamous cell carcinoma. *Oncol Rep* **2007**, *17*, 81-87.
71. Pissimissis, N.; Papageorgiou, E.; Lembessis, P.; Armakolas, A.; Koutsilieris, M. The glutamatergic system expression in human PC-3 and LNCaP prostate cancer cells. *Anticancer Res* **2009**, *29*, 371-377.
72. Chang, H.J.; Yoo, B.C.; Lim, S.B.; Jeong, S.Y.; Kim, W.H.; Park, J.G. Metabotropic glutamate receptor 4 expression in colorectal carcinoma and its prognostic significance. *Clin Cancer Res* **2005**, *11*, 3288-3295, doi:10.1158/1078-0432.Ccr-04-1912.
73. Teh, J.L.; Shah, R.; La Cava, S.; Dolfi, S.C.; Mehta, M.S.; Kongara, S.; Price, S.; Ganesan, S.; Reuhl, K.R.; Hirshfield, K.M.; et al. Metabotropic glutamate receptor 1 disrupts mammary acinar architecture and initiates malignant transformation of mammary epithelial cells. *Breast Cancer Res Treat* **2015**, *151*, 57-73, doi:10.1007/s10549-015-3365-8.
74. Speyer, C.L.; Hachem, A.H.; Assi, A.A.; Johnson, J.S.; DeVries, J.A.; Gorski, D.H. Metabotropic glutamate receptor-1 as a novel target for the antiangiogenic treatment of breast cancer. *PLoS One* **2014**, *9*, e88830, doi:10.1371/journal.pone.0088830.
75. Sexton, R.E.; Hachem, A.H.; Assi, A.A.; Bukhsh, M.A.; Gorski, D.H.; Speyer, C.L. Metabotropic glutamate receptor-1 regulates inflammation in triple negative breast cancer. *Sci Rep* **2018**, *8*, 16008, doi:10.1038/s41598-018-34502-8.
76. Zhang, C.; Xie, S.; Yuan, S.; Zhang, Y.; Bai, Y.; Chu, L.; Wu, Z.; Guo, N.; Wang, Q.; Zhang, J. Metabotropic Glutamate Receptor 8 Is Regulated by miR-33a-5p and Functions as an Oncogene in Breast Cancer. *J Oncol* **2021**, *2021*, 8002087, doi:10.1155/2021/8002087.
77. Xiao, B.; Chen, D.; Zhou, Q.; Hang, J.; Zhang, W.; Kuang, Z.; Sun, Z.; Li, L. Glutamate metabotropic receptor 4 (GRM4) inhibits cell proliferation, migration and invasion in breast cancer and is regulated by miR-328-3p and miR-370-3p. *BMC Cancer* **2019**, *19*, 891, doi:10.1186/s12885-019-6068-4.

78. Lunyak, V.V.; Burgess, R.; Prefontaine, G.G.; Nelson, C.; Sze, S.H.; Chenoweth, J.; Schwartz, P.; Pevzner, P.A.; Glass, C.; Mandel, G.; et al. Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* **2002**, *298*, 1747-1752, doi:10.1126/science.1076469.
79. Andrés, M.E.; Burger, C.; Peral-Rubio, M.J.; Battaglioli, E.; Anderson, M.E.; Grimes, J.; Dallman, J.; Ballas, N.; Mandel, G. CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc Natl Acad Sci U S A* **1999**, *96*, 9873-9878, doi:10.1073/pnas.96.17.9873.
80. Mahamdallie, S.S.; Hanks, S.; Karlin, K.L.; Zachariou, A.; Perdeaux, E.R.; Ruark, E.; Shaw, C.A.; Renwick, A.; Ramsay, E.; Yost, S.; et al. Mutations in the transcriptional repressor REST predispose to Wilms tumor. *Nat Genet* **2015**, *47*, 1471-1474, doi:10.1038/ng.3440.
81. Lee, N.S.; Evgrafov, O.V.; Souaiaia, T.; Bonyad, A.; Herstein, J.; Lee, J.Y.; Kim, J.; Ning, Y.; Sixto, M.; Weitz, A.C.; et al. Non-coding RNAs derived from an alternatively spliced REST transcript (REST-003) regulate breast cancer invasiveness. *Sci Rep* **2015**, *5*, 11207, doi:10.1038/srep11207.
82. Chong, J.A.; Tapia-Ramírez, J.; Kim, S.; Toledo-Aral, J.J.; Zheng, Y.; Boutros, M.C.; Altshuller, Y.M.; Frohman, M.A.; Kraner, S.D.; Mandel, G. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* **1995**, *80*, 949-957, doi:10.1016/0092-8674(95)90298-8.
83. Cavadas, M.A.; Mesnieres, M.; Crifo, B.; Manresa, M.C.; Selfridge, A.C.; Keogh, C.E.; Fabian, Z.; Scholz, C.C.; Nolan, K.A.; Rocha, L.M.; et al. REST is a hypoxia-responsive transcriptional repressor. *Sci Rep* **2016**, *6*, 31355, doi:10.1038/srep31355.
84. Yao, H.; Goldman, D.C.; Fan, G.; Mandel, G.; Fleming, W.H. The Corepressor Rcor1 Is Essential for Normal Myeloerythroid Lineage Differentiation. *Stem Cells* **2015**, *33*, 3304-3314, doi:10.1002/stem.2086.
85. Magin, A.; Lietz, M.; Cibelli, G.; Thiel, G. RE-1 silencing transcription factor-4 (REST4) is neither a transcriptional repressor nor a de-repressor. *Neurochem Int* **2002**, *40*, 195-202, doi:10.1016/s0197-0186(01)00091-2.
86. Khan, A.J.; LaCava, S.; Mehta, M.; Schiff, D.; Thandoni, A.; Jhawar, S.; Danish, S.; Haffty, B.G.; Chen, S. The glutamate release inhibitor riluzole increases DNA damage and enhances cytotoxicity in human glioma cells, in vitro and in vivo. *Oncotarget* **2019**, *10*, 2824-2834, doi:10.18632/oncotarget.26854.
87. Yoo, B.C.; Jeon, E.; Hong, S.H.; Shin, Y.K.; Chang, H.J.; Park, J.G. Metabotropic glutamate receptor 4-mediated 5-Fluorouracil resistance in a human colon cancer cell line. *Clin Cancer Res* **2004**, *10*, 4176-4184, doi:10.1158/1078-0432.Ccr-1114-03.
88. Liao, S.; Ruiz, Y.; Gulzar, H.; Yelskaya, Z.; Ait Taouit, L.; Houssou, M.; Jaikaran, T.; Schvarts, Y.; Kozlitina, K.; Basu-Roy, U.; et al. Osteosarcoma cell proliferation and survival requires mGluR5 receptor activity and is blocked by Riluzole. *PLoS One* **2017**, *12*, e0171256, doi:10.1371/journal.pone.0171256.
89. Levite, M. Glutamate, T cells and multiple sclerosis. *J Neural Transm (Vienna)* **2017**, *124*, 775-798, doi:10.1007/s00702-016-1661-z.
90. Long, Y.; Tao, H.; Karachi, A.; Grippin, A.J.; Jin, L.; Chang, Y.E.; Zhang, W.; Dyson, K.A.; Hou, A.Y.; Na, M.; et al. Dysregulation of Glutamate Transport Enhances Treg Function That Promotes VEGF Blockade Resistance in Glioblastoma. *Cancer Res* **2020**, *80*, 499-509, doi:10.1158/0008-5472.Can-19-1577.
91. Pacheco, R.; Ciruela, F.; Casadó, V.; Mallol, J.; Gallart, T.; Lluis, C.; Franco, R. Group I metabotropic glutamate receptors mediate a dual role of glutamate in T cell activation. *J Biol Chem* **2004**, *279*, 33352-33358, doi:10.1074/jbc.M401761200.

92. Chiocchetti, A.; Miglio, G.; Mesturini, R.; Varsaldi, F.; Mocellin, M.; Orilieri, E.; Dianzani, C.; Fantozzi, R.; Dianzani, U.; Lombardi, G. Group I mGlu receptor stimulation inhibits activation-induced cell death of human T lymphocytes. *Br J Pharmacol* **2006**, *148*, 760-768, doi:10.1038/sj.bjp.0706746.
93. Hansen, A.M.; Caspi, R.R. Glutamate joins the ranks of immunomodulators. *Nat Med* **2010**, *16*, 856-858, doi:10.1038/nm0810-856.
94. Ganor, Y.; Levite, M. Glutamate in the Immune System: Glutamate Receptors in Immune Cells, Potent Effects, Endogenous Production and Involvement in Disease. In *Nerve-Driven Immunity: Neurotransmitters and Neuropeptides in the Immune System*, Levite, M., Ed.; Springer Vienna: Vienna, 2012; pp. 121-161.
95. Fallarino, F.; Volpi, C.; Fazio, F.; Notartomaso, S.; Vacca, C.; Busceti, C.; Bicciato, S.; Battaglia, G.; Bruno, V.; Puccetti, P.; et al. Metabotropic glutamate receptor-4 modulates adaptive immunity and restrains neuroinflammation. *Nat Med* **2010**, *16*, 897-902, doi:10.1038/nm.2183.
96. Siegel, R.L.; Miller, K.D.; Fuchs, H.E.; Jemal, A. Cancer statistics, 2022. *CA Cancer J Clin* **2022**, *72*, 7-33, doi:10.3322/caac.21708.
97. Rebecca, V.W.; Sondak, V.K.; Smalley, K.S. A brief history of melanoma: from mummies to mutations. *Melanoma Res* **2012**, *22*, 114-122, doi:10.1097/CMR.0b013e328351fa4d.
98. Lee, C.; Collichio, F.; Ollila, D.; Moschos, S. Historical review of melanoma treatment and outcomes. *Clin Dermatol* **2013**, *31*, 141-147, doi:10.1016/j.clindermatol.2012.08.015.
99. Straume, O.; Sviland, L.; Akslen, L.A. Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma. *Clin Cancer Res* **2000**, *6*, 1845-1853.
100. Pasquali, S.; Spillane, A.J.; de Wilt, J.H.; McCaffery, K.; Rossi, C.R.; Quinn, M.J.; Saw, R.P.; Shannon, K.F.; Stretch, J.R.; Thompson, J.F. Surgeons' opinions on lymphadenectomy in melanoma patients with positive sentinel nodes: a worldwide web-based survey. *Ann Surg Oncol* **2012**, *19*, 4322-4329, doi:10.1245/s10434-012-2483-3.
101. Viros, A.; Fridlyand, J.; Bauer, J.; Lasithiotakis, K.; Garbe, C.; Pinkel, D.; Bastian, B.C. Improving melanoma classification by integrating genetic and morphologic features. *PLoS Med* **2008**, *5*, e120, doi:10.1371/journal.pmed.0050120.
102. Sample, A.; He, Y.Y. Mechanisms and prevention of UV-induced melanoma. *Photodermatol Photoimmunol Photomed* **2018**, *34*, 13-24, doi:10.1111/phpp.12329.
103. Watson, M.; Holman, D.M.; Maguire-Eisen, M. Ultraviolet Radiation Exposure and Its Impact on Skin Cancer Risk. *Semin Oncol Nurs* **2016**, *32*, 241-254, doi:10.1016/j.soncn.2016.05.005.
104. Hodis, E.; Watson, I.R.; Kryukov, G.V.; Arold, S.T.; Imielinski, M.; Theurillat, J.P.; Nickerson, E.; Auclair, D.; Li, L.; Place, C.; et al. A landscape of driver mutations in melanoma. *Cell* **2012**, *150*, 251-263, doi:10.1016/j.cell.2012.06.024.
105. Huang, F.W.; Hodis, E.; Xu, M.J.; Kryukov, G.V.; Chin, L.; Garraway, L.A. Highly recurrent TERT promoter mutations in human melanoma. *Science* **2013**, *339*, 957-959, doi:10.1126/science.1229259.
106. Lee, H.J.; Wall, B.A.; Wangari-Talbot, J.; Chen, S. Regulation of mGluR1 expression in human melanocytes and melanoma cells. *Biochim Biophys Acta* **2012**, *1819*, 1123-1131, doi:10.1016/j.bbagrm.2012.06.005.
107. Crepaldi, L.; Lackner, C.; Corti, C.; Ferraguti, F. Transcriptional activators and repressors for the neuron-specific expression of a metabotropic glutamate receptor. *J Biol Chem* **2007**, *282*, 17877-17889, doi:10.1074/jbc.M700149200.

108. Nikolaou, V.A.; Stratigos, A.J.; Flaherty, K.T.; Tsao, H. Melanoma: new insights and new therapies. *J Invest Dermatol* **2012**, *132*, 854-863, doi:10.1038/jid.2011.421.
109. Sullivan, R.J.; Flaherty, K.T. Resistance to BRAF-targeted therapy in melanoma. *Eur J Cancer* **2013**, *49*, 1297-1304, doi:10.1016/j.ejca.2012.11.019.
110. Sun, J.; Carr, M.J.; Khushalani, N.I. Principles of Targeted Therapy for Melanoma. *Surg Clin North Am* **2020**, *100*, 175-188, doi:10.1016/j.suc.2019.09.013.
111. Davies, H.; Bignell, G.R.; Cox, C.; Stephens, P.; Edkins, S.; Clegg, S.; Teague, J.; Woffendin, H.; Garnett, M.J.; Bottomley, W.; et al. Mutations of the BRAF gene in human cancer. *Nature* **2002**, *417*, 949-954, doi:10.1038/nature00766.
112. Tsao, H.; Chin, L.; Garraway, L.A.; Fisher, D.E. Melanoma: from mutations to medicine. *Genes Dev* **2012**, *26*, 1131-1155, doi:10.1101/gad.191999.112.
113. Robert, C.; Schachter, J.; Long, G.V.; Arance, A.; Grob, J.J.; Mortier, L.; Daud, A.; Carlino, M.S.; McNeil, C.; Lotem, M.; et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med* **2015**, *372*, 2521-2532, doi:10.1056/NEJMoa1503093.
114. Robert, C.; Long, G.V.; Brady, B.; Dutriaux, C.; Maio, M.; Mortier, L.; Hassel, J.C.; Rutkowski, P.; McNeil, C.; Kalinka-Warzocha, E.; et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* **2015**, *372*, 320-330, doi:10.1056/NEJMoa1412082.
115. Olson, D.J.; Eroglu, Z.; Brockstein, B.; Poklepovic, A.S.; Bajaj, M.; Babu, S.; Hallmeyer, S.; Velasco, M.; Lutzky, J.; Higgs, E.; et al. Pembrolizumab Plus Ipilimumab Following Anti-PD-1/L1 Failure in Melanoma. *J Clin Oncol* **2021**, *39*, 2647-2655, doi:10.1200/jco.21.00079.
116. Ascierto, P.A.; McArthur, G.A.; Dréno, B.; Atkinson, V.; Liszkay, G.; Di Giacomo, A.M.; Mandalà, M.; Demidov, L.; Stroyakovskiy, D.; Thomas, L.; et al. Cobimetinib combined with vemurafenib in advanced BRAF(V600)-mutant melanoma (coBRIM): updated efficacy results from a randomised, double-blind, phase 3 trial. *Lancet Oncol* **2016**, *17*, 1248-1260, doi:10.1016/s1470-2045(16)30122-x.
117. Long, G.V.; Flaherty, K.T.; Stroyakovskiy, D.; Gogas, H.; Levchenko, E.; de Braud, F.; Larkin, J.; Garbe, C.; Jouary, T.; Hauschild, A.; et al. Dabrafenib plus trametinib versus dabrafenib monotherapy in patients with metastatic BRAF V600E/K-mutant melanoma: long-term survival and safety analysis of a phase 3 study. *Ann Oncol* **2017**, *28*, 1631-1639, doi:10.1093/annonc/mdx176.
118. Robert, C.; Karaszewska, B.; Schachter, J.; Rutkowski, P.; Mackiewicz, A.; Stroiakovski, D.; Lichinitser, M.; Dummer, R.; Grange, F.; Mortier, L.; et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* **2015**, *372*, 30-39, doi:10.1056/NEJMoa1412690.
119. Samatov, T.R.; Wicklein, D.; Tonevitsky, A.G. L1CAM: Cell adhesion and more. *Prog Histochem Cytochem* **2016**, *51*, 25-32, doi:10.1016/j.proghi.2016.05.001.
120. Haspel, J.; Grumet, M. The L1CAM extracellular region: a multi-domain protein with modular and cooperative binding modes. *Front Biosci* **2003**, *8*, s1210-1225, doi:10.2741/1108.
121. Heiner Schäfer, S.S.M. L1CAM (L1 cell adhesion molecule). **2008**.
122. Silletti, S.; Yebra, M.; Perez, B.; Cirulli, V.; McMahon, M.; Montgomery, A.M. Extracellular signal-regulated kinase (ERK)-dependent gene expression contributes to L1 cell adhesion molecule-dependent motility and invasion. *J Biol Chem* **2004**, *279*, 28880-28888, doi:10.1074/jbc.M404075200.
123. Yang, M.; Li, Y.; Chilukuri, K.; Brady, O.A.; Boulos, M.I.; Kappes, J.C.; Galileo, D.S. L1 stimulation of human glioma cell motility correlates with FAK activation. *J Neurooncol* **2011**, *105*, 27-44, doi:10.1007/s11060-011-0557-x.
124. Ye, D.Z.; Field, J. PAK signaling in cancer. *Cell Logist* **2012**, *2*, 105-116, doi:10.4161/cl.21882.

125. Meier, F.; Busch, S.; Gast, D.; Göppert, A.; Altevogt, P.; Maczey, E.; Riedle, S.; Garbe, C.; Schittek, B. The adhesion molecule L1 (CD171) promotes melanoma progression. *Int J Cancer* **2006**, *119*, 549-555, doi:10.1002/ijc.21880.
126. Nagaraj, V.; Mikhail, M.; Baronio, M.; Gatto, A.; Nayak, A.; Theis, T.; Cavallaro, U.; Schachner, M. Antagonistic L1 Adhesion Molecule Mimetic Compounds Inhibit Glioblastoma Cell Migration In Vitro. *Biomolecules* **2022**, *12*, doi:10.3390/biom12030439.
127. Sebastianutto, I.; Goyet, E.; Andreoli, L.; Font-Ingles, J.; Moreno-Delgado, D.; Bouquier, N.; Jahannault-Talignani, C.; Moutin, E.; Di Menna, L.; Maslava, N.; et al. D1-mGlu5 heteromers mediate noncanonical dopamine signaling in Parkinson's disease. *J Clin Invest* **2020**, *130*, 1168-1184, doi:10.1172/jci126361.
128. Abreu, N.; Acosta-Ruiz, A.; Xiang, G.; Levitz, J. Mechanisms of differential desensitization of metabotropic glutamate receptors. *Cell Rep* **2021**, *35*, 109050, doi:10.1016/j.celrep.2021.109050.