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Ion Channels: Novel Functional Hubs in Leukemia

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1. Introduction

Leukemia (from the Greek "leukos" (white) and "haima" (blood) is a type of cancer of the blood or bone marrow, characterized by an abnormal increase of white blood cells. Leukemia is a broad term, covering a spectrum of hematologic diseases, which can be distinguished by peculiar clinical and pathological characteristics. The first distinction is between acute and chronic forms. Moreover, according to which kind of blood cell is affected, leukemias are subdivided into lymphoblastic (a terminology limited to address the acute forms), lymphocytic and myeloid leukemias. Combining these two classifications provides four main categories: Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML). These broad categories are further subdivided into several subcategories, classically identified through morphological criteria (Greer et al., 2008).

During the last thirty years, the widespread introduction of molecular biological concepts and methods in oncology has provided the important notion that cancer cells are mutants. These cells sometimes carry somatic mutations. In other cases they bear either amplified or inactivated tumor-related genes. These latter effects can be also caused by epigenetic mechanisms, instead of mutation events *per se*. However, the picture is considerably complicated by the observation that aberrant extracellular signals can promote oncogenesis even in a benign cell physiological context. In other words, the most malignant neoplastic phenotypes do not arise in a strictly cell autonomous manner, and their manifestation cannot be understood solely based on tumor cell genomes. The ability of malignant cells to proceed along the invasion-metastasis pathway may be acquired, at least in certain cases, through their interaction with the tumor microenvironment, without the requirement to undergo additional mutations beyond those that were needed for primary tumor formation (Hanahan & Weinberg, 2011).

This scenario also applies to hematologic malignancies, where analysing the genes comprising the expression signature has provided important insights into the biology of leukemias. This has led to reshape the classification criteria for subcategories identification and risk stratification, as well as develop novel drugs addressing leukemia-specific processes. Further insights came from the discovery that the strict relationship between leukemia cells and the bone marrow microenvironment strongly determines malignancy and response to therapy. This relies on the fact that bone marrow cells emit survival signals

that strongly determine the development of the leukemic disease. CML has been for long, and is still, the "poster child" of translational medicine. The discovery of the Philadelphia chromosome and the subsequent finding of the BCR-ABL chimeric gene led to a unique understanding of the biology of the disease that spurred the development of targeted therapy, as well as methods for the molecular monitoring of the disease. These achievements have shaped a therapeutic framework that is the envy of oncology (American Society of Hematology [ASH] Education Program Book, 2010). CLL, despite being the most common leukemia with a clinical description dating back to the mid-nineteenth century, still remains a rather enigmatic disease. Nonetheless, considerable progress in understanding the biology of CLL occurred in the last few years. In particular, important advances have been made in identifying inherited and acquired genetic mutations, the role of B-cell receptor signaling and the tumor microenvironment. CLL resulted to be a disease dependent on the interplay of inherited, environmental and host factors (ASH, 2010). The picture is even more complex in acute forms, where different types of gene alterations, mainly translocations, have been observed that often lack a clear biological and clinical interpretation (ASH, 2010). ALL is the most common childhood leukemia form, and its therapy has improved substantially with the use of risk-directed treatment and improved supportive care. Current ALL trials have focused on improving the outcome of a few subtypes that remain refractory to treatment, such as infant ALL with specific gene translocations, MLL rearrangements, hypodiploid ALL, or poor early responders (ASH, 2010). By contrast, most of the AML cases continue to pose a therapeutic challenge. AML forms show marked differences in survival following intense chemotherapy based on age, blast cell morphology and cytogenetic abnormalities. However, although therapeutic advances have lagged over the past two decades, recent work has provided an array of new prognostic factors in AML, which is driving our understanding of the disease biology and the development of new therapeutic targets (ASH, 2010).

Here, we review the growing experimental and preclinical evidence that indicates that ion channels should be included among the genes whose expression is altered in leukemias. Channel dysfunction can have a strong impact on hematopoietic cell physiology and signaling, with ensuing effects on the onset and progression of the leukemia disease. These effects depend on the widespread roles of ion channels in modulating cellular functions that contribute to determine the clinical features and the therapeutic responses of hematologic diseases, such as proliferation, differentiation and apoptosis. In addition, many ion channels are at the same time effective sensors of extracellular signals and transducers of these signals into cellular regulatory cascades. From a pharmacologic standpoint, because ion channels are membrane proteins, they can be easily accessed by extracellular ligands or peptides (toxins), which offers clear advantages for treatment. For these reasons, we believe that ion channels represent promising targets for cancer therapy, which may open a novel pharmaceutical and clinical field.

2. Overview of the relevant ion channel and aquaporin types

Ion channels are integral membrane proteins that provide an aqueous pathway for ions to cross the energetically unfavourable barrier constituted by the plasma membrane. Ion channels are generally either permeable to cations or anions. Moreover, they can be more or less specific for different ions. The main stimuli for channel opening (activation) are either change in membrane potential (voltage-gated channels) or ligand binding (ligand-gated

channels). In the following paragraphs, we summarize the main structural and physiological features of the channel types that will be mentioned subsequently.

2.1 Voltage-gated cation channels (VGC)

VGCs belong to a large molecular family that comprises K_V (Voltage-gated K^+ channels), Na_V (Voltage-gated Na^+ channels) and Ca_V (voltage-gated Ca^{2+} channels). The K^+ channel types are named K_V1 to K_V12, with subtypes named $K_V1.1$, etc. (Gutman et al., 2005). They are tetrameric channels, with each subunit containing six transmembrane domains (S1 to S6). S4 is rich of amino acid residues with alkaline side chains and is thought to be the voltage sensor (Böriesson & Elinder, 2008). S5 and S6 are linked by the so-called pore (P) loop, which gives a fundamental contribution to ion selectivity and contains the K^+ channel 'signature' GYG (Alam & Jiang, 2011). Both the N- and C-termini are intracellular. The cytoplasmic domains contain consensus sequences for phosphorylation and the N-terminus determines interaction with regulatory proteins and other subunits. Besides the classical role in mediating the repolarizing phase of action potentials, many other functions have been found to be exerted by K_V channels, which are a very diversified molecular family. These functions cannot be reviewed here, but a few relevant examples will be described later. The structure of Na_V and Ca_V is similar to that of K_V, except that the four independent subunits observed in K_V are instead four homologous repeated domains of the same polypeptide. The main physiological role of Na_V channels is shaping the rising phase of action potentials, whereas Ca_V channels control Ca^{2+} entry during action potentials, exocytosis, muscle contraction and the many other physiological processes that are modulated by $[Ca^{2+}]$. In all voltage-gated channels, auxiliary subunits regulate the channel properties and control both membrane targeting and interaction with other proteins (Catterall, 1992, 2000).

2.2 Inward rectifier K^+ channels (K_{IR})

These channels preferentially carry inward K^+ currents, because the outward currents are blocked by intracellular cations obstructing the pore, particularly cytosolic polyamines and Mg^{2+}. Block is increasingly more effective as V_m depolarizes. K_{IR} channels are tetrameric proteins structurally related to the VGC family. However, each subunit only contains two transmembrane domains (M1 and M2). These are respectively homologous to S5 and S6 and are connected by a P-loop (Nichols & Lopatin, 1997). K_{IR} channels contribute to regulate the resting V_m in a range not too far from the K^+ equilibrium potential, thus controlling for example excitability in resting conditions, the cardiac action potential repolarization and the extracellular K^+ buffering exerted by glial cells.

2.3 Ca^{2+}-activated K^+ channels (K_{Ca})

These tetrameric K^+ channels are formed by subunits collectively named K_{Ca} and structurally related to K_V, with the typical S1-S6 module (Wei et al., 2005). In $K_{Ca}1.1$, a transmembrane segment named S0 precedes the S1-S6 module. $K_{Ca}1.1$ are also named BK ('Big') because of a particularly high single-channel conductance. BK are activated by both depolarization and $[Ca^{2+}]$. $K_{Ca}1.1$ typically determines the Ca^{2+}-dependent after hyperpolarization observed in certain neurons. $K_{Ca}4$ and $K_{Ca}5$ present similar overall features, but their precise physiological roles are still matter of debate. $K_{Ca}4$ channels are not voltage-dependent, because of S4 neutralization and are activated by Na^+ instead of Ca^{2+}. $K_{Ca}2$ (or SK, after Small conductance K^+ channels) and $K_{Ca}3$ (or IK, after Intermediate

conductance K^+ channels) are also not voltage-dependent, because of partial neutralization of S4. They are activated by [Ca^{2+}] around 0.5 μM, through binding to the calmodulin proteins tightly associated with each subunit. These channels can regulate cell firing, the vascular tone and, as discussed below, are also implicated in cell proliferation and neoplasia.

2.4 Transient Receptor Potential (TRP) channels

TRP channels are homo- or hetero-tetramers of subunits structurally related to the VGCs, with the typical S1-S6, a P loop and cytoplasmic N- and C-termini. They are generally permeable to cations; the permeability to Ca^{2+} is extremely variable between subtypes. TRP are important sensors of the cell's environment and can respond to many chemical as well as physical stimuli (including temperature). In mammals, six main subfamilies are known: TRPA, TRPC, TRPM, TRPV, TRPP and TRPML (Venkatachalam & Montell, 2007). TRP channels are widely distributed in mammalian tissues, and are implicated in a wide spectrum of functions (Nilius et al., 2007). Besides the classic roles as sensory transducers, increasing evidence implicates TRP channels in developmental functions. Ca^{2+} influx through these channels can in fact regulate axon guidance and neuronal survival (Talavera et al., 2008).

2.5 Two-pore domain K^+ channels (K_{2P})

K_{2P} channels were divided into six subfamilies: TWIK (tandem of pore domains in a weak inward rectifying K^+ channel), TREK (TWIK-related K^+ Channel), TASK (TWIK-related Acid-Sensitive K^+ Channel), TALK (TWIK-related alkaline pH-activated K^+ channel), THIK (tandem pore domain halothane-inhibited K^+ channel) and TRESK (TWIK-related spinal cord K^+ channel). The systematic nomenclature is KCNK followed by a specific number for each subtype. Each subunit is formed by intracellular N- and C-termini that comprise four transmembrane domains (TMS1-TMS4). Each subunit contains two P loops (which explains the name K_{2P}), one between TMS1 and TMS2 and the other between TMS3 and TMS4. The functional channel is a dimer of two-pore domain containing subunits. K_{2P} are believed to behave as background K^+ channels, i.e. channels mostly open in resting conditions, and thus give a substantial contribution to V_{rest}. Accordingly, they present weak voltage-dependence and rectification. More specific physiological roles, including those in neoplastic cells, are still debated (Enyedi & Cziriák, 2010).

2.6 Ionotropic purinergic receptors

Purinergic receptors can be metabotropic or ionotropic receptors activated by adenosine (P_1) or ATP (P_2). In particular, P_2 receptors comprise the ionotropic P_{2X} receptors. Seven subunits have been identified (P_{2X1} to P_{2X7}) that can form homo- or heterotrimeric channels typically activated by extracellular ATP. P_{2X} receptors have intracellular N- and C-terminus and two transmembrane domains connected by a long extracellular loop involved in subunit association. The extracellular channel portion contains the ATP binding crevice plus modulatory sites. The C-terminus has very variable length and probably controls both the channel's desensitization kinetics and receptor trafficking. The open channel is permeable to cations, including Ca^{2+}. P_{2X} receptors exert physiological functions in many tissues, including the adult and developing nervous system, the respiratory, gastrointestinal, cardiovascular and genitourinary systems (Köles et al., 2007).

2.7 Voltage-gated Cl^- channels

These are ion channels permeable to anions and gated by membrane depolarization. Nine subunits (CLC-1 to CLC-7, plus ClC-Ka and ClC-Kb) are expressed in mammals. CLC-1, CLC-2, CLC-Ka, and CLC-Kb are certainly Cl^- channels, activated by membrane depolarization and permeable to different anions. The native channel comprises two identical subunits and contains two independent pores. Each subunit contains 18 α helical segments (A to R), with intracellular N- and C-termini. The segments A-I are homologous to J-R, but the two half-subunits have opposite orientation in the membrane. CLC channels are expressed in a variety of cells, where they regulate membrane excitability, cell volume, pH and transepithelial Cl^- flux. They are also expressed in the organelles' membranes. CLC-3, CLC-4 and CLC-5 are expressed on the membrane of intracellular vesicles and are thought to function as Cl^--H^+ antiporters. However, evidence about CLC-3 is controversial. The physiology of ClC-6 and ClC-7 is also unclear (Zifarelli & Pusch, 2007).

2.8 Interplay between channels expressed in the plasma membrane and intracellular compartments: The role of CRAC

Ion channels are also widely distributed in intracellular organelles and vesicles, where they control transmembrane fluxes implicated in neurotransmitter loading into synaptic vesicles, cytoplasmic Ca^{2+} homeostasis (and the related physiological processes), mitochondrial and nuclear function. Ion transport across the intracellular membranes is tightly coupled to the fluxes between the cytosol and the extracellular space. The CRAC channels (Ca^{2+} release-activated Ca^{2+} channels) have a pivotal role in such interplay in that they mediate calcium influx from the extracellular compartment depending on the state of intracellular Ca^{2+} stores. The full physiological response of CRAC channels depends on interaction between the plasma membrane protein ORAI1 (or CRACM1), which forms at least part of the Ca^{2+} pore, and STIM1 (stromal interaction molecule 1), which is expressed in the endoplasmic reticulum membranes. When Ca^{2+} decreases in the intracellular stores, STIM1 and ORAI1 form a complex that stimulates Ca^{2+} influx from the extracellular space. This process is implicated in the cellular processes that are regulated by Ca^{2+} and the related pathologies. For review see Parekh (Parekh et al., 2010).

2.9 Aquaporins

Aquaporins (or water channels) are integral membrane proteins that form a specific transmembrane pathway for water. Several aquaporins subtypes are known in mammals, formed by subunits named AQP0-AQP12, with different tissue distribution. They form tetrameric channels, with each subunit containing 6 transmembrane domains and one pore. Both the N- and C-termini are intracellular. Aquaporins control osmotic fluxes in a variety of physiological conditions, from cell volume alteration to transepithelial flux. Some subtypes form selective water channels, with scarce selectivity for ions and other small solutes. However, recent evidence shows that other AQP subtypes are also permeable to small solutes different from water, such as glycerol, urea, CO_2, NO, NH_3 and others. This considerably extends the range of possible functions of these membrane channels (King et al., 2004).

3. Expression and function of ion channels in leukemia cells

Work carried out in the early eighties led to the discovery of ion channels in lymphocytes and suggested specific channel roles in lymphocyte activation and function (Fukushima &

Hagiwara, 1983; DeCoursey et al., 1984; Matteson & Deutsch, 1984; Fukushima et al., 1984; Chandy et al., 1984). In particular, work done in M. Cahalan's research group indicated that K^+ channels could regulate mitogenesis, in T cells. Subsequent work from this and other groups clarified the differential expression of K^+ channels in T lymphocyte populations and how they control T cell activation (Cahalan et al., 1985; Beeton & Chandy, 2005; Krasznai, 2005). These cells turned out to express delayed rectifying K^+ channels ($K_v1.3$) and intermediate conductance Ca^{2+}-dependent K^+ channels ($K_{Ca}3.1$) (Douglas et al., 1990; Logdson et al., 1997; Wulff et al., 2000). The K^+ channel-dependent hyperpolarization facilitates the Ca^{2+} influx induced by antigen binding. The consequent stimulation of intracellular Ca^{2+}- and PKC-dependent pathways triggers proliferation (reviewed in (Chandy et al., 2004)). A similar scheme may apply to transformed cell lines. K^+ currents seem often to be necessary during proliferation, although which kind of channel is involved depends on the cell type and the stimulus inducing leukemia cell entry into the mitotic cycle. Early evidence was obtained in the myeloblastic leukemia cell line ML-1. When proliferating, these cells express functional K^+ channels sensitive to 4-amino-pyridine (4-AP), which are instead suppressed after inducing macrophage differentiation (Lu et al., 1993). Treatment with 4-AP makes ML-1 cells arrest in G1, with no evidence of cell differentiation (Xu et al., 1996). Therefore, K^+ channels in ML-1 cells appear to be strictly linked to the cell cycle control. Consistently, K^+ currents are inhibited when cells are arrested in G1 by serum deprivation, and restored on serum re-addition or EGF application (Wang et al., 1997). The process depends on channel phosphorylation (Xu et al., 1999). The possible effects of K^+ channel activation on Ca^{2+} fluxes have not been tested in ML-1 cells, but were demonstrated in a rat basophilic leukemia cell line, RBL-1, which expresses K_{IR} channels. These probably maintain a favourable driving force for Ca^{2+} influx through CRAC channels (Straube & Parekh, 2002), in agreement with the early hypothesis based on work in T cells. Besides CRAC channels, other Ca^{2+} permeable channels have been studied in leukemia, and their role in still under study. For example, K562 cells, i.e. a human cell line obtained from a patient with CML in blast crisis, have been recently shown to co-express TRPV5 and TRPV6, two channel proteins that physically interact in these cells (Semenova et al., 2009). The same two channels were also detected in the lymphoblastic leukemia Jurkat cell line. Their expression pattern and high Ca^{2+} permeability indicate an important role in controlling Ca^{2+} homeostasis and probably in malignant transformation of blood cells (Vasil'eva et al., 2008). In other cases, the relation between K^+ channels and Ca^{2+} flux is more complex, with Ca^{2+} producing feedback on K^+ currents themselves. For example, the human Daudi cell line, a model of B-lymphoma, expresses functional $K_v1.3$ and $K_{Ca}3.1$. Specific block of $K_{Ca}3.1$ inhibits cell cycle, whereas the opposite occurs when these channels are up regulated by serum addition (Wang et al., 2007).

An extensive study of the K^+ channel transcripts in primary lymphocytes and leukemias as well as several hematopoietic cell lines has been carried out by Smith and colleagues (Smith et al., 2002). In particular, they tested *$K_v1.3$, $K_v10.1$, $K_v11.1$* and *$K_v12.2$*. Among these, only *$K_v11.1$* turned out to be significantly up regulated in cancer cells. Expression was however not related to proliferation *per se*, because it was not observed in proliferating noncancerous lymphocyte types such as activated tonsillar cells, lymphocytes from Sjögren's patients and Epstein-Barr virus-transformed B cells. Conversely, our group has found the *$K_v11.1$* transcript and the corresponding channel protein (Kv11.1, better known as hERG1) and currents (I_{hERG1}) in AML cell lines and in a high percentage of primary blasts from AML

patients. In this case, the block of I_{hERG1} , by applying specific hERG1 blockers, led cells to pause in G1. However, this was not the sole effect of hERG1 blockers; in fact, hERG1-blocking drugs also impaired AML cell migration through fibronectin. Hence, hERG1 also regulates cell migration and invasiveness in myeloid leukemias. This effect was mediated by a signaling mechanism triggered by cell adhesion, centered on Akt and modulated by hERG1 channel activity (Pillozzi et al, 2007). Similar results were obtained in childhood B-acute lymphoblastic leukemia (B-ALL) (Pillozzi et al., 2007). Both B-ALL cell lines and primary B-ALL cells expressed functional hERG1 channels, and hERG1 inhibition impeded the bone-marrow induced, integrin-dependent, protection against chemotherapeutic drugs, thus restoring a substantial apoptotic cell death. The hERG1 role in cancer cell biology is thus very complex. Mechanistic hypotheses based on current evidence are discussed later. Another member of the same Kv family, Kv 10.1 or EAG1, has long been related to cancer biology. Although the physiological expression of EAG1 is restricted to the brain, this channel is frequently and abundantly expressed in many solid tumors (Stühmer & Pardo, 2010). Until recently, it was assumed that EAG1 was not expressed in hematologic malignancies; however, Pardo and coworkers found a significant EAG1 expression in myelodisplastic syndromes, CML and almost half of a cohort of AML samples. In these cells, EAG1 blockade inhibited both proliferation and migration, both in AML cell lines and cultured AML primary samples (Agarwal et al., 2010).

The regulatory complexity is considerably increased by the fact that, in other contexts, the effects of K^+ channels directly modulate cell differentiation, instead of cell cycle. This was formerly observed in Friend erythroleukemia cells (MELC), which express Ca^{2+}-dependent K^+ channels (BK_{Ca}) (Arcangeli et al., 1987a, 1987b). These are transiently activated when differentiation is stimulated by cell adhesion onto fibronectin (Arcangeli et al., 1991; Becchetti et al., 1992), or by application of classical inducers of erythroid differentiation (Arcangeli et al., 1989). Similar effects were observed in THP-1 human monocytic leukemia cells. Undifferentiated THP-1 cells express K_{DR} channels. When differentiation to macrophages is induced by phorbol esters, K_{DR} expression is turned off, whereas BK_{Ca} and IRK are turned on (DeCoursey et al., 1996). hERG1 was also shown to be relevant to mediate ostecoclastic differentiation in a pre-osteoclastic leukemia cell line, FLG 29.1 cells. In these cells differentiation may be induced by integrin-mediated adhesion to fibronectin as well as by treatment with phorbol esters. In both cases, the hERG1 blockade inhibited cell differentiation, which in these cells is witnessed by the increased expression of the calcitonin gene and by the up regulation of the $\alpha_v\beta_3$ integrin, both markers of osteoclastic differentiation (Hofmann et al., 2001). A full discussion of the K^+ channel effects on differentiation is outside the scope of the present review. We limit ourselves to exhort the reader to keep in mind the possible complementary effects exerted by channel modulation on the proliferation and differentiation branches of cell signaling.

While no study is available on K^+ channel expression in true hematopoietic stem cells (HSCs), K_{IR} currents have been observed in primitive hematopoietic precursor cells (HPCs) ($CD34^+$ $CD38^-$) stimulated with the combination of interleukin-3 (IL-3) and stem cell factor (SCF) (Shirihai et al., 1996). The biophysical features of whole cell currents suggested that several K_{IR} channel types were co-expressed. In fact, later work showed that both strongly rectifying (K_{IR} 4.3) and weakly rectifying (K_{IR} 1.1) channels are present in these cells. The expression of both K_{IR} types seems essential for the generation of committed progenitors *in vitro*, as inhibition of the expression of either suppresses the generation of progenitor cells

from IL-3 and SCF-stimulated umbilical cord blood $CD34^+$ $CD38^-$ cells (Shirihai et al., 1998). More recently, the *Kv11.1* transcript was detected in circulating $CD34^+$ cells upon cell cycle induction by IL3 (interleukin 3), GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor) and SCF (Pillozzi et al., 2002). As illustrated in more detail below, $K_v11.1$ (i.e. hERG1) associates with the β_1 integrin in cord blood $CD34^+$ cells. This interaction is essential for proper BM engraftment of these HPCs. Finally, the *$K_v11.1$* transcript was recently detected in $CD34^+/CD38^-/CD128^{(high)}$ leukemic cells (Li et al., 2008), i.e. in the stem cell population that is critical for perpetuation of the leukemia disease (Li et al., 2008). On the whole, it is conceivable that Kv channels, and in particular hERG1, is relevant for normal and leukemic hematopoietic stem cells.

A novel player in this field is represented by the family of aquaporins (AQPs). A member of the family, AQP5 turned out to be overexpressed in CML cells, where it promotes cell proliferation and inhibits apoptosis, perhaps through an effect on cell volume control. In addition, the AQP5 expression increased with the emergence of imatinib mesylate resistance (Chae et al., 2008) (see also the paragraph below). Another member of the family, AQP9, has been shown to play a role in drug uptake and modulation of drug sensitivity in leukemia (Bhattacharjee et al., 2004) (see Chapter 6). Another channel, which has been recently reported to be expressed in the Jurkat cell line (Pottosin et al., 2008), is the TWIK-related spinal cord K^+ (TRESK) channel, belonging to the double-pore domain K^+ channel family.

As detailed in the general session on ion channels, P_{2X7} receptors are widely distributed in a variety of cell types and are involved in diverse biological effects. A sustained high level of extracellular ATP was detected in a tumor microenvironment which implied the involvement of abnormal signaling in tumour cells mediated by P2 family receptors. Besides being detected in solid tumors (Solini et al., 2008), high expression of the P_{2X7} receptor was observed in B-cell CLL (Adinolfi et al., 2002), acute leukemias and myelodisplastic syndromes (Zhang et al., 2004; Chong et al., 2010). Moreover, a series of P_{2X7} polymorphisms have been discovered, and their impacts on P_{2X7} functions and prognosis were studied (see also paragraph 3.2). For example, a N187D substitution was found in the J6-1 leukemia cell line, which displayed a lack of P_{2X7}-mediated calcium response upon BzATP stimulation (Zhang et al., 2004). It was also shown that K562 leukemia cells bearing this hyposensitive mutant displayed a proliferative advantage over wild-type P_{2X7}, both *in vitro* and in a nude mouse model. Furthermore, an increased angiogenesis and intratumoral inflammation could be detected in tumor masses formed by K562 cells bearing this mutant (Chong et al., 2010). Finally, P_{2X7} receptors were also functionally expressed in murine erythroleukemia cells, and the activation of these receptors seems to be important in the induction of apoptotic death and release of microparticles by these cells (Costantinescu et al., 2010).

An interesting debate, partially solved by the work of Huber's group (Kasinathan et al., 2007), involves chloride channels, and in particular ClC-3 channels. In this work, fluorescence microscopy revealed an intracellular localization of ClC-3 protein in K562 cells. Oxidation, on the contrary, increased the expression of ClC-3 protein into the plasma membrane, suggesting a role of plasma membrane-inserted ClC-3 in the oxidation-stimulated anion current observed in these cells (Kasinathan et al., 2007). Oxidation not only affects anion currents in K562 cells but also activates the non-selective cation channel TRPM2, resulting in an increase of intracellular free Ca^{2+} concentration, which in turn activates SK4 K^+ channels on the plasma membrane and may trigger apoptosis. An oxidation-induced co-activation of the ClC-3-dependent anion permeability results in loss of

KCl (and osmotically obliged H_2O) and thus in cell shrinkage, suggesting that the ClC-3-dependent anion permeability *per se* may generate apoptotic volume decrease. Another intriguing role of anion conductance emerged from the work of Soriani and coworkers (Renaudo et al., 2007). In particular, they studied the relationships between sigma receptors and volume-regulated chloride currents (VRCC). Sigma receptors are intracellular proteins that were first postulated as opioid receptors on the basis of pharmacological and behavioural studies. Sigma-1 receptors were functionally coupled with membrane potassium channels in the pituitary (Aydar et al., 2002; Soriani et al., 1999a). Subsequently, it was reported that the activation of sigma-1 receptors by highly selective ligands provoked the arrest of the cell cycle progression in the G1 phase, in cancer cells. This effect was partly linked to the inhibition of voltage-dependent potassium channels (Renaudo et al., 2004). More recently, they also demonstrated that the sigma-1 receptor modulates VRCC and cell volume regulation properties in leukemic cells leading to an alteration of cell proliferation and apoptosis (Renaudo et al., 2007).

4. Genetics and epigenetics of channel expression in leukemias

It is clear that most human neoplastic cell types show altered expression of a variety of ion channels and that these exert different functional roles. However, there does not seem to be a specific channel-tumor correlation. This conclusion is in broad agreement with current genetic evidence, because no clear cancer-related mutation in any channel-encoding gene has been reported so far. Therefore, it would appear that ion channels are not so much involved in tumor causation, but are implicated in the different stages of neoplastic progression. A partial exception is *KCNRG*, which encodes a K^+ channel-regulating protein that has been proposed to be a tumor suppressor gene (Ivanov et al., 2003). A missense mutation at the codon 92 of *KCNRG* is often present in human hepatocellular carcinomas, positive for the Hepatitis B virus (Cho et al., 2006). In B cell-CLL *KCNRG* has been detected in the minimal common deleted region (CDR) of the 13q14 chromosomal deletions. The latter is the most common abnormality in CLL (Liu et al., 1997; Dohner et al., 1999); deletions at 13q14.3 are associated with the longest survival. Rearrangements and/or deletions in the region of 13q14.3 are also found in other types of hematopoietic malignancies, including mantle cell lymphomas (Rosenwald et al., 1999) and multiple myelomas (MM)(Harrison et al., 2003; Elnenaei et al., 2003). In the majority of these non-CLL cases, 13q14 deletions are associated with a poor chemotherapy response profile. The CDR encompasses an area containing *DLEU1, DLEU2, RFP2*, and *KCNRG* as well as microRNAs miR-15a and miR-16-1 (Liu et al., 1997; Tyybakinoja et al., 2007; Kapanadze et al., 1998; Calin et al., 2002; Baranova et al., 2003) *KCNRG* is located within the 3′ end of the largest transcript of *RFP2* (Ivanov et al., 2003). Due to its effect of interfering with the normal assembly of the K^+ channel proteins by binding to their tetramerization domain, thereby, causing the suppression of Kv currents, it has been hypothesized that *KCNRG* may exert a tumor suppressor effect relevant to CLL and MM. Another frequent genetic alteration in leukemia is represented by translocations. The *RUNX1* (previously known as *AML1* or *CBFA2*) gene, located on chromosomal band 21q22, encodes the alpha subunit of the heterodimeric core-binding factor (CBF). Located at the C-terminus of *RUNX1*, a transcriptional regulation domain is required for the transcriptional activation or repression of genes relevant to myeloid and lymphoid development. *RUNX1* acts as a key regulator of hematopoiesis and is frequently targeted by mutations and chromosomal translocations in leukemias (Redaelli

et al., 2004). The t (1;21)(p22;q22) was first reported in a case of a possible therapy-induced leukemia (Rosenwald et al., 1999); in this case the partner gene of *RUNX1* was identified in the *CLCA2* gene, which then resulted to be a novel fusion partner of *RUNX1* in adult patients with a therapy-related AML.

The frequent overexpression of channel-encoding genes in human cancers seems to be often caused by gene amplification. This has been demonstrated for *KCNK9,* in breast (Mu et al., 2003) and colorectal cancers (Kim et al., 2004), and *CACNA1E* (Ca_v 2.3), in Wilms' tumours (Natrajan et al., 2006). In other cases, epigenetic mechanisms have been invoked. Among these, a paradigmatic example is aberrant promoter methylation of the growth regulatory genes. This mechanism is probably a common alternative to gene inactivation, in human cancers. Evidence along this line is available for channel-related genes, such as *CLCA2*, whose promoter region is frequently inactivated by hypomethylation, in breast cancer (Li et al., 2004). Moreover, methylation of *KCNH5* is observed in about 80% of NSCLC tissue, but only in 14% of non-cancerous tissue (Feng et al., 2008). Finally, inactivation of *CACNA1G* by aberrant methylation of its 5' CpG island has been reported in AML, gastric cancers and colorectal cancers (Toyota et al., 1999). More puzzling remain the genetic mechanisms underlying AQP5 overexpression in CML. No genomic amplification was detected, whereas methylation analysis of the AQP5 promoter region suggested that promoter demethylation might be relevant, although this fact was proven in head and neck and lung cancer cell lines, while validated data are still lacking in leukemias.

Another genetic mechanism that could explain the alterations of ion channel encoding genes in leukemias, involves micro (mi) RNAs. MiRNAs are naturally occurring 18- to 25-nucleotide RNAs that are cleaved from 70- to 100-nucleotide hairpin precursors by a complex protein system that includes the RNase III Drosha and Dicer, members of the Argonaute family. Mature microRNAs typically hybridize to the 3'untranslated regions of protein-coding messenger RNAs (mRNAs) and cause their post-transcriptional repression and/or degradation (Ambros, 2004; Bartel, 2009). MiRNAs regulate normal cell homeostasis and are involved in many physiologic processes, including hematopoiesis (Garzon & Croce 2008; Vasilatou et al., 2010; Havelange & Garzon, 2010). Recently, dysregulation of miRNAs has been shown in many types of solid tumors and leukemias (Calin & Croce, 2006). Direct involvement of miRNAs in cancer has been suggested by a study demonstrating that several miRNAs are localized in genomic regions associated with cancer, such as breakpoint regions in chromosome aberrations involving oncogenes or tumor suppressor genes, minimal regions of loss of heterozygosity, minimal regions of amplification, and at loci close to fragile sites and integration sites of the human papilloma virus. Several functional studies confirmed the important role of miRNA deregulation in hematologic malignancies, mainly B-CLL and AML. Ion channel encoding genes are among the target genes of miRNAs: for example two miRNAs often dysregulated in CLL, miR15a and miR16-1 have, among their target genes, genes encoding K_v and water channels, in particular *KCNH8* (i.e. $K_v12.1$) and aquaporin 11, respectively. *KCNH3* (i.e. $K_v12.2$ or *elk-2*), another K_v encoding gene strictly related to *KCNH8* and functionally similar to $K_v11.1$, is also one of the target genes of miR221, often deregulated in CLL. $K_v11.1$ is also negatively regulated by miR133, which is one on the miRNAs down-regulated in a specific clinicopathological subgroup (t (8:21)) of AML. This fact could explain the frequent increased expression of $K_v11.1$ transcript in AML.

Growing evidence also suggests that tumors tend to express splice variants or alternative transcripts of channel-encoding genes, although the significance for cancer progression is still uncertain. The *hsloBK* splice variant of *gBK* has been detected in gliomas (Olsen et al.,

2005) and the *herg1b* alternative transcript of $K_v11.1$ is overexpressed in human leukemias and neuroblastomas (Pillozzi et al., 2007; Crociani et al., 2003). Another splice variant of the $K_v11.1$ transcript, which encodes for a C-terminus deleted K_v11.1 protein, named $herg1b_{USO}$, is also overexpressed in several leukemias cell lines, and exerts a post-translation control on the plasma membrane expression of the full length K_v11.1 protein (Guasti et al., 2008).

5. Ion channels in primary leukemias: A novel biomarker?

As better detailed in chapter 1, leukemia is a disease with marked heterogeneity in both response to therapy and survival. Cytogenetic, age, and performance status have long determined prognosis and therapy. The advent of molecular diagnostics has heralded an explosion in new prognostic factors: microarray technology can now identify unique gene expression signatures associated with prognosis. Similarly miRNA expression, single nucleotide polymorphism arrays, and DNA methylation signatures have recently described important new prognostic subgroups in the single leukemia cathegories. We may be close to a time when we will be able to use these prognostic factors and technologies to identify new targets for therapy and to determine who may benefit from that therapy, and ultimately change how we treat individual patients with leukemia. It is mandatory at this time, to transfer these concepts to the ion channels, after a 20 years work mainly focused to study ion channel expression and function identification. Indeed some recent papers aim at delineating the impact of single ion channels, or of an "ion channel signature" as biomarkers in leukemias. Following are some examples: $K_V11.1$ expression was correlated with a more aggressive AML phenotype both *in vitro* and *in vivo*. In a cohort of patients affected by AML, $K_v11.1$ expression was associated with a higher probability of relapse and a shorter overall survival (Pillozzi et al., 2007). This was one of the first clinical and prognostic applications of an expression screening for a K_v channels. Subsequently, EAG1 was found to be expressed in myelodisplastic syndromes, CML and AML. Interestingly, channel expression in AML patients strongly correlated with increasing age, higher relapse rates and a significant shorter overall survival. Multivariate Cox regression analysis revealed EAG expression levels in AML as an independent predictive factor for reduced disease-free and overall-survival; such association further stresses the impact of K_v channels of the EAG family as biomarkers in AML (Agarwal et al., 2010). Moon and co-workers (Chae et al., 2008) found evidence that AQP5 might be associated with the progression of CML. Indeed, CML patients diagnosed at accelerated or blast crisis phase showed significantly higher level of AQP5 expression than those diagnosed at chronic phase, while CML patients who gained imatinib mesylate resistance at chronic phase exhibited significantly higher level of AQP5 expression than those who gained resistance at accelerated or blast crisis phase. Furthermore, AQP5 expression increased with the appearance of imatinib mesylate resistance (see also chapter 7.2). A recent paper reported the results of a study in which the expression of P_{2X} receptors in blood mononuclear cells from Chinese pediatric leukemias patients was determined. P_{2X1}, P_{2X4}, P_{2X5} and P_{2X7} were simultaneously over expressed in leukemias compared to controls. The co-expression of P_{2X4} and P_{2X7} was a common feature of leukemic samples, and the highest expression of P_{2X7} was detected in relapsed patients, whereas a concomitant decrease of P_{2X4}, P_{2X5} and P_{2X7} was observed after chemotherapy (Chong et al., 2010). This aspect has a clear relevance also for the chemoresistance, which is described in chapter 6.

Some studies also reported altered expression of ion channels and transporters in primary lymphomas. For example upregulation of KCNN3 ($K_{Ca}2.3$) was observed in germinal center B-like diffuse large B cell lymphoma (DLBCL), whereas KCNA3 (*Kv 1.3*) was upregulated in activated B-like DLBCL (Alizadeh et al., 2000). Moreover, in the lymphoma cell line Daudi, expression of $K_{Ca}3.1$ has been described and its activity may account for cell malignant growth and proliferation (Wang et al., 2007). Data obtained by a microarray study in pediatric brain tumors indicate a marked deregulation of ion channels and transporters. Hence, we suggest to focus on the "ion channels and transporters" term when analyzing microarray gene expression data (Masselli et al., unpublished).

6. Outline of the functional role of ion channels in cancer

Ion channels are generally involved in many processes necessary for cell proliferation, adhesion to substrate and motility. For example Ca^{2+} fluxes control the cell cycle machinery and the secretion of cytokines and growth factors. Moreover, ion channels participate in regulating the cell volume changes that normally occur during mitosis, cell migration, etc. However, altered expression or function of different channel types are also thought to determine more specific aspects of malignancy. In leukemic cells, these alterations contribute to determine the poorly differentiated phenotype, invasiveness, transendothelial migration and chemoresistance (Arcangeli et al., 2009). In this context, a common occurrence is ion channel involvement in the signaling pathways that are related to the modulation of cell adhesion to the extracellular matrix.

6.1 V_m in cancer cells

As is well known, proliferating cells tend to be depolarized as compared to non cycling cells. Such a difference may depend on regulated channel expression. For instance, developing glial cells generally express outward rectifying K_V, subsequently substituted by inward rectifier K^+ channels that, in mature cells, determine the typical hyperpolarized state of adult glia (Sontheimer et al., 2008). Why expression of different K^+ channels produces such a different effect on V_m? The reason is that proliferating and tumor cells usually undergo slow and low amplitude V_m changes, compared to excitable cells. Therefore, what matters more are the steady state K^+ channel properties, which determine the fraction of open channels at a certain stable V_m. During the neoplastic transformation, the process exemplified by glia differentiation often reverts in that cancer cells tend to express a variety of K^+ channels that either carry little current at strongly negative V_m, such as $K_v10.1$ and $K_v1.3$ (unless they undergo overexpression, see below) or channels whose maximal steady state probability of being open is obtained at relatively depolarized V_m (e.g. -40 mV for $K_V11.1$). In some instances, the neoplastic condition seems a partial reversion to a state normally occurring during development, but the evidence about this is still limited.

However, considering the average V_m is simplistic, because oscillations are observed in either the expression or regulation of several ion channel types during the cell cycle phases, with ensuing alterations in V_m (Arcangeli & Becchetti, 2006). In general, it is unclear if these V_m changes regulate the downstream signals or are by products of alterations in channel activity/expression that the cell controls for reasons not necessarily linked to V_m. For example, specific channel subtypes can exert specific signaling roles. Current evidence indicates that there is probably no unique explanation. Several examples suggesting a spectrum of possible mechanisms are illustrated below.

6.2 Hyperpolarization can stimulate Ca^{2+} influx

Intracellular Ca^{2+} signals are known to regulate cell cycle in both normal and cancer cells. Ca^{2+} channels are in fact widely expressed in cancer cells, in which they are also likely to modulate cell movement and migration. Original work in T lymphocytes indicated that a hyperpolarization produced by increased K^+ channel function facilitates the Ca^{2+} influx necessary for T cell activation. Analogous results were more recently obtained in breast cancer cells, where growth factors applied in G1 can lead to such an over-expression of $K_v10.1$ to lead to cell hyperpolarization and G1-S transition. Such a hyperpolarization triggers a positive feedback in that Ca^{2+} influx stimulates $K_{Ca}3.1$, which maintains a tonic hyperpolarization that further sustains the Ca^{2+} signal that regulates the cyclins and the cyclin-dependent kinases (Ouadid-Ahidouch & Ahidouch, 2008).

6.3 Control of cell volume and motility

Mammalian cells undergo oscillatory volume changes during both cell cycle and migration (Habela & Sontheimer, 2007; Boucrot & Kirchausen, 2008). The interplay of K^+ and Cl^- channels is very important in the early phases of cell volume regulation. A full review of this topic cannot be given here (Chandy et al., 2004; Nilius, 2007) but it is clear that altered control of these processes may affect proliferation as well as tumor invasiveness, as has been shown in gliomas.

6.4 Ion channels and intracellular signaling

Ion channels are also implicated in different aspects of leukemia malignancy, such as the lack of differentiation, invasion and transendothelial migration, chemoresistance (see also Table 1). The role of ion channels in such processes can be attributed to signaling mechanisms, which are often related to the modulation of adhesive interactions with the extracellular matrix.

6.4.1 Signaling mechanisms

Cell proliferation in mammalian cells can be triggered by growth factor (GF) binding to specific receptors, usually protein tyrosine kinases that autophosphorylate upon ligand binding. This process typically turns on a kinase cascade that converges onto phosphorylation of the extracellular signal-regulated protein kinase 2 (ERK2) mitogen-activated protein (MAP) kinase. Once activated, this protein translocates to the nucleus and phosphorylates an array of specific transcription factors (Arcangeli & Becchetti, 2006). A similar mechanism is also triggered by cell adhesion. In particular, the integrin-mediated cell adhesion to the extracellular matrix modulates, in proper context, cell migration, proliferation, differentiation and prevents apoptosis (Juliano, 2002; Arcangeli & Becchetti, 2006). The integrin linked kinase (ILK) and the focal adhesion kinase (FAK) are pivotal factors in these cascades. Once activated, they recruit further signaling components, thus leading to the activation of MAP kinases, of the phosphoinositide-3 kinase (PI3K), and small GTPases such as Rho A, Rac 1 and CDC42 (Arcangeli & Becchetti, 2006). Adhesion signals greatly overlap with those activated by GF and cytokine receptors. In some cases, such an overlap depends on the formation of macromolecular complexes between integrins and the other membrane receptors, to form signaling platforms at the adhesive sites. These platforms can also include ion channels, with ensuing crosstalk between the different components (Arcangeli & Becchetti, 2006). For example, T lymphocyte activation leads $K_v1.3$

channels to associate with β_1 integrins and activate them (Levite et al., 2000), an interaction also observed in melanoma cells (Artym et al., 2002). A macromolecular complex between β_1 integrin subunit and hERG1 (K_v 11.1) forms in several neoplastic cells (Cherubini et al., 2005). The β_1/hERG1 complex localizes at the adhesion sites, probably within caveolae/lipid rafts, and recruits FAK, Rac1 and PI3K. FAK phosphorylation, Rac1 and PI3K activities turned out to depend on hERG1 currents (Cherubini et al., 2005). Moreover, we noticed that in AML cells the β_1/hERG1 complex also includes the VEGF receptor 1 (Flt-1). The macromolecular complex regulates signaling downstream to Flt-1 (MAP kinase and PI3K) and thus AML proliferation and migration (Pillozzi et al., 2007). In childhood B-ALL, the β_1/ hERG1 complex is triggered by adhesion onto human bone marrow stromal cells (MSC), and comprises the chemokine receptor CXCR4. We determined the signaling pathways activated by the components of the β_1 integrin/hERG1/CXCR4 complex in B-ALL cells and we found that ILK was the first effector to be activated after engagement of CXCR4, integrin activation and/or culture of leukemia cells on MSC. MAP kinase and PI3K/pAkt pathways (downstream effectors of ILK activity) were also activated in B-ALL cells cultured on MSC The activation of both signaling pathways depended on β_1 integrin activation, as it was inhibited by a blocking antibody. Importantly, both ILK activity and ERK1/2 and Akt phosphorylation were strongly reduced in B-ALL cells cultured on MSC after blocking hERG1 channels by two specific blockers. When cultured on MSC, leukemia cells are protected from apoptosis induced by chemotherapeutic drugs (see below). Another example, involves the K_v channels regulated by GFs in ML-1 myeloblastic leukemia cells (Xu et al., 1999; Guo et al., 2005), as well as for the VGSCs controlled by NGF (Brackenbury et al., 2007) and EGF (Uysal-Ongonen et al., 2007; Ding et al., 2008). But signals can also flow in the opposite direction: for example the 4-AP-sensitive K^+ channels control ML-1 proliferation through multiple signal transduction processes, such as phosphorylation of ERK 1/2 and Akt (Guo et al., 2005).

6.4.2 Non-conductive roles

As discussed above, some effects of ion channels on neoplastic progression are clearly a consequence of changes in ion fluxes. Less attention has been so far devoted to possible modulation of intracellular pathways by enzymatic roles of channel proteins or conformational coupling with their proteins, ultimately converging onto the transcriptional regulation of cancer-related genes. These mechanisms may well accompany the typical effects on ion flow. Some VGCs have been in fact shown to behave as bifunctional proteins that, besides gating ion fluxes as usual, exert an ion conduction-independent control of several intracellular responses (Iwasaki et al., 2008; Wang et al., 1999). An example with oncological implications is the voltage sensor-containing phosphatase (Ci-VSP) of *Ciona intestinalis*. This consists of an ion channel-like transmembrane domain, followed by a phosphatase domain highly homologous to PTEN, a tumor suppressor of human cancers (Iwasaki et al., 2008). Recent review of some of these non-conducting functions is found in (Levitan, 2006). Although a similar behaviour has not been clearly demonstrated to occur in cancers and leukemia, it could be relevant in prospecting new therapeutic strategies.

7. Ion channels as therapeutic targets in leukemia

Using extracellular ion channel blockers for oncologic therapy would limit harmful metabolic effects and allow relatively easy calibration of doses. Moreover, thanks to the

modern electrophysiological methods and recent insight into ion channel structure, the mechanism of action of channel blockers is often understood in depth, which facilitates rational therapy and design of new compounds. These can be tested both in heterologous systems and *in vivo*, which allows to predict or study some of the side effects. Although ion channels are very suitable targets for drug screening and rational therapeutic strategies, they are still scarcely used to target non excitable cells such as the neoplastic, because of the risk of serious side effects. A classical example is K_v11.1. As fully discussed elsewhere (Arcangeli et al., 2009), several indications suggest that K_v11.1 could be an effective target for cancer therapy. However, this channel type also regulates the repolarization phase of the cardiac action potential. Therefore, K_v11.1 block can lead to the so-called *torsade de points* (TdP), i.e. ventricular arrhythmia that may lead to lethal ventricular fibrillation (Witchel et al., 2000). Therefore, hERG1 blockers are generally considered unsafe for pharmacological treatment in humans. Another drawback of the most common hERG1 blockers is that they act on the cytoplasmic face of the channel.

However, even in cases as unfavourable as this, a way out of the trouble is provided by the availability of many different inhibitors that act with different mechanisms. More explicitly, several K_V11.1 blockers are not torsadogenic, although the reasons for such differences between different compounds are still poorly understood. In general, recent work suggests several methods to obtain better tissue specificity even when selective blockers for channel subtypes are not available. Alternatively, ion channels can be targeted to deliver tracers or cytotoxic compounds. The following paragraph summarizes such evidence with a focus on leukemic cells.

7.1 Targeting ion channels in leukemias: Experimental evidences

As described above, different papers have reported that ion channels inhibitors can affect different biological aspects of leukemic cells, both *in vitro* and *in vivo*. Such evidences are summarized in Table 1, in which are shown the specific drug and ion channel targeted as well as the biological aspect of leukemia influenced.

	LEUKEMIA	*ION CHANNEL*	*DRUG (or molecular tools)*	*References*
PROLIFERATION	AML	K_V 11.1	E4031, WAY 123.398	*Pillozzi, 2002* *Pillozzi, 2007*
	AML, CML myelodisplastic syndrome	EAG1	astemizole, imipramine, mAb56	*Agarwal, 2010*
	CML	AQP5	siRNA	*Chae, 2008*
	AML (ML-1 cell line)	K+ currents	4-AP	*Xu, 1996*
	B-lymphoma	KCa 3.1	TRAM-34	*Wang, 2007*
MIGRATION/TEM	AML	K_V 11.1	E4031, WAY 123.398	*Pillozzi, 2007*
DIFFERENTIATION	AML-M5A	K_V 11.1	WAY 123.398	*Hofmann, 2003*
CHEMIORESISTANCE	B-ALL	K_V 11.1	E4031, WAY 123.398, sertindole, erythromycin.	*Pillozzi, 2011*
APOPTOSIS	CML	AQP5	siRNA transfection	*Chae, 2008*
	APL	AQP9	overexpression	*Bhattacharjee, 2004*

Table 1. Pharmacological and molecular modifiers able to affect ion channels involved in different biological aspects of leukemia disease. (AML: acute myeloid leukemia; CML: chronic myeloid leukemia; ALL: acute lymphoblastic leukemia; APL: acute promyelocytic leukemia).

Several types of K^+ and Cl^- channels have been shown to be potential targets for cancer treatment (Arcangeli et al., 2009). In leukemia cells, most of the relevant results concern EAG and hERG1, both of which belong to the K_V channel family and share 47% of the amino acid sequence. They are expressed in different forms of leukemias and have been implicated in cell cycle progression and proliferation in these and other cancers (Arcangeli et al., 2009). Inhibition of these channels reduces proliferation both *in vitro* and *in vivo,* which indicates that modulating their activity could produce beneficial effects on patients. In fact, several studies on immunodepressed mice show that blocking EAG and hERG1 channels inhibits progression of the disease. In fact, we also determined whether hERG1 inhibitors could improve leukemia treatment in murine models of B-ALL. In a first set of experiments, NOD-SCID mice were inoculated with 697 cells and treated daily with E4031 (20mg/Kg) for two weeks, starting one week after the inoculum. At the end of treatment, some of the mice were sacrificed and the degree of bone marrow, peripheral blood and extramedullary organs invasion by B-ALL cells was quantified. E4031 treatment significantly reduced the leukemia burden and the liver and spleen infiltration by leukemic cells, with significant prolongation of mice survival. In a second set of experiments, we tested the effects *in vivo* of the combined treatment with E4031 and dexamethasone on REH cells, which have been reported to be resistant to corticosteroids *in vivo*. Mice were treated for two weeks with dexamethasone, E4031, or both. E4031 reduced bone marrow engraftment of REH cells, similarly to what was observed for 697 cells. This effect was related to an increased apoptosis of B-ALL cells, and was higher than that produced by dexamethasone. Treatment with dexamethasone and E4031 together nearly abolished bone marrow engraftment while producing substantial apoptosis. A marked reduction in leukemic cell infiltration of the spleen in mice treated with dexamethasone plus E4031 was also observed. These data clearly indicate that hERG1 blockers can treat the leukemia disease *in vivo*, both alone and in combination with classical chemotherapeutic drugs and are capable of reverting drug resistance in vivo. These drugs also exerted clear antileukemic activity (see details below) and thus represent promising candidates for further studies aimed at paving the way to clinical trials.

Because EAG and hERG1 channel are structurally related, any drug acting on EAG channel may also block hERG1 channels and thus have arrhythmogenic effects. Therefore, irrespective of whether one intends to use pharmacological blockers of EAG and hERG1, to avoid side effects it will be necessary to either develop specific inhibitors for different subtypes or carefully test each inhibitor for the effects on each channel type. These and other molecular tools have been already applied to specifically target Eag in cancer cells, and particularly 1) chemical blockers; 2) monoclonal antibodies; 3) small interfering RNA (siRNA). The challenge with the latter approach is designing an appropriate vehicle for transport and delivery of siRNA to the target organ, something that is currently the subject of intense research. All of these methods can be used in conjunction with chemotherapeutic agents or can be exploited to improve survival in chemoresistant diseases.

As to Cl^- channels, the activation and the subsequent hyperpolarization triggered by the antiparasitic ivermectin at low micromolar concentrations preferentially induces cell death in AML cell lines and primary patient samples compared to the normal hematopoietic cells. Ivermectin also delayed tumor growth in 3 independent mouse models of leukemia and synergized with cytarabine and daunorubicin, that also increase reactive oxygen species production. Although the specific channel activated by ivermectin has not been clearly

identified, the thorough knowledge about the toxicology and pharmacology of ivermectin, this compound could be rapidly advanced into clinical trial for leukemia. (Sharmeen et al., 2010).

7.2 Ion channels and leukemia chemoresistance

Chemoresistance is recognized clinically as the development of tumor resistance to a wide variety of anticancer drugs following exposure to a single drug. Resistance to multiple drugs could arise from cellular defenses that broadly limit access of the agent to a cellular target, or prevent the cell from entering apoptosis following injury. The development of resistance to a wide spectrum of cytotoxic drugs frequently impedes the successful treatment of acute and chronic leukemias either at the initial presentation or following primary or subsequent relapses (O'Gorman et al., 2001). The majority of leukemias in fact respond to initial treatment; however, relapse is common, indicating resistance of leukemic cells to current therapies. Several mechanisms may account for this phenomenon, including failure of the drug to reach and/or affect its intracellular target or failure of the cell to undergo apoptosis in response to chemotherapy (Ross et al., 2000). There is emerging evidence that also extrinsic components mediated by the microenvironment play a pivotal role in survival and drug resistance of leukemic cells. It is believed that environment-mediated drug resistance is a transient state whereby leukemic stem cells are protected through signals from the niche, which eventually leads to the selection of secondary genetic changes and outgrowth of cells that acquired multiple mechanisms of pharmacologic resistance (Meads et al., 2009).

In addition to the direct relationship between transporters and drug substrates, indirect mechanisms may also modulate chemosensitivity. For example, transporters and channels can affect chemosensitivity by providing nutrients to cancer cells or modulating the electrochemical gradient across membranes, thereby, modifying apoptosis pathways or the efficiency of drug diffusion along electrochemical gradients into cells (Huang et al., 2006).

Several genes that encode subunits of Na^+, Cl^-, K^+, and other cation channels correlated with drug activity, confirming that ion channels can modulate drug response—possibly by affecting the cell's resting potential or by providing key metal ion cofactors. We do not know at this point whether these gene products mediate drug transport directly or affect sensitivity and resistance by indirect mechanisms. Ion channels modulate electrochemical gradients generated by ion pumps and ion exchangers. Maintenance of a strong electrochemical gradient is vital to the cell and a potentially strong influence on drug activity. K^+ and Cl^- leakage currents tend to polarize cells, whereas Ca^{2+} and Na^+ channels depolarize them. These two types of flux would be expected to have opposite effects on drug equilibration across cell membranes. However, Ca^{2+} flux is also important in apoptotic signaling, as noted above, so the net effect on drug potency is difficult to predict.

The main ion channels whose activity is related to the establishment of chemoresistance in leukemia cells are mitochondrial voltage-dependent anion channels (VDAC), aquaporins and hERG1. The VDAC, located at the outer mitochondrial membrane, play a central role in the regulation of apoptosis. VDAC is the constituent of the mitochondrial permeability transition pore (PTP), which mediates the release of apoptogenic factors such as cytochrome c from the intermembrane space into the cytosol (Shimizu et al., 1999). Three VDAC genes have been identified in human, *VDAC1*, *VDAC2* and *VDAC3*. *VDAC1* binds to Bcl-2 and its homologues, such as Bax, Bak and Bcl-XL to regulate the opening of PTP (Abu-Hamad et al., 2009). Cells deficient in *VDAC2* but not cells lacking the more abundant VDAC1 are more

susceptible to apoptotic death. VDAC has been suggested to be one of the biological targets of arsenic trioxide (As_2O_3), an anticancer drug for acute promyelocytic leukemia (APL) (Zheng et al., 2004). One member of the aquaporin family, AQP9, has been shown to play a role in drug uptake and modulation of drug sensitivity in leukemia (Bhattacharjee et al., 2004). AQP9 has broad transport specificity, including water, glycerol, urea, carbamides, purines, and pyrimidines, but its physiological function is still unknown. Increased expression of AQP9 in leukemic cells K562 and HL60 increases uptake of and sensitivity to As (III) and Sb (III), the trivalent arsenic drug trisenox. Moreover primary APL cells expressed AQP9 significantly (2-3 logs) higher than other AML, which might explain their exquisite As_2O_3 sensitivity. AQP7 also transports As (III) and Sb (III), but to a lesser extent than AQP9. Arsenic drugs often cause significant toxicity during treatment, showing marked individual variability. Individual variability in expression of AQP7 and AQP9 may partly explain the differential response and toxicity to arsenic therapy (Liu, 2002). Based on these observations, we suspect that AQP5 may be conferring a growth advantage in the process of CML progression. Furthermore, it has been reported that AQP5 may play a role in developing imatinib mesylate resistance, irrespective of other known major resistance mechanisms such as *bcr-abl* mutation or amplification (Chae et al., 2008).

As anticipated in paragraphs 6.4.1 and 7.1 our group studied the molecular mechanisms underlying the protection by MSC in B-ALL. We evidenced that coculture with MSC induced the expression of a plasma membrane signaling complex in B-ALL cells, constituted by hERG1 channels, the β_1 integrin subunit and the chemokine receptor CXCR4. Interaction of integrins with their ligands on MSC layers is critical to the formation of the complex. We next tested the effects of coculturing B-ALL cell lines with MSC on chemosensitivity and the role of the β_1 integrin/hERG1/CXCR4 complex. B-ALL cell lines cultured with or without MSC were exposed to doxorubicin, prednisone and methothrexate, drugs commonly used to treat ALL. Classical hERG1 blockers, E4031 or Way 123,398, were tested on cells cultured on MSC and treated in combination with each drug. It emerged that MSC protected B-ALL from the apoptosis induced by all the drugs tested, although to different degrees depending on the drug and cell line. MSC-induced resistance was almost completely abrogated when ALL were treated with hERG1 blockers. We also tested the effects of sertindole (an antipsychotic) and erythromycin (an antibiotic), which are known to block hERG1 channels but do not cause cardiac arrhythmia and can be used clinically. The two drugs were added to B-ALL cells cultured on MSC, along with doxorubicin. Both drugs reverted MSC-drug induced chemoresistance in all the B-ALL cell lines tested, at levels even greater to those obtained with the hERG1 blockers E4031 and Way 123,398. Hence, the activity of hERG1 channels inside the β_1 integrin/hERG1/CXCR4, whose activation is triggered by culture on MSC, mediates the MSC- induced drug resistance to apoptosis, and that different hERG1 blockers can overcome drug resistance. These results were corroborated by the studies in murine models of B-ALL reported above. We can conclude that hERG1 channels are upstream regulators of the MSC-triggered pro- survival signals in B-ALL, and that administration of hERG1 blockers could improve chemotherapy responses in patients with ALL (Pillozzi et al., 2011).

8. Conclusion

The evidence we have reviewed shows that certain ion channel types exert important regulatory roles in leukemic cell physiology. These functions are implicated in the neoplastic

progression and thus appear to be potential target for therapy, as is also suggested by recent work in murine models. For the reasons discussed above, particularly the possibility of serious side effects, ion channels are still somewhat neglected as pharmacologic targets in oncology. However, we believe they should receive more attention because they present considerable advantages in terms of thorough mechanistic understanding and clinical potential, not only for leukemias. In fact, clinical trials are currently in progress for testing the efficacy of targeting specific channel types in several tumors such as glioblastoma and urinary bladder carcinoma. Therefore, more widespread efforts should bring novel pharmacological applications of ion channel for treating oncohematologic diseases as well as other cancers

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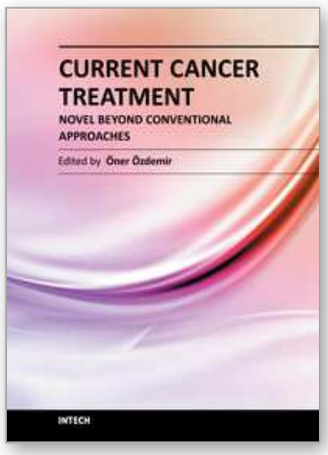

Current Cancer Treatment - Novel Beyond Conventional Approaches

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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book "Current Cancer Treatment - Novel Beyond Conventional Approaches", consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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