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Enhanced antitumor activity by the combination of Dasatinib and Selinexor in Chronic Myeloid Leukemia

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List of abbreviations			
Abbreviation	**Definition**	**Abbreviation**	**Definition**
ΔΨm	Mitochondrial membrane potential	**IMA**	Imatinib
$2^{-\Delta\Delta Ct}$	Delta-delta CT method	**IPO**	Importin
2gTKI	Second generation TKI	**JAK**	Janus Kinase
A	Adenine	**K562**	Cell line derived from 53-years old female with CML in blast crisis
AKT	Protein kinase B or AK strain trasforming	**LAMA84**	Cell line derived from 29-years old female with CML one month before blast crisis
AP	Accelerated Phase	**LMB**	Leptomycin B
ATCC	American Type Culture Collection	**LSC**	Leukemia Stem Cell
ATP	Adenosine Triphosphate	**Maf**	Musculoaponeuretic Fibrosarcoma
B2M	Beta-2-Microglobulin	**MARE**	Maf recognition elements
Bach	Broad complex tramtrack bric a brac A Cap and collar Homology	**MFN**	Mitofusin
BAK	BCL2 Antagonist or Killer	**MMR**	Major Molecular Response
BAX	BCL2-Associated X protein	**MR^3**	Molecular Response 3 log reduction from the standard baseline
BCL2	B Cell Limphoma 2	**MR^4**	Molecular Response 4 log reduction from the standard baseline
BMT	Bone Marrow Transplantation	**$MR^{4.5}$**	Molecular Response 4.5 log reduction from the standard baseline
BOS	Bosutinib	**MR^5**	Molecular Response 5 log reduction from the standard baseline
BP	Blast Phase	**mRNA**	messenger Ribonucleic Acid
BU	Busulfan	**mtDNA**	mitochondrial DNA
C	Cytosine	**mTOR**	mammalian Target Of Rapamycic
CCyR	Complete Cytogenetic Remission	**NADPH**	Nicotinamide Adenine Dinucleotide Phosphate
CML	Chronic Myeloid Leukemia	**nDNA**	nuclear DNA
CO	Carbon monoxide	**NES**	Nuclear Export Sequence
CO_2	Carbon dioxide	**NIL**	Nilotinib
CP	Chronic Phase	**NLS**	Nuclear Localizing Signal
CytB	Cytochrome B	**NPC**	Nuclear Pore Complex

DAS	Dasatinib	**Nrf2**	Nuclear factor erythroid 2-related factor
DiOC2(3)	3,3-Diethylozacarbocyanine Iodide	**O_2**	Diatomic oxygen
DLI	Donor Lymphocyte Infusion	**OPA1**	Optic Atrophy 1
DNA	Deoxyribonucleic Acid	**PBS**	Phosphate Buffered Saline
EIF5A	Eukaryotic translation Initiation Factor 5A	**PCR**	Polymerase Chain Reaction
ELN	European LeukemiaNet	**PEG-ZnPP**	pegylated zinc protoporphyrine
Fe^{2+}	Ferrous ion	**Ph**	Philadelphia chromosome
FISH	Fluorescence In-Situ Hybridization	**Pi3K**	Phosphatidylinositol-3-kinase
Fw	Foward	**PINK1**	PTEN-induced kinase 1 protein
G	Guanine	**PLT**	Platelet
GAB2	GRB2-Associated Binding protein 2	**PON**	Ponatinib
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**RAN**	Ras-like nuclear GTPase
GDP	Guanosine Diphosphate	**RIC**	Reduced-Intensity Conditioning
GRB2	Growth factor Receptor-Bound protein 2	**ROS**	Reactive Oxygen Species
GTP	Guanosine Triphosphate	**RPMI 1640**	Roswell Park Memorial Institute 1640
GVHD	Graft-versus-Host Disease	**RT**	Real Time
GvL	Graft-versus-Leukemia	**Rw**	Reverse
HIF1α	Hypoxia-Inducible Factor 1α	**SINE**	Selective Inhibitors of Nuclear Export
HLA	Human Leukocyte Antigens	**SLX**	Selinexor
HO	Heme Oxygenase	**SOS**	Son of Sevenless
HyU	Hydroxyurea	**STAT**	Signal Transducer and Activator of Transcription
IFN-α	Interferon α	**T**	Thymine
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1	**TKI**	Tyrosine Kinase Inhibitor
IL	Interleukin	**WBC**	White Blood Cell
ILD	Interstitial Lung Disease	**WHO**	World Health Organization

Abstract

Introduction. Chronic Myeloid Leukemia (CML) is a neoplasm characterized by the uncontrolled increase in the number of leukemic progenitors in bone marrow and in peripheral blood. The discovery of the pivotal role of BCR-ABL has allowed the development of tyrosine kinase inhibitor (TKI) drugs that have changed the history of this disease making it substantially controllable. Despite the impressive therapeutic successes, TKI-resistance, the persistence of Leukemia Stem Cells (LSC), and the high rate of relapse upon discontinuation of treatment call for the identification of new therapeutic strategies. Recent evidences demonstrated a crucial role of mitochondrial activity and Heme Oxygenase 1 (HO1) in the development of TKI-resistance. Selinexor (SLX) is an inhibitor of Exportin 1 recently approved for the treatment of refractory multiple myeloma. Its antitumor action seems to be linked to mitochondrial impairment and to the nuclear accumulation of tumor suppressors, but no data are currently available on CML.

Aim of this study is to evaluate the efficacy of SLX treatment alone and in combination with Dasatinib (DAS) on CML cell lines and the effects of treatment on mitochondrial activity and HO1 expression.

Material and methods. SLX and DAS were tested on human CML cell lines K562 and LAMA84. In order to evaluate mitochondrial mass and apoptotic population, flow cytometry was performed. Gene expression was investigated with Real Time Reverse Transcription Polymerase Chain Reaction. Western blot analysis and immunofluorescence were used in order to evaluate HO1 expression and its nuclear translocation. Unpaired T test or ANOVA, where appropriate, were used to analyze the data and a p value <0.05 was considered statistically significant.

Results. Data from flow cytometry show that 50nM and 100 nM SLX treatment did not affect LAMA 84 cell viability after 48 hours. On the contrary, a significative reduction in cell viability was observed using 1μM, 2μM and 5μM SLX (45±32%, 55±39% and 60±43% compared to untreated cells, respectively; $p<0.0001$). The dose dependent reduction on cell viability was also observed after 72h of treatment. No significant variation in cell viability were observed after 48h of treatment in K562 cell line. A significant increase in cell apoptosis was observed in K562 cells after 72h of treatment with 1μM, 2μM and 5μM SLX (17±12,5%, 36±26% and 68±48,2% compared to control, respectively; $p <0.0001$). A significant percentage of mitochondrial depolarized cells after 48h of 50nM and 100nM SLX was observed in LAMA 84 cell line (3±1,8% and 5±3,6%, respectively; $p<0.0001$). Mitochondrial depolarization increased massively with 1μM, 2μM and 5μM SLX compared to control of about 64±45%, 68±47% and 71±49%, respectively ($p<0.0001$). In K562 cell line, according to previous results, no increase in mitochondrial depolarization was observed after 48h treatment and the increase was evident after 72h of treatment in the groups 1μM, 2μM and 5μM SLX in a dose dependent manner (37%, 45%, and 70% respectively; $p<0.0001$).

In order to investigate the efficacy of the combination treatment DAS/SLX, K562 and LAMA84 cell lines were treated with DAS 2nM alone or in combination with SLX 500nM

and 1μM for 24h, 48h and 72h. In K562 cell line DAS alone was sufficient to significantly reduce cell viability after 48h and 72h (28±20,3% and 62±44,1% compared to untreated cells, respectively; $p<0.001$) and combination with SLX did not improve significatively the TKI-induced apoptosis, therefore the subsequent experiments took into account LAMA84 cell lines. DAS and SLX alone decreased cell viability of LAMA84 cells of 5±3,4% and 10±6,2%, respectively, compared to untreated cells. Their combination significantly increased apoptosis of 24±17,3% in DAS/500nM SLX group and 33±23,2% in DAS/1μM SLX group, compared to untreated cells ($p<0.0001$). DAS treatment caused a significant mitochondrial depolarization with a reduction of diethyloxacarbocyanine iodide mean fluorescence intensity (MFI) of 20±13,6% after 24h compared to untreated cells ($p<0.0001$). DAS/SLX treatment significantly increased the percentage of DAS-induced depolarized cells of 78±44,9%, compared to DAS alone treated cells ($p<0.0001$).

Since a strong depolarization of mitochondria could be accompanied by a drastic reduction of mitochondrial mass, mitochondria of CML cells were evaluated by using MitoTracker staining and flow cytometry in DAS alone, SLX alone and DAS/SLX groups. DAS/SLX increased the reduction of MitoTracker-MFI of 16±9.2% compared to DAS alone ($p<0.0001$). As expected from these data, an increased expression of PTEN-induced kinase 1 protein was observed compared to untreated cells ($p<0.05$). To respond to mitochondrial stress, CML cells treated with DAS alone promoted compensatory upregulation of mitochondrial dynamic-related genes Mitofusin (MFN) 1, MFN2, Optic Atrophy 1 (OPA) ($p<0.0001$, $p<0.001$ and $p<0.0001$ compared to untreated cells, respectively), as well as the oxidative phosphorylation related gene as Cytochrome B (CytB) and ATP synthase (ATPsynt) ($p<0.0001$ and $p<0.01$ compared to untreated cells, respectively).

On the contrary, DAS-induced expression of MFN1, MFN2, OPA, CytB and APTsynt expression was significantly downregulated in cells treated with DAS/SLX ($p<0.0001$, $p<0.01$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.001$, compared to DAS alone group, respectively), highlighting a close link between SLX treatment and mitochondrial impairment. Moreover, SLX alone significantly reduced all the aforementioned genes ($p<0.0001$, $p<0.01$, $p<0.0001$, $p<0.0001$ and $p<0.001$, compared to untreated cells).

Finally, levels of HO1 were evaluated in DAS, SLX and DAS/SLX groups. Western blot analysis showed that DAS treatment significantly increased HO1 levels compared to untreated cells ($p<0.0001$); on the contrary, both SLX alone and DAS/SLX decreased HO1 expression compared to DAS treated cells ($p<0.0001$). Immunofluorescence showed that DAS treatment significantly induced HO1 nuclear translocation, which, conversely, resulted decreased in DAS/SLX group.

Conclusion. SLX affected the viability of CML cells. DAS/SLX treatment increased the DAS-induced apoptosis in LAMA84 cell causing a strong mitochondrial depolarization associated to a significant decrease of mitochondrial mass. In the DAS-group, a compensatory upregulation of mitochondrial dynamic-related genes was observed. This overexpression was downregulated in the DAS/SLX group. DAS treatment significantly increased HO1 expression and its nuclear translocation, both resulted decreased in

DAS/SLX group. DAS/SLX combination seems to be effective in CML cells disrupting mitochondrial dynamics and mitochondrial fitness, which are potential active mechanisms of LSCs resistance. Further studies are needed to clarify whether combination therapy with TKI and SLX may be useful in the treatment of refractory forms of CML or in eradicating LSCs.

1. Introduction

1.1. Definition of Chronic Myeloid Leukemia.

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder whose hallmark is the increased proliferation of granulocytic cell line and subsequent marked production of cells that maintain the possibility to differentiate. Therefore, an increased number of granulocytes and their precursors can be observed in the peripheral blood [1].

In 1960, Nowell and Hungerford discovered that the neoplastic clone had a chromosomal anomaly characterized by a reciprocal translocation t(9;22) (q34.1;q11.2) [2]. The chromosome took the name of the city in which it was discovered and today it is commonly known as "Philadelphia chromosome" (Ph). In the Ph chromosome the BCR-ABL1 fusion gene is created and the presence of Ph chromosome and BCR-ABL1 clearly differentiates CML from myeloproliferative disorders (Fig. 1) [3].

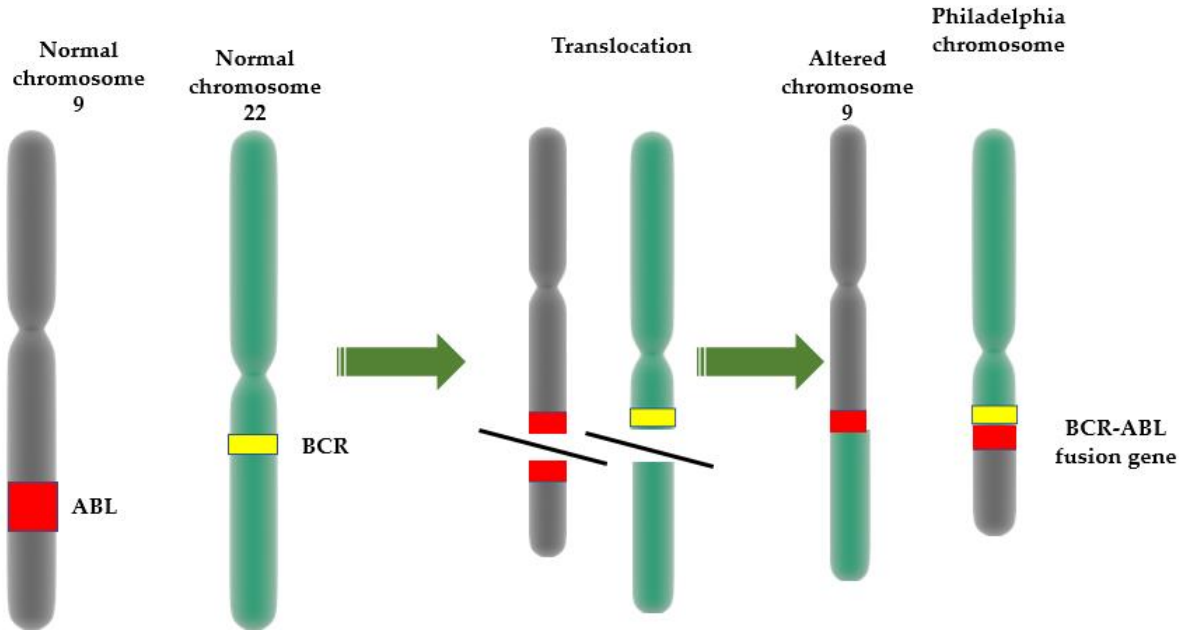

Fig. 1. Genesis of Philadelphia chromosome. The reciprocal translocation between chromosome 9 and 22 generates the Philadelphia chromosome with BCR-ABL1 fusion gene.

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1.2. Epidemiology of CML.

CML represents 15% of the new diagnosis of leukemia [4]. The data available from the European registries show an incidence of 0.7-1/100000 with a slightly higher involvement for male, as shown by a male/female ratio 1.2-1.7 [5]. The incidence increases with the age, and the mean age at diagnosis is 57-60 years.

The pediatric form is extremely rare, with an incidence of 0.7/1000000 in children under 14 years old and 1.2/1000000 in adolescents [6].

No increase in the incidence of CML has been observed since 1970 [7-8], contrary to what happens with regard to the prevalence which is markedly influenced by the advent of the tyrosine kinase inhibitors (TKIs), responsible of the dramatic increase in survival and,

consequently, in the prevalence. In France, an increase in the prevalence of CML has been observed, from 3/100000 inhabitants in the 1960 to 17 in the 2016. From these data, a mathematical model has been extrapolated that predicts a prevalence of 30/100000 by 2040 [9].

1.3. Pathophysiology of CML.

Experiments on mouse models allow us to state beyond any reasonable doubt that the presence of the BCR-ABL1 oncogene is decisive in triggering the processes that will give rise to CML and it is probably sufficient to initiate the chronic phase of the disease [10].

In normal conditions, ABL moves from the nucleus to the cytoplasm. When the BCR-ABL1 fusion gene is formed, it loses this ability and it carries out a predominantly cytoplasmatic action on a plethora of pathways involved in the neoplastic genesis [11]. The binding with BCR results in an activation of ABL which plays the role of tyrosine kinase. Studies on murine models showed that in BCR, the NH_2-terminal coiled-coil domain was essential and sufficient to activate ABL to induce the neoplastic evolution. Furthermore, the phosphorylated tyrosine 177 in the Grb2-binding site is required for efficient induction of CML [12]. Adenosine triphosphate (ATP) provides the phosphate groups necessary to perpetuate the phosphorylation of the proteins. Several pathways have been shown to be triggered in this leukemogenesis mechanism. The phosphorylation of tyrosine 177 creates a high-affinity binding site for Growth factor Receptor-Bound protein 2 (GRB2) which binds BRC-ABL to SH2 domain and Son of Sevenless (SOS) and GRB2-Associated Binding protein 2 (GAB2) to SH3 domain [13]. The binding with SOS and GAB2 activates the Ras-

Mitogen Activated Protein Kinase pathway which promotes cell survival and proliferation directly or in two indirectly mechanisms: the activation of Phosphatidylinositol-3-kinase/Protein kinase B/mammalian target of rapamycin (Pi3k/AKT/mTOR) pathway, which in turn stimulates cell survival and proliferation, and the antagonism with JUNB pathway, which, on the contrary provides an inhibition to cell survival and proliferation [14]. Pi3K/AKT/mTOR is also connected to autophagy mechanism. Autophagy is a mechanism of defense in which cytoplasmatic constituents, protein aggregates or damaged mitochondria are degraded and recycled in order to avoid the development of an environment that can favor the development of neoplasm, such as the accumulation of reactive oxygen species (ROS) or damage to DNA. Pi3K/AKT/mTOR is shown to inhibit autophagy in several conditions, including CML [15]. Mitochondria are one of the most important sources of endogenous ROS in normal conditions, as products of mitochondrial respiration. Physiologically, a group of enzyme prevents the accumulation of ROS. It is widely known in literature that ROS accumulation is related to the development of cancer due to damage to DNA, proteins and lipids [16]. The nature of mitochondrial DNA makes itself particularly prone to developing DNA damage which results in a reduction in oxidative phosphorylation, but in an increase of ROS [17]. Under normal conditions, the intrinsic pathway of apoptosis is activated where the pro-apoptotic proteins of B-Cell Limphoma 2 (BCL2) family, BCL2-Associated X protein (BAX) and BCL2 Antagonist or Killer (BAK) form a channel on the surface of the mitochondrial membrane which makes it permeable to cytochrome C, hence the formation of the apoptosome, cascade of caspases and apoptosis. In CML, this pathway is antagonized by the anti-apoptotic proteins of

BCL2 family which has been shown to play a crucial role in the survival of leukemia cells and leukemia stem cells [18]. Several literature studies have observed how leukemic stem cells are able to escape the first-line treatment with TKI using the increased expression of mitochondrial respiration. They have also observed that combination therapy with action on mitochondrial respiration may be able to achieve the eradication of cancer stem cells [19]. In parallel, the leukemogenic stimulus appears to use the Janus Kinase (JAK) 1 and JAK2 pathway on Signal Transducer and Activator of Transcription (STAT) 3 and STAT5 at the beginning and then maintain itself on STAT5 via tyrosine 693 phosphorylation in a JAK-independent manner [20-21]. STAT5 exerts its pro-survival action promoting the expression of BCL2 family proteins [22].

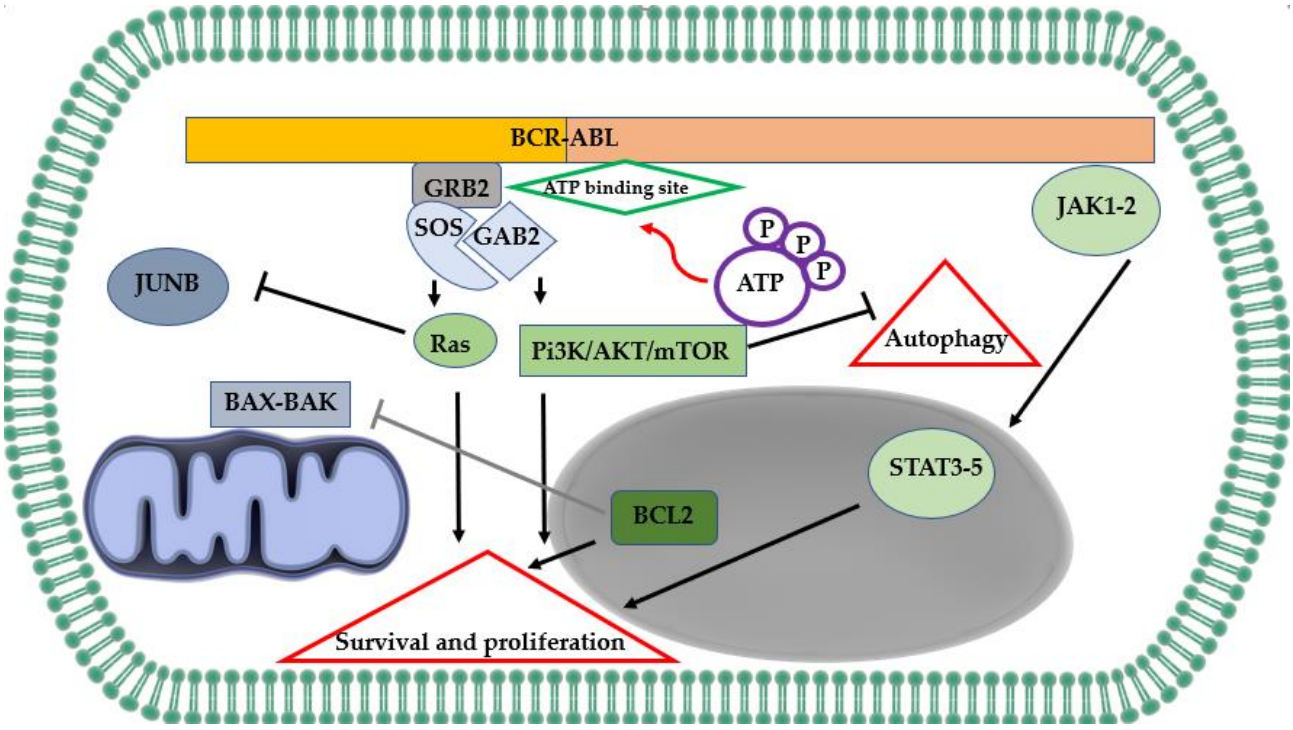

Fig. 2. Pathways of BCR-ABL. A simplified and incomplete graphical representation of the pathways triggered by BCR-ABL.

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1.4. Clinical presentation and stages of CML.

CML is completely asymptomatic in approximately 50% of the patients and the suspicion arises from the occasional finding during routine blood tests. Some interesting data on the clinical presentation derive from a paper published on a case series derived from an economically disadvantaged country that does non implement screening policies and, therefore, all patients refer to the local hematological center as symptomatic. In these patients, the most common clinical presentation was splenomegaly (91.2%) and related symptoms, followed by abdominal swelling (79.4%), anemia and related symptoms (61.8%), fever (50%), weight loss (50%), general malaise (41.2%), infection (8.8%), and hepatomegaly (8.2%) [23]. In some cases, a bleeding due to platelet disfunction was described. These cases are more common in patients with severe thrombocytosis. Symptoms of hyperviscosity (priapism, neurological features, visual disturbances, fatigue, etc.) are seen when white blood cell (WBC) count exceeds 250000/μL [24].

The clinical presentation of CML is divided into 3 phases. At the diagnosis, 85% of the patients are in the so-called "chronic phase" (CP). The patient is usually asymptomatic or with non-specific symptoms such as fatigue or general malaise. Leukocytosis is evident in the peripheral blood smear, due to the overproduction of granulocytes at various stage of maturation. Less than 2% of WBC are blasts. An increase in eosinophils and basophils and a slight increase in monocytes is commonly observed. Platelets (PLT) are usually in the normal range of increased, decreased in a minority of patients. Similarly, at this phase, the bone marrow biopsy shows marked granulocytic proliferation, blasts <5%, decreased

erythroid precursors and megakaryocytes variable (increased in about 50% of patients) [25]. In its natural history, the disease can evolve towards an accelerated phase (AP), where the number of blasts reaches up to 19%, then a blast phase (BP), potentially fatal, characterized by more than 20% blasts in peripheral blood and/or in the bone marrow with the possibility of extramedullary proliferation of blasts [26]. In Table 1, the main differences between the phases encoded by World Health Organization (WHO) and European LeukemiaNet (ELN).

Table 1: Characteristics of the chronic, accelerated, and blast phase in CML encoded by WHO [27] and ELN [28].

Phase	WHO	ELN
Chronic	No criteria for accelerated or blast phase are met	
Accelerated	Blast cells [A] 10-19%. Basophils [A] >20%. PLTs <100 x 10^9/L unrelated to therapy or > 1000 x 10^9/L uncontrolled by therapy. Clonal chromosomal abnormalities in Ph+ cells on treatment. WBC >10 x 10^9/L persisting or increasing unresponsive to therapy. Splenomegaly persisting or increasing unresponsive to therapy.	Blast cells [A] 15-29%. Basophils [A] >20%. PLTs <100 x 10^9/L unrelated to therapy. Clonal chromosomal abnormalities in Ph+ cells on treatment.
Blast	Blast cells [A] ≥20%. Extramedullary involvement [B].	Blast cells [A] ≥30%. Extramedullary involvement.

Definitions
A: in peripheral blood or in bone marrow.
B: lymph nodes, skin, Central Nervous System, bone, lung.
PLTs: Platelets; WBC: white blood cells.

1.5. Risk stratification in CML.

Several scores were created to stratify the risk in course of CML. The first of these is the Sokal score [29]. The score takes into account the age of the patient, the size of the spleen, the PLT count and the peripheral blood blasts with the formula:

$$\exp [0.0116 \times (\text{age in years} - 43.4)] + 0.0345 \times (\text{spleen size in cm} - 7.51) + 0.188 \times [(\text{platelets in } 10^9/L/700)^2 - 0.563) + [0.0887 \times (\% \text{ of blasts} - 2.1)]$$ [30].

According to this formula, a point below 0.8 is considered as low risk, 0.8-1.2 as intermediate and over 1.2 as high risk.

In 1998, Hasford created a new score specifically focused on patients treated with interferon alpha where, in addition to the details considered by Sokal, the count of eosinophils and basophils in the peripheral blood were added [31]. This score uses the following formula [32]:

$$[0.6666 \times \text{age (0 if <50; 1 if >50)}] + (0.042 \times \text{spleen size in cm}) + (0.0584 \times \% \text{ of blasts}) + (0.0413 \times \% \text{ of eosinophils}) + [0.2039 \times \% \text{ basophils (0 if <3\%; 1 if >3\%)}] + [1.0956 \times \text{platelet count (0 if <1500 x } 10^9/L\text{; 1 if >1500 x } 10^9/L)] \times 1000.$$

In this case, a score ≤780 is considered as low risk, 781-1480 as intermediate and ≥1481 as high risk.

In the imatinib era, the EUTOS score was created [33]. This score takes into account spleen size and percentual of basophils in peripheral blood, recognizing a low risk when score <87 and a high risk when ≥87. The formula of EUTOS score is

(7 x % of basophils) + (4 x cm of spleen) [34].

These 3 scores are used in a substantially interchangeable way. A recent study observed the following concordance rate: Sokal vs Hasford 53%, Sokal vs EUTOS 64%, Hasford vs EUTOS 98% [35].

More recently, the ELTS score was created, showing a better performance than the previous 3 in defining the long-term prognosis of patients with CML [36]. This score considers the age in completed years, spleen size in cm below costal margin, percentual of blasts in peripheral blood and platelet count in 10^9/L with the formula

$$[0.0025 \times (age/10)^3] + (0.0615 \times \text{spleen size below costal margin}) + (0.1052 \times \% \text{ of blasts}) + [0.4104 \times (\text{platelet count}/1000)^{-0.5}].$$

An ELTS score ≤1.568 is considered as low risk, 1.5681-2.2185 as intermediate risk, >2.2185 as high risk [37].

1.6. Diagnosis and baseline work-up in CML.

Clinical suspicion is placed on the basis of the suggestive abnormalities to the blood count. The presence of Ph chromosome must be determinated with Real Time-Polymerase Chain Reaction (RT-PCR) on peripheral blood. The bone marrow analysis for cytology and karyotype should be performed in order to avoid the underestimation of the disease phase. Mutation analysis at the baseline is limited to AP and BP [38].

According to the collected data, bone marrow aspiration should be performed for morphometry and cytogenetics. Morphometry is crucial to define the phase of the disease.

Cytogenetics is performed in order to identify Ph chromosome with Chromosome banding analysis of Giemsa-stained metaphase from bone marrow cells.

Bone marrow biopsy can identify bone marrow fibrosis, which has an impact on the prognosis, or eventual nests of blasts lost with the aspirate.

Qualitative reverse transcriptase PCR on peripheral blood is mandatory to state the BCR-ABL1 transcripts that will be searched in the follow up to establish the response to treatment, in order to avoid false negatives linked to atypical transcripts that cannot be recognized by routine probes.

Fluorescence in-situ hybridization (FISH) should be performed in case molecular examination shows BCR-ABL1, but cytogenetics is negative for Ph chromosome. FISH is useful in monitoring patients with atypical transcripts.

Then, an evaluation of liver and spleen size, electrocardiogram and standard biochemical profile, including B-hepatitis serology, are recommended.

Finally, the risk should be stratified with the previously presented scores [39].

1.7. Definition of molecular response.

Molecular response is assessed according to International Scale as the ratio between BCR-ABL1 transcripts and ABL1 transcripts, or other accepted control transcripts as beta glucuronidase. These data are expressed as BCR-ABL1% on a logarithmic scale where 1%, 0.1%, 0.01%, 0.0032% and 0.001% correspond to a decrease of 2, 3, 4, 4.5, and 5 logs respectively. Hence the following definitions:

- Complete cytogenetic remission, CCyR. BCR-ABL1 ≤1%.
- Major molecular response, MMR or MR^3. BCR-ABL1 transcripts level BCR-ABL1 ≤0.1%.
- MR^4. BCR-ABL1 transcripts level BCR-ABL1 ≤0.01% or undetectable disease in cDNA with >10000 ABL1 transcripts.
- $MR^{4.5}$. BCR-ABL1 transcripts level BCR-ABL1 ≤0.0032% or undetectable disease in cDNA with >32000 ABL1 transcripts.
- MR^5. BCR-ABL1 transcripts level BCR-ABL1 ≤0.0001% or undetectable disease in cDNA with >10000 ABL1 transcripts [39].

1.8. Timing and goals of the treatment.

From the start of the treatment to the complete hematologic response, blood cell counts should be performed every 2 weeks. BCR-ABL1% should be performed with PCR on peripheral blood cells every 3 months, even when MMR is achieved. The ELN 2020 recommendations highlight the milestones for treating CML where "optimal response" indicates the good response to the treatment, "warning" urges a re-evaluation of the therapy on the basis of the patient's characteristics, and "failure" makes the change of the treatment mandatory. In the table below, the re-elaboration of the milestones proposed by ELN 2020 on the response in BCR-ABL1% [39].

Table 2. Milestones of treatment of CML expressed in BCR-ABL1%			
Entry level: consider as a warning the presence of high-risk additional chromosome abnormalities and high-risk to the prognostic scores.			
Time	**Optimal**	**Warning**	**Failure**
3 months	≤10%	>10%	>10% if confirmed within 1-3 months
6 months	≤1%	>1-10%	>10%
12 months	≤0.1%	>0.1-1%	>1%
Any time	≤0.1%	>0.1-1%, loss of MMR	>1%, resistance mutations, high-risk atypical chromosome abnormalities

1.9. Treatment of CML before the TKIs era.

The first treatment used against CML was the Fowler's solution, a 1% solution of arsenic trioxide, in 1865 [40].

In 1954, busulfan (BU) was developed. BU is a chemotherapy with a cytotoxic action. Its action is carried out by inserting alkyl groups in the DNA causing errors and breaking of the strands. This results in a direct cytotoxic action involving the neoplastic cells. To date, its role is limited to conventional conditioning treatment prior to hematopoietic progenitor cell transplantation in association with cyclophosphamide. BU can be administrated orally or intravenously, but intravenous administration is preferred. The dose commonly used is 0.8 mg/kg every 6 hours for 4 days. Side effects are common to all alkylating agents and include mucositis, alopecia, pancytopenia, risk of malignancy. Hepatic veno-occlusive disease is a commonly life-threatening side effect associated to busulfan. Typically, it occurs between the 10th and 20th day post transplantation, but it is possible until the 75th day. Hepatic veno-occlusive disease is characterized by jaundice, abdominal pain, liver tenderness, fluid accumulation, elevated hepatic enzyme in serum with a fatality rate

among 50%. Other side effects related to busulfan are interstitial lung disease (ILD) and seizure, generally tonic-clonic due to its rapid distribution into the cerebrospinal fluid. In some cases, a premedication with benzodiazepine is recommended [41].

Hydroxyurea (HyU) was available since 1964 and it was officially introduced into CML therapy in 1972 [42]. The primary target of HyU is ribonucleotide reductase, an enzyme involved in the catalyzing of the reduction of ribonucleoside diphosphates to their corresponding deoxyribonucleotides necessary in DNA replication and repair processes [43]. HyU inhibits the enzyme on the M2 unit and this results in damage to DNA repair and cell death in the S phase of the DNA replications. The dose of 40 mg/kg in chronic phase of CML showed a rapid reduction of WBC and platelets. When WBCs drop below 20000, the dosage is titrated to maintain a WBC count between 5000 and 15000. The main side effect is related to bone marrow depression with consequent cytopenia which can involve all cell lines. Other side effects are skin ulcers and rashes, impaired kidney function, liver enzyme movement, fever, neurological symptoms, and lupus-like syndrome. These reactions appear to resolve upon temporary withdrawal of the drug. Rarely, the use of HyU has been linked to the onset of new hematological malignancies or ILD. Comparative studies have demonstrated the superiority of hydroxyurea vs busulfan in terms of response, survival and safety [44]. Based on these studies, HyU became the first line of treatment, reserving busulfan for non-responders or intolerants to hydroxyurea. It is important to note, however, that neither hydroxyurea nor busulfan have been shown to be useful in avoiding progression to blast crisis in CML [45].

In 1982, the first cases of heterologous bone marrow transplantation (BMT) in CML were reported [46]. This was the first therapy capable to induce a state of Ph-negativity and today is considered the only therapy potentially able to recover from CML. Over time, BMT became the therapy of choice in the treatment of young people and in 1990 CML was the most common indication for bone marrow transplantation worldwide [47]. More commonly, the transplant was performed in subjects in the CP, but was also considered in the AP and BP, despite significantly lower results in terms of relapse and survival [48]. The protocol generally included a myeloablative conditioning with Cyclophosphamide and Total Body Irradiation. The best performance in terms of 5-years survival (74%) was observed in patients with age <50 years who received a transplant from Human Leukocyte Antigens (HLA)-matched donor within 1 year after the diagnosis and with the prophylactic use of fluconazole and ganciclovir. Overall data showed a relapse rate of 10% in 5 years, non-relapse mortality rate of 44% and overall survival rate of 57%. The majority of deaths occurred in the first year after transplant, due to treatment failure or the toxicity of myeloablative therapy. Particularly, acute Graft-versus-Host Disease (GVHD) grade II-IV was observed in 77% of patients and chronic extensive GVHD in 67% [49]. Subsequent studies, conducted on patients transplanted in CP with at least 5 year of post-transplant survival, showed an overall survival of 94% at 10 years and 87% at 15 years, with a 2.5 higher risk of death compared with general population due to organ failure, infection, disease relapse (4% at 10 years and 7% at 15 years) and secondary malignancy. The transplanted subjects who survived beyond 15 years showed a survival comparable to the general population [50]. The toxicity of myeloablation has opened several debates about

the advisability of BMT: firstly, because young subjects eligible for BMT were a minority of subjects diagnosed with CML, and moreover, over time treatments with moderate efficacy emerged such as Interferon α (IFN-α) or IFN plus cytarabine which, although not curative, certainly showed a greater safety than BMT. Several strategies have been studied to improve transplant safety [51]. *In vitro* T-cell depleted transplant showed significantly higher safety, especially in preventing GVHD, but patients who developed severe acute GVHD showed the same case-fatality of those who performed a non-T-cell depleted BMT. Even with regard to ILD, there were no differences between T-cell depleted and non-T-cell depleted marrow either in terms of incidence or in terms of case-fatality. Above all, subjects who performed T-cell depleted BMT showed a higher incidence of graft failure and the disease relapse in 3 years in nearly 50% of cases, versus 9% of non-T cell depleted marrow, probably due to the absence of graft-versus-leukemia (GvL) [52]. Another strategy was characterized by the use of reduced-intensity conditioning (RIC). RIC correlated with greater security that allowed access to transplantation even for older subjects, but considerable possibilities of failure and transplant related mortality [53]. An alternative approach is represented by autologous transplant of stem cells obtained from the patient during CCyR. This approach was influenced by the contamination of stem cells with malignant clones. It showed a good safety profile and it had the potential to be curative, although the majority of surviving subjects still showed persistence of disease [54]. In cases of relapse like these, Donor Lymphocyte Infusion (DLI) can produce durable remission [55]. These data, in association with the recognized importance of GvL in disease control, have pointed toward the use of regimens with lower condition dose [56].

In the TKI-era, BMT is limited to patients (i) in AP or in BP; (ii) failing or intolerant to TKI; (iii) with TKI-resistant mutations such as T315I mutation. There does not appear to be any significant differences in survival between unrelated donor and HLA-matched sibling donor, but advanced stages of disease are still related to a worse prognosis. Considering these patients are often elderly and polytreated, the RIC regime is commonly favored with *in vivo* T-cell depletion. After BMI, in order to treat cytogenetic or molecular relapse, TKI, low dose DLI or a combination of the two is used [51].

As mentioned above, IFN-α emerged as a preferable alternative to BMT in a large number of patients. IFN-α has been available in CML since the second half of the 1980s. This treatment has been shown to be able to determine a complete cytogenetic and molecular response in a small, but statistically significant part of treated patients. In a prospective study, it was observed that 27% of the patients achieved CCyR. In this study, among CCyR patients, the 10-year survival rate was 9% and, among surviving patients, 88% were treatment-free for a median of 50 months with a range 11-139 [57]. A meta-analysis of 7 different trials demonstrated superiority of IFN-α with respect to treatment with BU or HyU [58]. The treatment starts with 3 million units administrated intramuscularly or subcutaneously (high doses can be administered intravenously with slow infusion) with a gradual increase up to 9 million units, on the basis of tolerability, and, once a response on WBC is achieved, the dosage can be administered 3 times per week. A limitation to the treatment is represented by side effects. A flu-like manifestation is described in almost all patients at the start of the treatment, with very marked asthenia that, in some cases, forced the drug to be discontinued. The possibility of hypotensive crises and cardiac arrhythmias

raises serious concerns about the advisability of treating patients with history of cardiovascular disease, particularly myocardial infarction. In rare cases, pulmonary arterial hypertension or central nervous system adverse events have been reported.

1.10. The TKIs era.

TKIs have dramatically changed the natural history of CML by marking a dividing line between a pre-TKI era and the current treatment of CML.

Imatinib (IMA) was the first TKI approved for the treatment of CML. IMA exerts an ATP-mimetic action and binds to the ATP binding site. This prevents ATP from providing the phosphate groups necessary for the phosphorylation which results in inhibition of the proliferation and apoptosis of BCR-ABL positive cells [40,59] (Fig. 3). Available since 2001, IMA was initially limited to patients who had failed IFN-α treatment. Subsequently, a comparative study between IMA vs IFN-α + cytarabine demonstrated beyond reasonable doubt the superiority of IMA both in terms of efficacy and safety [60]. This allowed for the approval of treatment in the new diagnoses of CML in 2003 at dosage of 400 mg/die orally. A dose of 800 mg/die was used in patients in AP, but current guidelines recommend using a second generation TKI (2gTKI) in these cases [39].

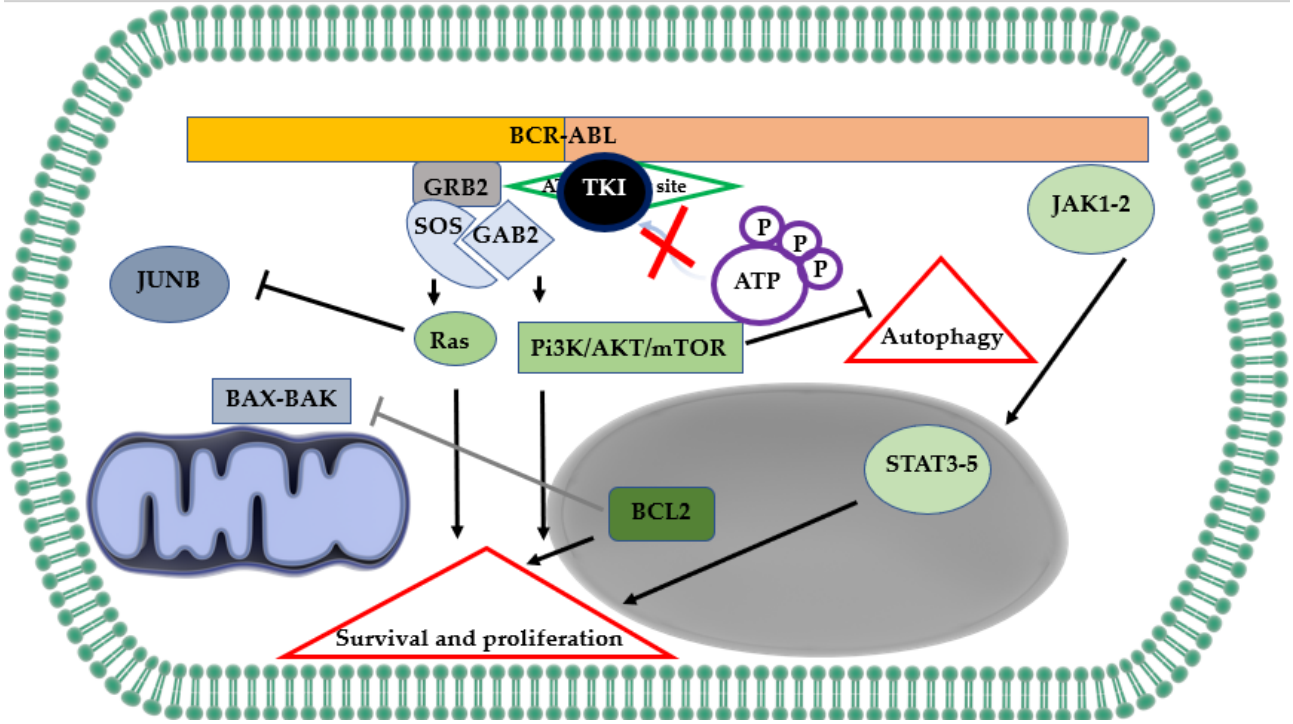

Fig. 3. Site of action of TKIs in the presented pathogenetic scheme.

* * *

A 10-years randomized prospective study showed in the IMA-group a 10-years overall survival of 82%, a 10-years progression-free survival of 80% and a 10-years relative survival of 92%. Patients defined as responders showed a normal life expectancy [61].

IMA is generally well tolerated. Apart from the cytopenia, which can involve all blood line, the most common adverse events are skin rash, joint pain, anorexia, muscle cramps, insomnia, fatigue, although patients with low cardiac ejection fraction or renal failure should be carefully monitored during treatment for eventual organ toxicity [62].

In 2004, Dasatinib (DAS) was available. DAS is a 2gTKI who proved efficacy also against BCR-ABL1 resistance mutation, such as Y253H, E255V/K, F359V/I/C [39]. Compared to IMA in treatment-naïve patients, DAS was superior to IMA in terms of MMR, MR^4, $MR^{4.5}$,

progression-free survival, overall survival and rate of transformation to AP or BP. Up to 37% of subjects in DAS-group experience recurrent pleural effusion and it is important to note that a concomitant pleuro-pulmonary or pericardial disease contraindicates treatment. Other adverse events observed were no different from what was observed in the IMA-group [63]. DAS is commonly used at the dosage of 100 mg daily or 70 mg twice daily in the AP/BP [39].

In the same period of DAS, Nilotinib (NIL) was developed. NIL is the second 2gTKI who proved efficacy against BCR-ABL1 resistance mutation such as F317L/V/I/C, T315A, V299L [39]. A large study with follow up of 10 years demonstrated superiority of NIL compared to IMA in terms of MMR, MR^4, $MR^{4.5}$ and rate of transformation to AP or BL, although not regarding overall survival. On the other hand, the safety profile is clearly in favor of IMA, having described adverse cardiovascular events at 10 years in 20% of subjects in the NIL-group and 5% in the IMA-group [64]. NIL is used at the dosage of 300 mg twice daily in treatment-naïve patients and 400 mg twice daily as successive lines of treatment. Dosages higher than 300 mg twice daily correlate with higher incidence of cardiovascular events. A history of cardiovascular events or peripheral arterio-occlusive disease are strong contraindications for NIL as first line treatment. Arterial hypertension, diabetes mellitus and dyslipidemia are relative contraindications. Pancreatitis was described in 5% of patients in NIL-group, so a previous pancreatitis is considered a contraindication to treatment [39].

From 2006, Bosutinib (BOS) was available. BOS is the third 2gTKI and it responds to the need for effective action against BCR-ABL1 resistance mutation (F317L/V/I/C, T315A, Y253H, E255V/K, F359V/I/C) with a good safety profile [39]. BOS is used at 400 mg daily in treatment-naïve patients and at 500 mg in successive lines of treatment. It proved to be superior to IMA in terms of MMR and CCyR at 12 months. The main adverse events described in the BOS-group were mainly diarrhea involving approximately 30% of the enrolled subjects and a movement of transaminase, especially in the first weeks of treatment [65]. To date, there are no absolute contraindication in the treatment of CML with BOS.

From 2017, Ponatinib (PON) was available. PON is a third generation TKI which stands as the most effective of all TKIs. It is effective against all the BCR-ABL1 resistance mutations where DAS or BOS, NIL, and NIL or BOS proved efficacy. It is also effective against the BCR-ABL1^{T315I} mutation at the dosage of 45 mg daily [66]. PON proved to reach the main efficacy endpoints also in third or fourth line of treatment and it is recommended to prefer it as a second line therapy instead of another 2gTKI, if no contraindications coexist [39,67]. The main contraindication is related to cardiovascular adverse events which involve 30% of subjects. The correction of modifiable cardiovascular risk factors is mandatory. The cardiovascular toxicity is dose-related. The dosage of 45 mg daily should be limited to BCR-ABL1^{T315I} subjects, other patients should be treated with 30 mg daily and the dosage should be reduced to 15 mg daily in subjects who achieved MMR or CCyR. There are no reliable data on the usefulness of preventive therapy with acetyl salicylic acid or anticoagulants [39,68].

For completeness, it should be noted that, only in South Korea, is available in the first line of treatment the 2gTKI Radotinib, a drug extremely similar to NIL, with the same efficacy and safety profile [39,69].

1.11. New insights in pathogenesis.

In recent years, several new insights have emerged regarding the pathogenesis of CML. Some of these appear to play a crucial role in the genesis of CML and in the resistance to treatment.

1.11.1. The nuclear transport.

The nuclear transport of proteins is arousing increasing interest in different types of neoplasia, particularly in hematological ones.

A characteristic of eukaryotic cell is the presence of the nuclear membrane which represents a barrier between the nuclear transcription of the proteins and their cytoplasmatic translation. An extremely rigorous control of the transport of the proteins across the nuclear membrane has a pivotal role in a large number of physiological mechanisms. This pathway is mediated by nuclear transport receptors included in the karyopherin family of which the importin (IPO) α and IPOβ are part, the latter one subdivided in IPOs and exportins (XPO), with diametrically opposite roles [70]. The main components of this mechanisms are (i) the Nuclear Pore Complex (NPC), a complex formed by nucleoporins; (ii) the nuclear transport receptors; (iii) Ras-like nuclear (RAN) Guanosin Triphosfatase (GTPase) that provide energy for the transport.

IPOβ recognizes the cargo and binds to it, and it can carry it autonomously. Alternatively, IPOα binds to the Nuclear Localizing Signal (NLS) of the cargo facilitating the transport through NPC forming the IPOβ-α-NLS-cargo complex [71]. Once the load is released, RAN-GTP provides the energy needed to export the IPOβ-α complex that is no longer useful. Some of the imported cargoes have already been shown to have a pathogenetic role, such as Retinoic Acid Receptor α (involved in the promotion of proliferation and inhibition of differentiation in acute promyelocytic leukemia), p65 (promoting anti-apoptotic effects in multiple myeloma, chronic lymphocytic leukemia and nonHodgkin lymphoma), Janus Kinase 1 (involved in the cellular proliferation during diffuse large B-cell lymphoma), and β-catenin (with a role in regulation of stemness, differentiation and cellular proliferation in course of non-Hodgkin lymphoma and acute leukemia) [70]. The mechanisms of export are similar. XPO1, the best-known XPO, recognizes the Nuclear Export Sequence (NES) of the cargo and binds to it. Again, Ras-GTP provides the energy needed to export the complex through NPC (Fig. 4).

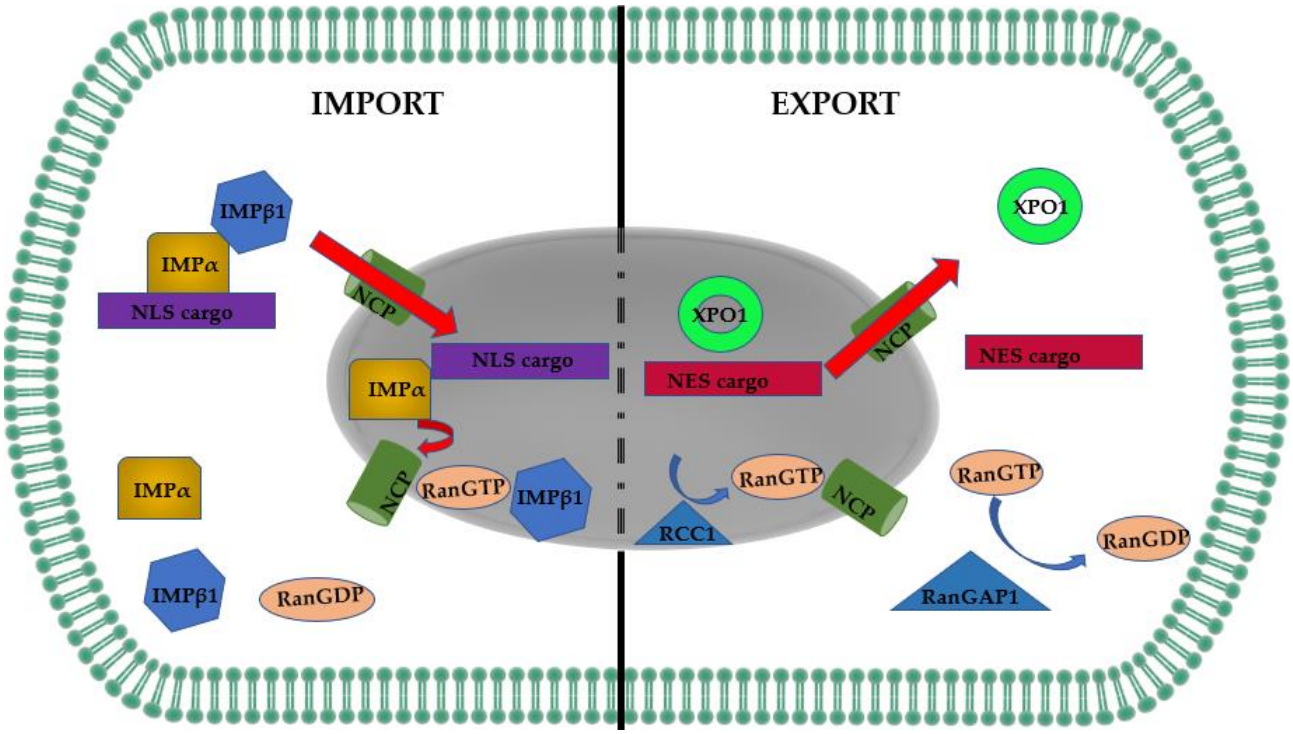

Fig. 4. Simplified graphic representation of the mechanisms of import and export through the nuclear membrane.

* * *

XPO1 can export a plethora of proteins with tumor-suppressor activity (i.e., p53, p21, p27, etc.) and proto-oncogene (i.e., BCR-ABL). High levels of XPO1 were observed in many tumors, both solid and hematological, and correlated with a worse prognosis [72]. The mechanisms inducing high expression of XPO1 are not yet known. We know that, for example, BCR-ABL and p53 have opposite effects: upregulation the first and downregulation the second. What seems to happen is that the interrelation between XPO1 and oncogenes may increase the activity of XPO1, triggering a stimulus to its greater expression [70].

In CML, XPO1 has been related to treatment resistance mechanisms and leukemia stem cells (LSC) survival [73]. To date, in fact, only a small minority of patients with CML

treated with TKIs achieve a drug-free remission. Most patients remain committed to chronic treatment, and this suggests the existence of pathogenic pathways BCR-ABL-independent. It is known in the literature that, in CML, only LSCs with a more mature phenotype undergo apoptosis during treatment with TKIs, but most LSCs are insensitive to treatment, despite inhibition of BCR-ABL activity [74]. Some BCR-ABL-independent pathways that promote LSC survival and resistance to TKIs treatment have been identified, such as the activation of the wnt-β catenin and the inhibition of the oncosuppressive Protein Phosphatase 2A. It has not yet been definitively clarified which are the determinant cargoes in the pathogenetic role of XPO1, but the effects observed in the *in vitro* studies on inhibition of XPO1 candidate it as a promising therapeutic target [75].

1.11.2. Heme Oxygenase 1.

Heme Oxygenases (HOs) are enzymes deputed to the catalyzation of the catabolism of heme. HOs work with cytochrome P450, nicotinamide adenine dinucleotide phosphate (NADPH) and three molecules of diatomic oxygen (O_2) per molecule of heme to obtain the catalyzation of the oxidative cleavage of heme to free iron (Fe^{2+}), carbon monoxide (CO), and biliverdin, this converted to bilirubin in cytosol by biliverdin reductase [76]. Previously identified as 3 isoforms, HO3 was subsequently believed to be a processed pseudogene derived from HO2 transcripts [77]. HO1 is inducible and represents the predominant isoform in liver and spleen, HO2 is constitutive and present in brain and

testis, both HO-isoform are anchored to the outer membrane of the endoplasmic reticulum [78].

The products of heme catabolism have cytoprotective effects. Fe^{2+} stimulates the synthesis of ferritin heavy chain and the activation of Fe-ATPase, allowing the efflux of cytosolic iron, causing the reduction of the intracellular content of Fe^{2+} and the consequent ROS generation from the Fenton reaction [79]. Among the products of heme catabolism, CO seems to be the most important in the regulation of immune system. CO reduces the production of proinflammatory cytokines, such as Interleukin (IL) 1, IL6 and Tumor Necrosis Factor α and increases the production of anti-inflammatory ones, such as IL10. CO also shows an antiapoptotic role by modulating Fas/Fas ligand and BCL2 family proteins [80]. Immediately after the production of biliverdin, biliverdin reductase catalyzes the conversion to bilirubin that has a potent anti-inflammatory and antioxidant action, acting as a scavenger of ROS and with an antiapoptotic effect [81-82].

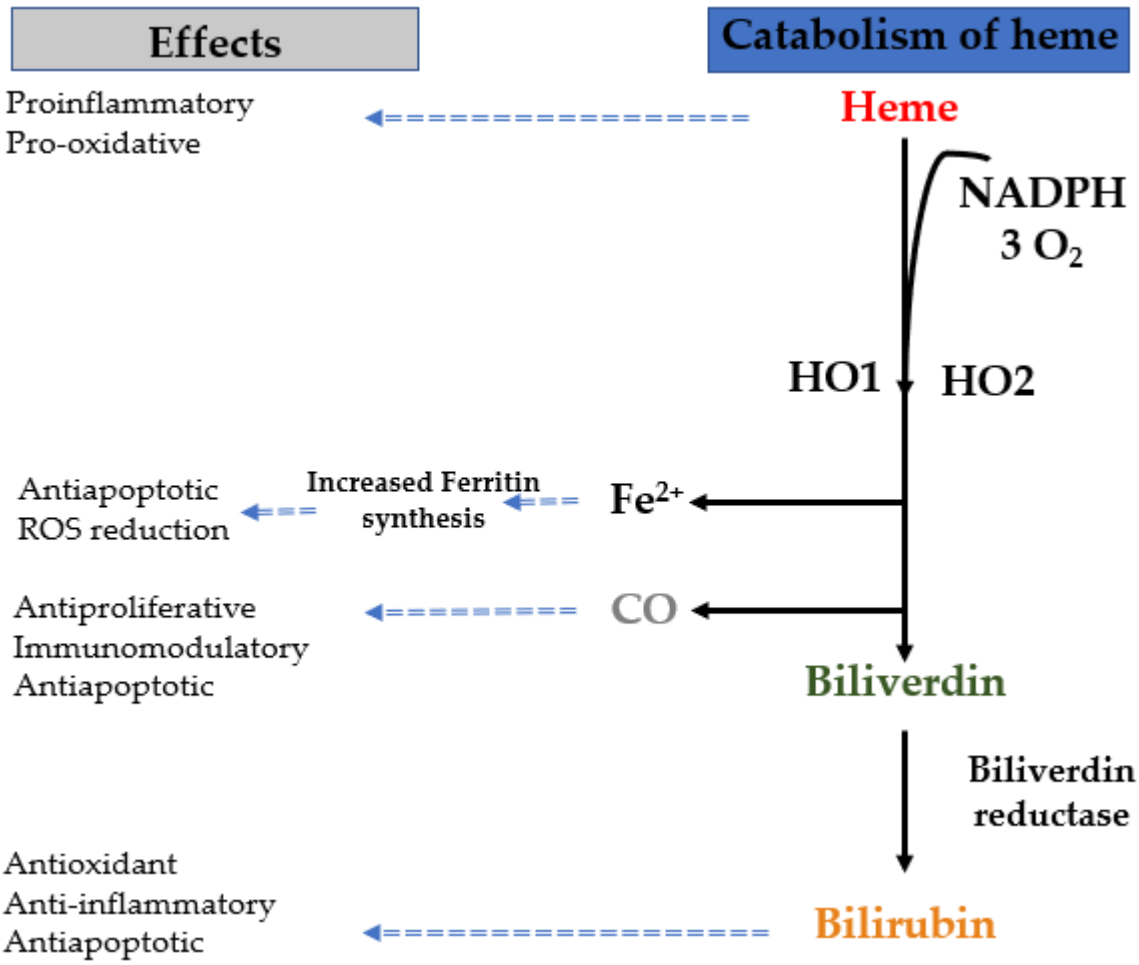

Fig. 5. Schematic presentation of the products of heme catabolism and their effects.

* * *

Oxidative stress highly promotes the expression of HO1 through the activation of several pathways, but the most important role is recognized in the Nuclear factor erythroid 2-related factor (Nrf2) [83]. In normal condition, heme levels are coordinated by a feedback mechanism which involves the proteins of the Broad complex tramtrack bric a brac A Cap and collar Homology family (Bach) Bach1 and Bach2 [84]. When the levels of heme are low, Bach1 binds to Musculoaponeuretic Fibrosarcoma (Maf) recognition elements (MARE), acting as suppressor of HO1, with consequent accumulation of heme. Heme is a suppressor

of Bach1. When the levels of heme are high, Nrf2 (and not Bach1) binds to MARE, which results in the promotion of expression of HO1, with its antioxidant effects [85].

These data assume particular importance if we consider subjects with neoplasms, where the increase in ROS-induced by chemotherapy is a desired effect and, therefore, the cytoprotective action of HO1 is found to stimulate neoplastic progression [86]. In CML, the action of HO1 is one of the mechanisms that BCR-ABL exploits to achieve cell survival. HO1 is constitutively expressed in CML with significantly greater levels in IMA-resistant than in IMA-sensitive cells [87]. As described in the previous chapters, BCR-ABL activates the Pi3K/AKT/mTOR pathway which leads to the production of the antiapoptotic proteins of the Bcl2 family. These inhibit the nuclear translocation of the proapoptotic proteins [88]. In normal conditions, Bach2 is localized in cytoplasm. The ROS stimulus causes the nuclear accumulation of Bach2 that activates an apoptotic response. In CML, the Pi3K pathway phosphorylates Bach2 preventing nuclear translocation and causing cytoplasmatic accumulation with consequent antiapoptotic effect [89].

In parallel, the Pi3K pathway phosphorylates a serine included in the Neh2 domain of Nrf2 causing its activation [90]. This induces the expression of HO1. Over the years, the recognized importance of heme catalysis products has decreased. To date, at least with regards to IMA-resistance of the CML cells, it appears that the main role of HO1 is linked to its nuclear translocation. This could be due to the fact that intranuclear HO1 could activate transcription factors leading to resistance to IMA and cell proliferation [91]. This observation was confirmed by subsequent studies and therefore HO1 inhibition has been

proposed as a therapeutic target in several neoplasms, including CML [92]. Growing evidences demonstrate that HO1 inhibition reduces the viability of IMA-resistant CML cells, proposing to integrate this new strategy with current therapies [93].

1.12. Inhibition of nuclear export.

Although treatment with TKI has substantially changed the natural history of CML, it has limitations, mainly in cases of resistance to treatment and in the fact that it is unlikely to obtain a drug free remission. On these aspects, the inhibition of nuclear export has been proposed as a promising therapeutic target.

The first molecule developed was leptomycin B (LMB), an antibiotic with antitumor activity which *in vitro* was shown to be able to inhibit XPO1 [94]. The inhibition was irreversible and, when tested *in vivo*, the trial was suspended due to the unacceptable frequency of adverse events [95]. The new generation of Selective Inhibitors of Nuclear Export (SINE) have been developed to achieve reversible inhibition with encouraging results.

The SINE actually developed are KPT-330 (Selinexor, SLX), KPT-335 (verdinexor), KPT-8602 (eltanexor), SL-801 (felezonexor), KPT-185, KPT-251, KPT-276, KPT-330: they have shown better safety profile and they are currently being studied in several hematological malignancies [96].

In CML, a combination of IMA + LMB was used. IMA caused BCR-ABL nuclear entry and LMB trapped it within the nucleus due to its inhibition of XPO1. This resulted in the death of cells expressing BCR-ABL [97]. More recent studies have shown that SEL-induced

inhibition of XPO1 was able to increase sensitivity to IMA in resistant cells and the preferential target was represented by LSC CD34+, without effect against CD34+ from normal cord blood [98]. The mechanisms of action are not fully understood, and it is likely that they involve multiple pathways. There are still few studies available on CML and therefore we borrow the experiences obtained on other cancers. An interesting model has been proposed in ovarian cancer where an important role seems to be played by mitochondria. In this model, XPO1 exports Insulin-like growth factor 2 mRNA-Binding Protein 1 (IGF2BP1) and XPO4 exports Eukaryotic translation Initiation Factor 5A (EIF5A). Once in the cytoplasm, IGF2BPI binds to EIF5A and they accumulate in the cytoplasm, inducing cell survival. If inhibited XPO1, only EIF5A would be exported in the cytoplasm. This, without binding to IGF2BPI, is able to penetrate the mitochondrion and induce apoptosis [99]. Even in the case of CML, an emerging role of the mitochondria is observed. CML cells possess increased mitochondrial oxidative functions compared to normal hematopoietic stem cells [100]. Given that mitochondrial oxidative metabolism has a pivotal role for energy production,- and emerging evidences suggest that metabolic plasticity in cancer cells driven by mitochondrial activity is also responsible for the onset of relapses after chemotherapy and may mediate drug resistance, containment of mitochondrial functions may have a therapeutic role [101].

2. Aim of the study

Aim of this study was to evaluate the efficacy of treatment with SLX alone and SLX + DAS on CML cell lines focusing on mitochondrial activity and HO1 nuclear translocation in order to define potential vulnerabilities that could be pharmacologically associated with more precise therapies for CML patients.

3. Materials and Methods

3.1. Cell culture.

Human CML cell line K562 and LAMA84 were obtained from ATCC. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1% L-glutamine at 37 °C in 5% CO_2.

3.2. Flow cytometry.

A membrane potential probe, the 3,3-Diethylozacarbocyanine Iodide {DiOC2(3)}, was used to evaluate the mitochondrial membrane potential. Cells were incubated with 10uM DiOC2(3) (Thermo Fisher Scientific, Milan, Italy) for 30min at 37°C, washed twice, resuspended in PBS and analyzed by flow cytometry through the detection of the green fluorescence intensity of DiOC2(3). In order to measure changes in the mitochondrial mass, cells were reacted with 200 nM MitoTracker Red CMXRos probe (Thermo Fisher Scientific) for 30min at 37°C, according to the 3 manufacturer's instructions. After being washed twice, labelled mitochondria were analyzed by flow cytometry. To evaluate apoptosis after drug treatment, cells were resuspended in PBS after centrifugation and were stained with annexin AV FITC/7-ADD assay kit (Beckman Coulter) according to the manufacturer's instructions. The apoptotic population was immediately evaluated by flow cytometry. The percentages of early apoptotic cells (annexin V+/7-ADD−) and late apoptotic cells (annexin V+/7-ADD+) were calculated and graphed.

3.3. Real time RT-PCR for gene expression.

After RNA extraction, reverse transcription was performed by using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Then the relative transcription of specific genes was determined by RTqPCR using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Milan, Italy) and 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Expression of the following human genes was evaluated: OPA1 (Fw: GTGCTGCCCGCCTAGAAA; Rw: TGACAGGCACC CGTACTCAGT); MNF1 (Fw: GGCATCTGTGGCCGAGTT; Rw: ATTATGCTAAGTCTCCGCTCCAA); MNF2 (Fw: GCTCGGAGG CACATGAAAGT; Rw: ATCACGGTGCT CTTCCCATT); ATP-synthase (Fw: AGCTCAGCTC TTACTGCGG; Rw: GGTGGTAGT CCCTCATCAAACT); CytB (Fw: TCCTCCCGTGAGGCCAAATATCAT; Rw: AAAGAATCGTGTGAGGGTGGGACT); B2M (Fw: AGCAGCATCA TGGAGGTTTG; Rw: AGCCCTCCTA GAGCTACCTG); GAPDH (Fw: AATGGGCAGC CGTTAGGAAA; Rw: GCCCAATAC GACCAAATCAGAG). For each sample, the relative expression level of the mRNA of interest was determined by comparison with the control housekeeping genes B2M and GAPDH using the $2^{-\Delta\Delta Ct}$ method.

3.4. Western blot analysis.

Briefly, for Western blot analysis 10μg of protein was loaded onto a 8% polyacrylamide gel Mini-PROTEAN TGXTM (BIO-RAD, Milan, Italy) followed by electrotransfer to nitrocellulose membrane Trans- Blot TurboTM (BIO-RAD) using Trans-Blot SE Semi-Dry Transfer Cell (BIO-RAD). Subsequently, membrane was blocked in Odyssey Blocking Buffer (Licor, Milan, Italy) for 1h at room temperature. After blocking, the membrane was

three times washed in PBS for 5min and incubated with primary antibodies against human PINK1 and β-actin (ab23707, ab8226, Abcam, Milan, Italy), overnight at 4°C. Next day, membranes were three times washed in PBS for 5min and incubated with Infrared antimouse IRDye800CW (1:5000) and antirabbit IRDye700CW secondary antibodies (1:5000) in PBS/0.5% Tween-20 for 1h at room temperature. All antibodies were diluted in Odyssey Blocking Buffer. The blots were visualized using Odyssey Infrared Imaging Scanner (Licor, Milan, Italy) and protein levels were quantified by densitometric analysis of antibody responses. Data were normalized to protein levels of β-actin.

3.5. Immunofluorescence.

After drug treatment, cells were adhered to slides by cytospin and subsequently fixed with 4% formaldehyde for 20min at room temperature. After three washings in PBS for 5min, cells were fixed using 4% paraformaldehyde, permeabilized using 0.1% Triton X, and blocked to prevent nonspecific antibody binding using 2% bovine serum albumin. The slides were then incubated overnight at 4°C with the primary antibody against HO1 (anti-mouse; ab13248, Abcam, Milan, Italy) at dilution 1:100. Next day, cells were washed three times in PBS for 5min and incubated with anti-mouse FITC secondary antibody at 1:200 dilution for 1h at room temperature. The slides were mounted with medium containing DAPI (4,6-diamidino-2-phenylindole) to visualize nuclei. The fluorescent images were obtained using a Zeiss Axio Imager Z1 Microscope with Apotome 2 system (Zeiss, Milan, Italy).

3.6. Statistical analysis.

Statistical analyses were made with Prism Software (Graphpad Software Inc., La Jolla, CA, USA). Data were expressed as mean or SD. Statistical analysis was carried out by unpaired t-test or ANOVA test. A p-value of 0.05 was considered to indicate a statistically significant difference between experimental and control groups.

4. Results

4.1. SLX induces apoptosis in CML cell lines favoring mitochondrial depolarization.

Flow cytometric analysis was carried out to determine the effect of SLX treatment on the cell viability in both LAMA84 and K562 cell lines. Particularly, cell staining with Annexin V/PI was performed to determine the percentage of apoptotic cells 48h and 72h post-treatment with different doses of SLX (50nM, 100nM, 1μM, 2μM and 5μM). As shown in Fig. 6 A-B, 50nM and 100nM SLX treatment did not affect LAMA84 cell viability after 48h; instead, a significant reduction in cell viability was observed using 1μM, 2μM and 5μM SLX (respectively of about 45±32%, 55±39% and 60±43% compared to untreated cells; $p<0.0001$). The dose dependent reduction of the cell viability was also observed after 72 hours of treatment (Fig. 6 C-D). On the contrary, no significant variations in cell viability were observed after 48 hours of treatment in K562 cell line (data not showed). A significant increase of cell apoptosis was observed in this cell line only after 72h of treatment with 1μM, 2μM and 5μM SLX (respectively of about 17±12,5%, 36±26% and 68±48,2% compared to control; $p<0.0001$; Fig. 6 E-F).

To better investigate the mechanisms by which SLX treatment induces apoptosis in CML cells, we evaluated mitochondrial fitness after drug treatment. Our data demonstrated a significant increase in the percentage of mitochondrial depolarized cells after 48h of 50nM and 100nM SLX in LAMA84 cell line (respectively of about 3±1,8% and 5±3,6%; $p<0.0001$, Fig. 6 G). Mitochondrial depolarization increased massively with higher doses of 1μM, 2μM and 5μM of about respectively 64±45%, 68±47% and 71±49% compared to control

($p<0.0001$). In accordance with previous results on K562 cells, SLX-induced mitochondrial depolarization in this cell line only after 72h of treatment with 1µM, 2µM and 5µM in a dose dependent manner (±25%; $p<0.0001$; Fig. 6 H).

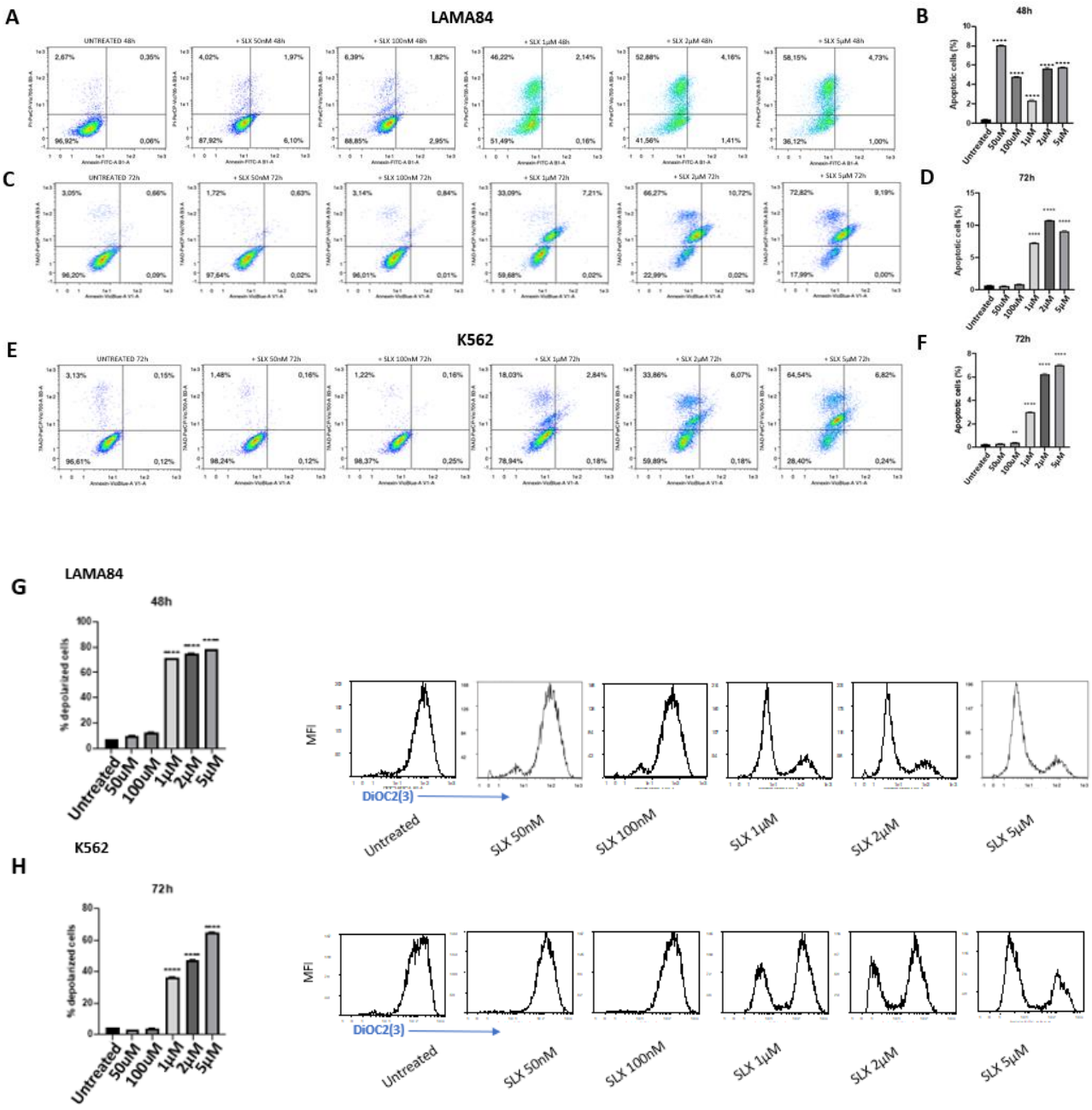

Fig. 6. SLX treatment induces apoptosis in CML cells damaging mitochondria. A-F. Apoptosis was evaluated after 48h and 72h SLX treatment by flow cytometry. Representative dot plots of SLX effect on the viability of LAMA84 and K562 cells are shown. The right panels B, C, E show the mean values of the percentage of apoptotic cells after of Annexin V-FITC and 7-ADD. **G-H**. Mitochondrial membrane potential was assessed following 48h and 72h incubation with SLX. Representative histograms of a flow cytometric analysis of DiOC2(3) staining are shown for both CML cell lines.
All results shown represent the means of four independent experiments. Bars indicate the standard error means (****p<0.0001).

* * *

4.2. SLX increases cytotoxicity of DAS in LAMA84 cell line.

Given the efficacy of the treatment with SLX against the viability of CML cell lines, we investigated a potential synergy of SLX with DAS. LAMA84 and K562 cell lines were treated with 2nM DAS alone or in combination with SLX (500nM and 1μM) for 24h, 48h and 72h. Whereas DAS and SLX alone respectively decreased LAMA 84 cell viability only of about 5±3,4% and 10±6,2 %, their combination significantly increased apoptosis of about 24±17,3% and 33±23,2%, respectively after treatment with DAS and 500nM or 1μM SLX ($p<0.0001$ compared to untreated cells; Fig. 7 A-B). This efficacy of DAS/SLX combination was not observed in K562 cell line (Fig. 7 C-H). Indeed, treatment with DAS alone was sufficient to significantly reduce cell viability after 48h and 72h (respectively of about 28±20,3% and 62±44,1% compared to untreated cells; $p<0.001$; Fig. 7 E-H); its combination with SLX did not improve the TKI-induced apoptosis. For this reason, only LAMA-84 cells were used for the next experiments.

As it has been suggested that perturbing mitochondrial function could improve elimination of leukemic stem cells [102], we subsequently evaluated the effects of DAS/SLX combination on mitochondrial fitness. Our data demonstrated that DAS treatment caused a significant mitochondrial depolarization with a reduction of DiOC2(3) MFI of about 20±13,6% after 24h ($p<0.0001$ compared to untreated cells; fig. 7 I). Interestingly, DAS/SLX combination significantly increased the percentage of DAS-induced depolarized cells of about 78±44,9% ($p<0.0001$ compared to cells treated with DAS alone).

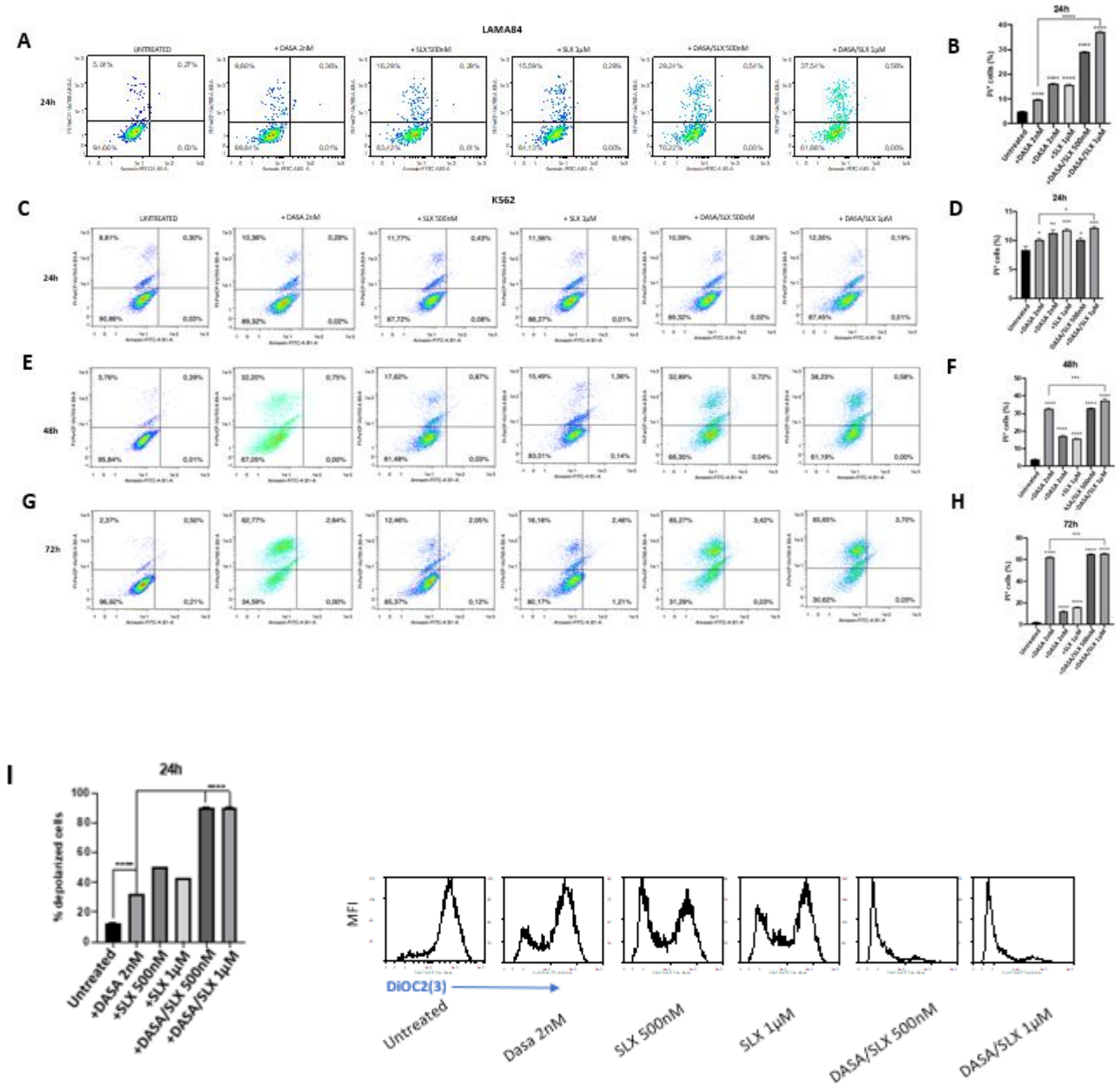

Fig.7. SLX increases the efficacy of DAS in LAMA84 cells. A-H. Apoptosis was evaluated after 24h, 48h and 72h of treatment with DAS, SLX or their combination by flow cytometry. Representative dot plots are showed. **I.** Mitochondrial membrane potential was assessed following 24h incubation with DAS, SLX or DAS/SLX. Representative histograms of a flow cytometric analysis of DiOC2(3) staining are shown. All results shown represent the means of four independent experiments. Bars indicate the standard error means (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

* * *

4.3. DAS/SLX combination decreases mitochondrial mass and dynamic.

Since a strong depolarization of mitochondria could be accompanied by a drastic reduction of mitochondrial mass [103], mitochondria of CML cells were evaluated after treatment with DAS, SLX or their combination. By using MitoTracker staining and flow cytometry analysis, we found that drug combination increased the reduction of MitoTracker-MFI value of about 16±9,2% compared to cells treated with DAS alone ($p<0.0001$; fig. 8 A). In accordance with these data, we also observed increased expression of PTEN-induced kinase 1 protein (PINK1) ($p<0.05$ compared to untreated cells; Fig. 8 B), a protein involved in the removal of damaged mitochondria [104]. To respond to mitochondrial stress, CML cells treated with DAS alone promoted a compensatory upregulation of mitochondrial dynamic-related genes Mitofusin 1 (MFN1), Mitofusin 2 (MNF2) and Optic Atrophy 1 (OPA) (respectively $p<0.0001$, $p<0.001$ and $p<0.0001$ compared to untreated cells; Fig. 8 C-E), as well as of the OXPHOS-related gene Cytochrome B (CytB) and ATP synthase (ATP synt) ($p<0.0001$ and $p<0.01$ compared to untreated cells; Fig. 8 F-G).

On the contrary, DAS-induced MFN1, MNF2, OPA, CytB and ATPsynt expression was significantly downregulated in cells treated with DAS/SLX (respectively $p<0.0001$, $p<0.01$, $p<0.0001$, $p<0.0001$ and $p<0.001$ compared to DAS alone; Fig. 8 C-G), highlighting a close link between SLX treatment and mitochondrial impairment. Moreover, SLX alone significantly reduced all analyzed mitochondrial dynamic and OXPHOS-related genes (respectively, $p<0.0001$, $p<0.01$, $p<0.0001$, $p<0.0001$ and $p<0.001$ for MNF1, MNF2, OPA, CytB, ATPsynt compared to untreated cells).

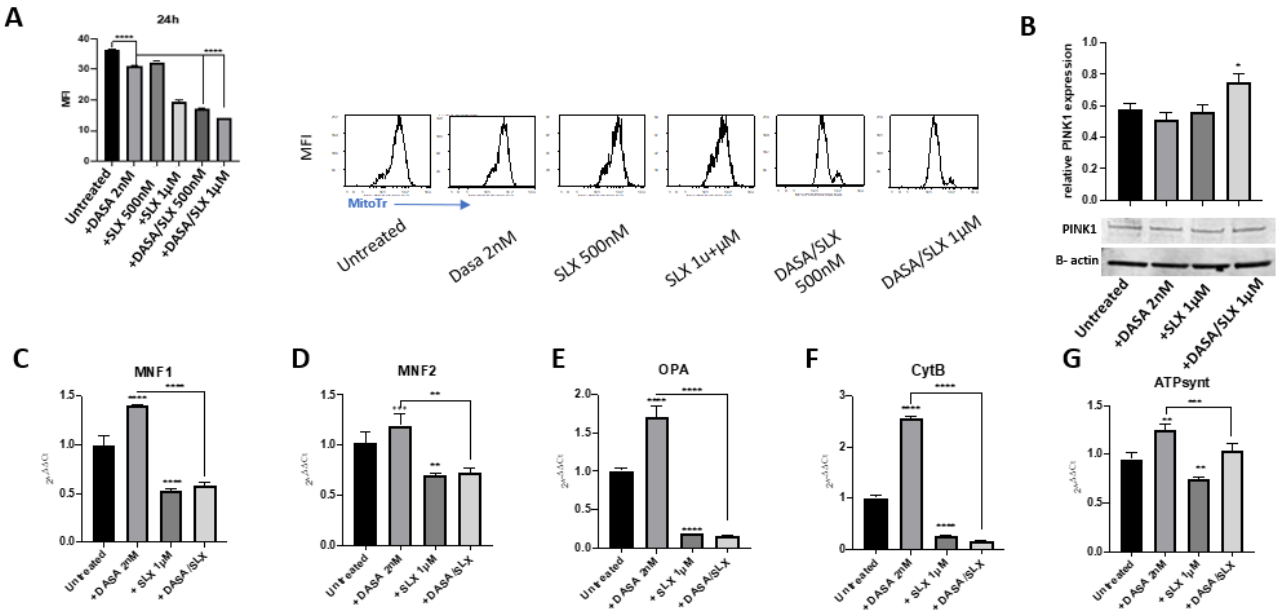

Fig. 8. DAS/SLX treatment decreases mitochondrial mass and mitochondrial dynamic. A. Flow cytometric analysis of Mitotracker Red CMXRos staining after treatmet with DAS, SLX or their combination for 24h. Representative flow cytometry histograms are showed**. B.** Western blot analysis of PINK1 expression. β-actin protein was used as total protein loading reference. For analysis, the optical density of the bands was measured using Scion Image software. **C-G.** Gene expression analysis of mitochondrial dynamic markers and OXPHOS-related genes. B2M gene was used as housekeeping gene. Calculated value of $2^{-\Delta\Delta Ct}$ in untreated cells was 1. All results shown represent the means of four independent experiments. Bars indicate the standard error means (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

* * *

4.4. DAS/SLX combination decreases HO1 nuclear translocation.

As it has been demonstrated that induction HO-1 expression is a mechanism of TKI-resistance [91], we finally evaluated its levels of expression after treatment with DASA and SLX alone or in combination. Western blot analysis showed that DAS treatment significantly increased HO1 protein levels compared to untreated cells (p<0.0001, Fig. 9 A). On the contrary, SLX exposure both alone and in combination with DAS significantly decreased

HO1 expression compared to DAS treated cells ($p<0.0001$). To better evaluate if DAS-induced HO1 expression was also associated to its increased nuclear translocation, an immunofluorescence analysis was performed. Our data showed that DAS treatment significantly induced HO1 nuclear translocation, which resulted decreased in CML cells treated with DAS/SLX combination (Fig. 9 B).

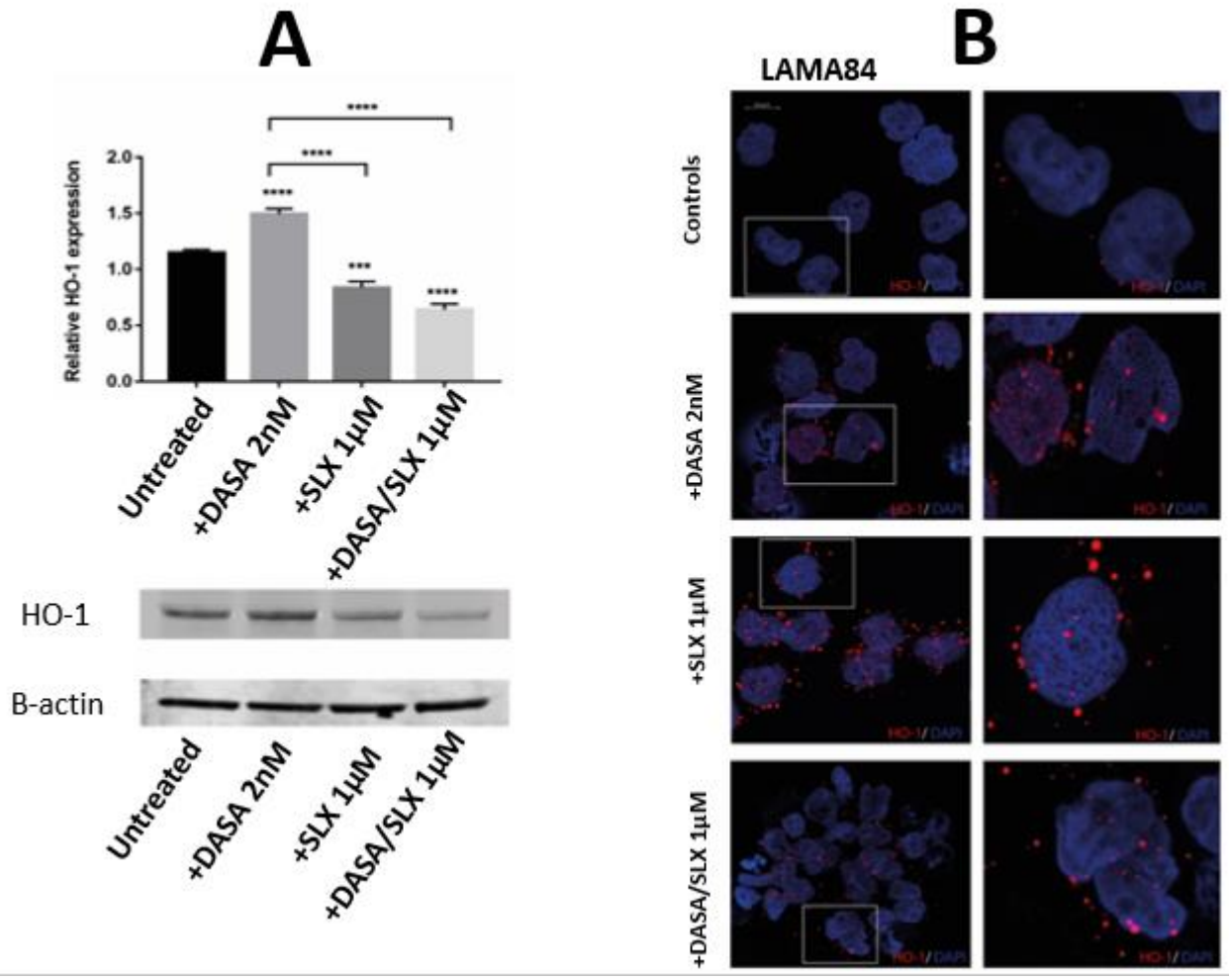

Fig. 9. Combination of DAS with SLX decreased TKI-induced HO-1 nuclear translocation. A. Western blot analysis of HO1 expression. β-actin protein was used as total protein loading reference. For analysis, the optical density of the bands was measured using Scion Image software. **B**. Analysis of HO1 localization by immunofluorescence. Bars indicate the standard error means (***$p<0.001$; ****$p<0.0001$).

* * *

5. Discussion

Comparing the impact that TKIs have had on the natural history of CML to that of antibiotics on bacterial disease is probably less bold than it may seem. The patients who respond to the treatment show substantially a normal life expectancy [61]. This is confirmed by the dramatic increase of the prevalence of the disease influenced by the improved survival [9]. The main issues related to the treatment are drug toxicity, with or without acquired resistance, the cost of the prolonged treatment and the adherence to the treatment, considering that about half of the patients in prolonged remission show relapse of the disease when TKI is discontinued [105-107]. This is probably related to the persistence of LSCs. TKI are not effective in eradicating LSCs [108]. Even considering patients who had achieved sustained undetectable molecular residual disease with TKI treatment, LSCs expressing BCR-ABL were identified [109]. A more recent study showed that, considering patients with deep molecular response who discontinued treatment with TKI, a molecular relapse was described in subjects with detectable and undetectable persistence of LSCs [110].

This stimulated the individuation of new therapeutic strategies.

In our study, the cytotoxic role of SLX in CML cell lines was initially investigated. Previous studies had shown that SLX selectively inhibits XPO1 by forming a slowly reversible covalent bond with cysteine 528 in the XPO1 cargo-binding pocket [111]. The inhibition of XPO1 results in the increase of tumor suppressor proteins in the nucleus and the decrease of oncoproteins, arrest of cell cycle and apoptosis [112]. In addition, XPO1

inhibition causes the disruption of the three-dimensional nuclear organization of the telomers preferentially in the tumor cells, sparing non-affected cells [113]. Considering this mechanism of action, SLX has been used successfully in multiple myeloma and in Waldenstrom macroglobulinemia [114-115]. European Medicines Agency considered that the benefits of SLX treatment in refractory multiple myeloma outweigh the risk and granted conditional authorization on May 27, 2021, pending the safety and efficacy data derived from the treatment Bortezomib + SLX + low-dose dexamethasone [116].

In this study, we observed that treatment with SLX affected the viability of CML cells and promoted mitochondrial membrane depolarization, a process triggered by the overexpression of the pro-apoptotic proteins BAX and BAK, probably due to XPO1 inhibition [117].

Recent studies have demonstrated that primitive CML cells rely on upregulated oxidative metabolism for their survival [100]. Although an extensive literature on the role of mitochondria in CML is not yet available, several works theorizing the importance of these organelles are emerging by borrowing the experience from other neoplasms. In CML, mitochondria seem to have the dual role of disease maintenance and resistance to TKIs [17]. The BCR-ABL TK activity is an important source of ROS [118]. Beyond a certain amount, ROS can cause DNA damage, first to mitochondrial one (mtDNA), which lacks the protein defenses to damage and has a lower repair capacity, then the nuclear one (nDNA) [119]. Normally, DNA damage would stimulate an attempt at repair or activate the intrinsic pathway of apoptosis via BAX-BAK channels, but in CML this pathway is antagonized by

the action of the antiapoptotic proteins of BCL2 family [120]. The damage to mtDNA causes mitochondrial dysfunction with accelerated production of ROS and a reduction of oxidative phosphorylation with inefficient ATP generation [17,121]. To meet the need for ATP, neoplastic cell enhances glycolysis mediated by Hypoxia-Inducible Factor α (HIF1α) [122]. BCR-ABL induces the glucose transporter GLUT1 to the cell surface via the Pi3K pathway in order to increase glucose intake [123]. A high level of glycolysis was observed in TKI-resistant cells, independently of the presence of IMA, suggesting a role of glucose metabolism in TKI resistance [124]. An increase in the glucose metabolism with activation of the Pi3K pathway leads to a further increase in ROS [125]. It has been hypothesized that damage to mitochondrial respiration, the role of HIF1α and the increase in glucose metabolism may be responsible for TKI-resistance via the inhibition of p53 activity, supporting the role of mitochondria in CML [126]. Moreover, the accumulation of ROS can induce DNA damage with consequent genomic instability, already characteristic of CML cells per se, which can induce mutation capable of conferring TKI resistance [127-128].

Considering these data, we evaluated the efficacy of treatment with SLX in combination with DAS. Our data demonstrated that XPO1 inhibition increased DAS-induced apoptosis in LAMA84 cells causing a strong mitochondrial depolarization associated to a significant decrease of mitochondrial mass.

Mitochondria respond to environmental changes by fusing together to form an interconnected reticulum, and by dividing to either increase mitochondrial number or to segregate portions of the organelle for degradation via mitophagy [129]. The interplay

between mitochondrial dynamics of repetitive cycles of fusion and fission and mitophagy assures cell homeostasis. To protect them from mitochondrial dysfunction, cells treated with DAS alone activated mitochondrial fusion and biogenesis, as demonstrated by the upregulation of MNF1, MNF2 and OPA1. Mitochondrial fusion has the role of preventing removal of impaired mitochondria thanks to the accumulation of the mitofusin proteins, which allows them to reengage in the fusion process despite their deprived energetic state, and in doing so, improves mitochondrial activity. Mitochondria can fuse only if their activity is above a certain threshold: depolarization below a certain mitochondrial membrane potential ($\Delta\Psi m$) is a prerequisite for mitophagy which has a role at the end of the axis of quality control of mitochondria [130]. Therefore, the low levels of depolarized mitochondria allow cells treated with DAS alone to coordinate mitochondrial fusion, biogenesis and mitophagy, leading to the maintenance of mitochondrial fitness. In accordance, PINK1 protein levels were not increased in DAS treated cells. Moreover, since OPA1 is involved in the remodeling of mitochondrial ridges and in the regulation of OXPHOS linked to Electron Transport Chain, its upregulation in cells treated with DAS alone has a certain impact on mitochondrial energy production and helps to allow for high oxidative metabolism and generation. In accordance with these observations, DAS treatment alone also induced a significant upregulation of CytB and ATPsynt. On the contrary, CML cells treated with DAS/SLX decreased DAS-induced MFN1, MFN2 and OPA1 upregulation as well as CytB and ATPsynt. This strong mitochondrial impairment observed in SLX/DAS treated cells was also accompanied by increased levels of PINK1, a protein that accumulates in the outer mitochondrial membrane (OMM) and recruits Parkin

to initiate mitophagy [131], a cellular process that selectively removes the damaged mitochondria.

As presented in previous chapters, HO1 has recently been recognized to have an important role as a mechanism by which BCR-ABL promotes cell survival [87]. Initially, the antiapoptotic effect of HO1 was thought to be due to the heme catalysis product [132]. This data has not been confirmed by subsequent works [87,91]. More recent literature data show that the increase in ROS stimulated the expression of HO1 and its cleavage in the c-terminal domain. HO1 can migrate to the nucleus where, as mechanism not yet elucidated, it plays a cytoprotective role [133]. Treatment with IMA increases ROS, already markedly present in the CML cell, increasing the expression of HO1 which, migrating into the nucleus, could be responsible of the TKI-resistance mechanisms [91]. HO1 overexpression was observed in BCR-ABL T351I mutant cells showing resistance to all available TKIs. In this case, targeting HO1 by pegylated zinc protoporphyrine (PEG-ZnPP) or styrene maleic acid-micelle-encapsulated ZnPP led to the growth inhibition of CML cells [134]. Moreover, a reduction in autophagy and an increase in IMA-sensitivity have been observed during HO1 inhibition, thus providing evidence that one of the mechanisms by which HO1 causes TKI-resistance is the promotion of autophagy [135]. These observations have been confirmed by several *in vitro* model studies, which recognized HO1 as a promising therapeutic target [136]. In this study, we observed that DAS treatment caused an increase of HO1 expression and its nuclear translocation. Combining SLX with DAS significantly decreased HO1 expression and its nuclear localization.

In conclusion, our data demonstrated the efficacy of the DAS/SLX combination in CML cells disrupting mitochondrial dynamics and mitochondrial fitness, which are potential active mechanisms of LSCs resistance. Further studies are needed to clarify whether combination therapy with TKI and SINE may be useful in the treatment of refractory forms of CML or in eradicating LSCs.

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