UNIVERSITA' DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SCIENZE DEL FARMACO

CORSO DI LAUREA MAGISTRALE IN PHARMACEUTICAL BIOTECHNOLOGIES - BIOTECNOLOGIE FARMACEUTICHE

TESI DI LAUREA

Development of new pharmacological approaches to trigger Cell death in a Mycosis Fungoides Cell line

RELATORE**:** Prof. Susanna Mandruzzato

Co-RELATORE: Dr. Micol Silic-Benussi

LAUREANDO**:** Neda Ordoodary

ANNO ACCADEMICO 2022/2023

Index

ABSTRACT

Background

Mycosis fungoides (MF) is the most common form of primary cutaneous T-cell lymphoma (CTCL) and represents about 50% of all primary cutaneous lymphomas (PCL). Although the initial stages of MF present an indolent clinical course and can benefit from "skin-directed" treatment, patients who relapse or are resistant to treatment are destined to progress to the advanced stages of cancer. These latter are considered incurable, and available therapies are primarily palliative. Cancer cells present high levels of reactive oxygen species (ROS.), which renders them potentially vulnerable to compounds that further increase ROS to cytotoxic levels. The present study investigated this possibility by testing response of the MF cell line MYLA to ROS-inducing drugs.

Aim of the study

The studies described in this thesis evaluate the effects of a few compounds capable of altering ROS homeostasis in mycosis fungoides cells grown "in vitro". For this purpose, we used CBR-5884 and Everolimus (RAD-001). These components block the metabolic pathways that limit ROS levels, the serine and the glucose-6-phosphate dehydrogenase pathways, respectively. We analysed cytosolic and mitochondrial ROS production as well as the effects on cell vitality that treatment with one or both compounds produced. We also attempted to make MMAE cytotoxicity specific for MF cells by conjugating it to a monoclonal antibody (ab) recognizing Cutaneous Lymphocyte Antigen (CLA), a characteristics homing receptor expressed by MF cells, in order to evaluate the ability of the antibody-drug conjugate (ADC) to induce cell death.

Materials and Methods

The experiments featured in this study were conducted on an MF cell line named MyLa CD4+ (ECACC95051032). Cell death and cytosolic and mitochondrial ROS production were measured by flow cytometer with markers: propidium iodide (PI) and reagents that emit a fluorescent signal when oxidized by ROS, respectively. death by apoptosis was measured by flow cytometer with the markers Annexin V and PI.

Results

The results obtained demonstrate that co-treatment with CBR-5884 and Everolimus leads to a significant increase in cell death when compared with the control group and with treatments using only one of the compounds. We demonstrated that cotreatment with these compounds leads to a significant increase of both mitochondrial and cytosolic ROS and that this increase seems to correlate with increased cell death. Also, results showed that MMAE was able to induce death in MyLa cells in a dose-dependent manner, however, the ADC exhibited lower cytotoxic activity compared to MMAE or CLA-ab alone.

Conclusion

We have shown that use compounds capable of modifying ROS homeostasis is a potential therapeutic method for inducing cell death in mycosis fungoides in vitro. The data collected is a starting point for further research targeted at creating therapeutics based on ROS level manipulation in the treatment of patients with relapsing or refractory MF.

Using an antibody-drug conjugate (ADC) composed of the drug MMAE and a monoclonal antibody (ab) targeting Cutaneous Lymphocyte Antigen (CLA), we discovered that MMAE could induce death in MyLa cells in a dose-dependent manner, though the ADC had a lower cytotoxic effect than MMAE or CLA-ab alone.

1. INTRODUCTION

1.1 Primary cutaneous lymphomas (PCL)

1.1.1 General

The skin is the largest organ in the human body, with a barrier function against external chemical, physical and infectious agents. Keratinocytes, fibroblasts, Langerhans cells, dendritic cells, memory T lymphocytes, polymorphonuclear leukocytes, mast cells and endothelial cells interact and cooperate to form the skin's immune system (Abbas, et al., 2000).

Rich in lymphocytes, the skin represents a possible site for the development of lymphomas. Lymphomas are a group of neoplasms that arise from lymphocytes at different stages of maturation and differentiation. They are divided the broad categories Hodgkin's lymphoma and non-Hodgkin's lymphomas (NHL). Hodgkin's lymphoma has clinical and histological characteristics and mainly affects the lymph nodes of the cervical districts. NHL is divided into two sub-categories, nodal and extra nodal, depending on their location. Primary cutaneous lymphomas (PCLs) are a heterogeneous group of extra nodal NHL with initial cutaneous presentation. They differ both clinically and prognostically from nodal NHL (Willemze R, et al., 2019; Willemze R, et al., 2005).

PCL can arise from T- or NK lymphocytes (primary cutaneous T and NK-cell lymphomas, CTCL) or B lymphocytes (primary cutaneous B-cell lymphomas, CBCL). The current and most widely used classification system for PCLs was introduced by the WHO / EORTC introduced in 2005 and updated in 2018 (Willemze R, et al., 2018). This system divides cutaneous lymphomas into two large groups: cutaneous T-cell lymphomas (CTCL) and cutaneous B-cell lymphomas (CBCL). Further classification is based on clinical, histopathological, immunohistochemical, and molecular characteristics.

In Western countries, the frequency of PCL is about 1/100,000. CTCLs comprise about 75-80% of all PCLs (Willemze R, et al., 2005). Mycosis fungoides, Sézary syndrome, and CD30+ lymphoproliferative disorders represent approximately 90% of the CTCL group, with MF being the most prevalent (39%) (Willemze R, et al., 2018). The overall incidence of CTCL has increased in recent years (Wilcox RA., et al., 2017).

1.1.2 Etiopathogenesis of CTCL

It is generally accepted that PCLs arise through a multiphasic and multifactorial process characterized by the progressive accumulation of mutations affecting oncogenes, tumor suppressors, and genes involved in the repair of DNA damage. These alterations seem to be driven by a hyperreactive-inflammatory process caused by chronic antigenic stimulation, which is supported by various genetic, infectious, immunological, and environmental factors (Burg G., et al., 2006).

The process that leads to PCLs occurs in 3 general phases.

1) the chronic activation of lymphocytes with genetic instability leads to a potentially reversible "pre-neoplastic" lymphoproliferative condition. In this phase, there is an increase in the activity of transcription factors such as c-MYC and STAT5 and increased expression of genes coding for antiapoptotic BCL2-family proteins (e.g., BCL2, MCL1), and augmented production of growth factors and cytokines (Qin JZ, et al., 2001).

2) The continuous stimulation of endogenous tyrosine kinases leads to the activation of additional transcription factors (NF-κB proteins, STAT2, STAT3) and their target genes. This sets up the ideal condition for lymphoma development, which is initially low-grade. The proliferation of neoplastic T cells is maintained by self-co-stimulation, and is favoured by cell-to-cell contact and contact with dendritic cells (Willemze R, et al., 2005).

3) During the third stage the neoplastic cells proliferate independently of cytokines and microenvironmental factors in the context of continuous activation of tyrosine kinases, Ras GTPase, and transcription factors and inactivation of tumor suppressors such as RB, and TP53. Proliferation and survival are favoured by downregulation of cell cycle regulatory proteins p15 and p16 and apoptosis-promoting BCL2 family proteins such as Bad-Bax (Burg G., et al., 2006).

The neoplastic lymphoid cells begin to express new non-skin-targeting homing receptors that permit them to spread and proliferate outside the skin, such as in the bloodstream, lymph nodes, bone marrow, and visceral organs (Armerding D, et al., 1999; Ferenczi K, et al., 2002).

1.1.3 Immunological aspects of CTCL development

CTCL presents a typical immunophenotype of the Th-2 lymphocyte population, with production of cytokines such as IL-4, IL -5, IL-6, IL-10, and IL-13 (Mosmann TR and Sad S., 1996) and expression of lineage-specific transcription factors (Stolearenco. V, et al., 2020; Kim. E.J., et al., 2005). Effects of IL-4, IL-5 and IL-13 on IgE production and consequent activation of eosinophils produce some of the typical clinical manifestations of CTCL, including changes in IgE and IgA serum levels, eosinophilia, pruritus, skin edema, and delayed hypersensitivity reactions (Iyer.A, et al., 2019).

The neoplastic lymphocytes also produce IL-15, a cytokine usually expressed by the basal layer of keratinocytes and dendritic cells. This results in the autocrine stimulation of tumor cells (Dobbeling, et al., 1998, Thode.C, et al., 2015). In normal conditions, IL-10 induces the downregulation of the antigen presentation process and of the accessory functions of monocytes, macrophages, Langerhans cells, thus indirectly inhibiting the activation of specific antigen T lymphocytes Asadullah K, et al., 1996). IL-10 secretion by malignant T cells weakens anti-tumor immune surveillance (Wilcox.R.A, et al., 2009) and thus favors disease progression.

Early lesions contain an abundant infiltrate of normal immune cells and rare tumor cells (Stolearenco V, et al., 2020; Thode. C, et al., 2015). In this phase, the infiltrate presents a Th1 pattern, which allows an antitumor response locally. In the later stages, cells with the Th2 phenotype accumulate and cytokine expression decreases, with loss of immunosurveillance function and T cell exhaustion (Chong BF, et al., 2008).

1.1.4 Genetic factors

Recent high-definition transcriptomic studies employing next-generation sequencing (NGS) have provided a comprehensive overview of the genomic and epigenetic landscape of CTCL and uncovered the complex mutational spectrum of mycosis fungoides and Sézary syndrome, with a solid link to their occurrence and progression; more than 50 disease-related driver mutations have been identified (Iyer A, et al., 2020).

The role of genetic factors in the development of CTCLs has to be fully understood. TP53, a DNA damage-responsive tumor suppressor, appears to play an essential role in CTCL development (Choi J, et al., 2015; Da Silva Almeida AC, et al., 2015). Constitutive activation of the nuclear factor kappa B (NF-κB) pathway, downstream of T cell receptor (TCR) signaling, has been described to play a key role in resistance to apoptosis in CTCL (Sors A, et al., 2006). The JAK3/STAT3 signaling

pathway also has a role in CTCL cell survival and proliferation (Bagherani N, et al., 2016; Sommer VH, et al., 2004).

MicroRNAs (miRNAs) are non-coding RNA involved in epigenetic mechanisms implicated in essential cellular processes (Garzon R, et al., 2010). Profiling of miRNAs in CTCL cell lines indicated that their increased expression of miR-213, miR-486, and miR-21 promotes apoptotic resistance (Narducci MG, et al., 2011).

1.1.5 Infectious Agents

Infectious agents are also implicated in CTCL. Epstein-Barr virus (EBV), a herpes virus with a worldwide distribution, is associated with lymphoproliferative diseases such as Burkitt's lymphoma, post-transplantation lymphoproliferative disorders, Hodgkin's lymphoma, nasal T-cell lymphoma, and some peripheral T-cell lymphomas (Zhou, A.D, et al., 2007; Thorley-Lawson D.A, 2001). In 2016, Chronic Active Epstein–Barr Virus Disease (CA-EBV) was classified under EBV-positive T- or NK-cell neoplasms. Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL), which in some cases shows cutaneous homing (*Futch N et al., 2017). The cutaneous subtype of ATL is included in the WHO/EORTC classification of CTCL (see below). Chlamydia infections can contribute to the development of erythrodermic types of CTCL (Abrams JT, et al., 1999).

1.1.6 Clinical features and diagnosis of CTCL

The clinical presentation of cutaneous lymphomas is highly varied. They can manifest as hypo- and hyper-pigmented patches, papules, plaques, and nodular lesions with erosion or ulceration (Prince HM, et al., 2009). This heterogeneous presentation complicates diagnosis.

Histologically, cutaneous lymphoma appears as a lymphocytic infiltrate involving the epidermis, dermis and/or subcutaneous tissue. Immunohistochemistry on tumor samples provides information on the phenotype, degree of maturation, and function of neoplastic cells, and whether the neoplastic clone derives from T, B or NK cells and permits classification (Fung MA, et al., 2002).

1.2 Mycosis fungoides

The CTCL mycosis fungoides (MF) was the first PCL to be described (in 1806). MF is indolent for an extended period and is clinically defined by progression through three phases: an eczematous "premycotic" phase, a phase with plaques, and a tumor phase. MF is considered to be “the great imitator,” as the possible clinical presentations are very varied and often attributable to other skin diseases (Zackheim HS, et al., 2002). These features complicate diagnosis and can lead to a delay in treatment.

MF is most frequently diagnosed in adults, with onset occurring between the ages of 55 and 60, and is more frequent in men (male/female ratio of 2:1); however, cases of MF have been reported in childhood and adolescence (Bradford PT, et al., 2009). About two-thirds of MF cases are diagnosed in Caucasian persons, followed by African and African / American, Hispanic, and Asian ethnicities.

MF patients may have an increased risk of developing a second form of cancer such as Hodgkin's lymphoma and non-haematological forms of cancer (Mahalingam, et al., 2015). As described in Section 1.2.3, early stages of MF are treated with conservative approaches such as skin-directed therapies, while advanced-stage patients are often treated with targeted and systemic chemotherapies, the effects of which are short-lived and associated with severe adverse reactions (Wilcox RA, et al. al., 2016).

1.2.1 Biology and pathogenesis

Cell of origin

All lymphocytes originate from precursors residing in the bone marrow, which, upon reaching the thymus, undergo maturation. Naïve mature T lymphocytes circulate in peripheral blood and lymphoid organs, where they can recognize antigens from skin presented by antigen-presenting cells (APCs). The activated T lymphocytes proliferate and differentiate into memory T lymphocytes (effector- or central memory).

Antigen-specific central memory T cells form a reservoir within the lymph nodes to provide long-term immune surveillance, whereas effector memory T cells express the E-selectin ligand cutaneous lymphocyte antigen (CLA) and chemokine receptors (such as CCR4, CCR8, and CCR10) that facilitate extravasation of T cells into the skin (2005; Wilcox, R. A., et al., 2017; Watanabe, R ., et al., 2015., Kim, E. J., et al., 2005). CLA, a well characterized ‘homing receptor’, participates in the

homing process by interacting with E-selectin expressed by the endothelial cells of the post-capillary venules present at the dermal level (Storz M et al., 2001).

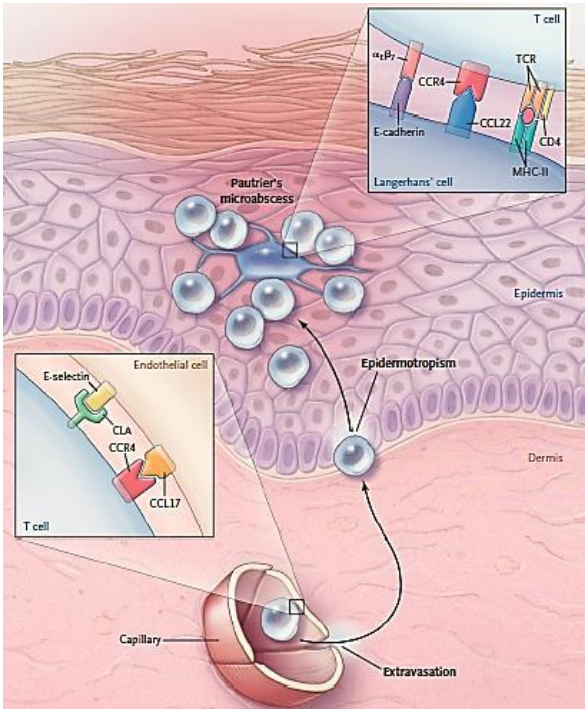

Figure 1.1: In mycosis fungoides, the neoplastic cells reside in the skin thanks to the interaction with the endothelial cells of the post capillary venules present in the dermal level. The neoplastic T lymphocytes express CLA antigen on their surface which allows lymphocyte "rolling" by interacting with E-selectin molecules expressed by endothelial cells. The receptors for chemokines present on the surface of neoplastic T lymphocytes, such as CCR4, bind to chemokines (e.g.: CCL17) produced at the epidermal level and present at the level of the luminary surface of the endothelial cells. This interaction facilitates the extravasation of the lymphocytes in the dermis. From here the neoplastic lymphocytes migrate to the epidermis and surround the Langerhans cells forming Pautrier's "micro-abscesses", a histological finding typical of MF (Girardi M, et al., 2004).

The immunophenotypic profile of MF indicates that they are derived from mature skin-resident memory T cells (Figure 1.1). [Sézary syndrome (SS) long regarded as a leukemia form of MF, is actually a separate disease that is derived from central memory cells, as they express the specific markers, CCR7 and L-selectin (Campbell JJ, et al., 2010)].

Neoplastic lymphocytes that accumulate in plaque and tumor lesions of MF predominantly express a typical phenotype of Th2 cells characterized by the production of IL-4, IL-5, IL-10 and IL-13. Clinically, the predominance of Th2 cytokines is associated with eosinophilia, erythroderma, and

high IgE levels. As shown in Figure 1.2, the chronic cytochine production by neoplastic T cells represents one of the mechanisms to escape the antitumor immune response (Kim EJ, et al., 2005).

It remains to be determined whether MF neoplastic cells are derived from a clone of Th2 cells or acquire this phenotype to the activation of cellular signaling pathways such as, NFAT, NF-κB, and JAK / STAT (Vaque JP, et al., 2014).

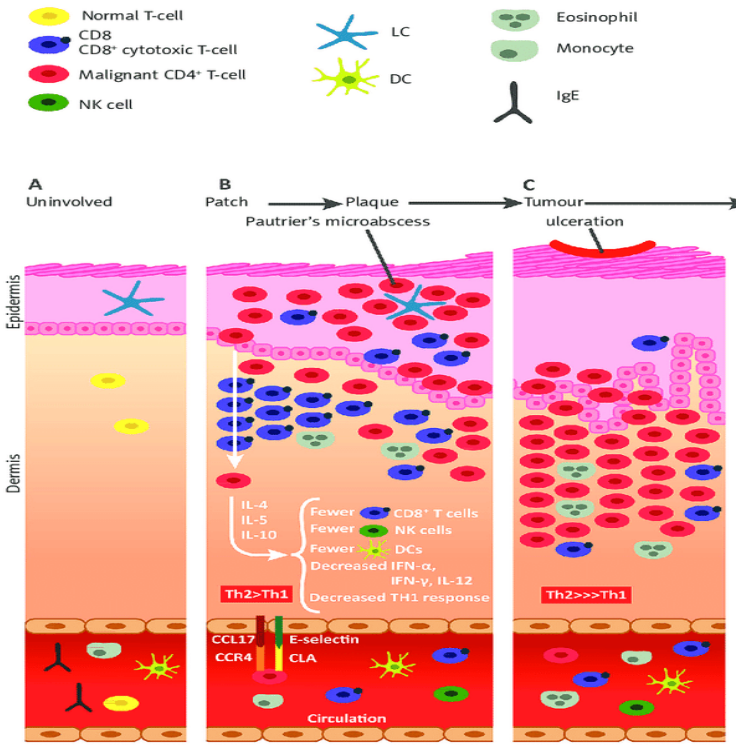

Figure 1.2: The mechanisms involved in the progression of MF. (A) Normal skin with only skin-homing T cells in dermis and in circulation. (B) Patch and plaque MF: more, especially malignant, T cells home to the skin via interaction of expressed CCR4 and CLA with CCL17 and E-selectin, instigating an immune response. These CTCL cells influence their microenvironment: Th2 cytokine production increases and Th1 cytokine production decreases. The Th2 increase leads to an increase in IL-4, IL-5, and IL-10 which in turn leads to a decrease in CD8+ T cells, NK cells, DCs, IFN-α, IFN-γ and IL-12, as well as other cytokines. (C) In MF, this effect escalates, so that the infiltrate is comprised mainly of malignant T cells and some CD8-positive cells, an increase in eosinophils, and greatly enhanced Th2 cytokine production. (Kim E J, et al, 2005).

Pathogenesis

Several pathogenic factors and potentially altered signaling pathways have been identified in MF (van Doorn R, et al., 2009), (Salgado R, et al., 2010). Amplification of the MYC gene and mutations of TP53, PTEN, CDKN2A or CDKN2B (p15/p16) tumor suppressor genes have been described in advanced stages of MF (Raess PW, Bagg A., 2012; Nicolae-Criesta AR, et al., 2015). Mutations have also been described in the GATA-3, AIRD1A, DNMT3A, ZEB1 and RAG genes (Choi J, et al., 2015). Other mutations affect STAT 3/STAT5, IL-2, and the death receptor family gene FAS. Deletions in 5q, 9p, and 13q and insertions in 1q, 7p, and 7q are also observed (Raess Pw, et al.,2012) (van Kester MS, et al., 2012) (Nicolae-Cristea AR, et al.,2015).

The PI3K / AKT / mammalian target of rapamycin (mTOR) pathway is a critical regulator of cell growth, proliferation, migration, apoptosis, cell cycle progression, and cytoskeletal rearrangement (Vivanco, C.L, et al., 2002), and is dysregulated in MF (Kremer. M, et al., 2010).

The tumor microenvironment (TME) plays a fundamental role in the pathogenetic process of MF by supporting tumor cell growth and suppressing the antitumor immune response. Understanding the interactions between tumor cells and TME could help find new treatments to eliminate the microenvironment component and thus eliminate tumor cells (Yamanaka K, et al., 2006; Berger CL, et al., 2002).

1.2.2 Clinical features, diagnosis and staging

Presentation and histopathology

MF shows a variety of clinical manifestations. The ‘classic’ form of MF is characterized by the progressive appearance of patches, plaques, and tumor lesions (Willemze et al., 2005), but this is not a constant in all patients, who may remain in the plaque phase without other signs of progression, or present from the earliest manifestations tumors or nodules associated with patches and plaques (Mu˜nozGonzález H, et al., 2016).

The first phase of MF is characterized by irregular, erythematous, and asymmetric patches, mainly localized on the back and lower limbs (Figure 1.3), which are generally asymptomatic or cause mildly pruritic. This phase can last for many years, and the lesions can regress and disappear. Some patients eventually progress to the erythrodermic stage, characterized by exfoliative erythroderma and intense itching.

As shown in Figure 1.3, patchy phase histology is characterized by a few lymphocytes arranged in the basal layers of the epidermis, associated with a focal parakeratosis and by a more significant number of lymphocytes arranged "in a band" along the dermo-epidermal junction. The lymphocytes found in the epidermis usually appear larger and more pleomorphic than those present at the dermis surface (Willemze et al., 2005). These morphological features suggest MF, but they are not always present and may resemble other skin diseases.

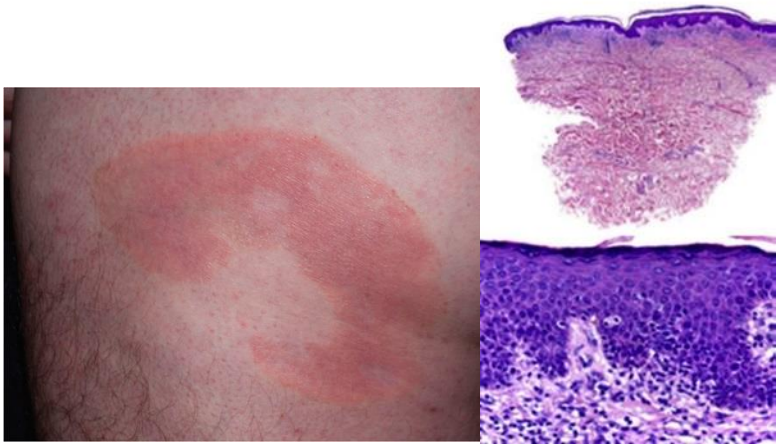

Figure 1.3: Clinical and histopathological findings typical of the "patchy" phase of MF. The images show a patchy lesion with histology is characterized by an infiltrate of atypical lymphocytes in the papillary dermis (Mu˜noz-González H, et al., 2016).

The second phase of MF is characterized by the presence of well-defined, itchy, erythematous/brownish plaques, detectable by touch and often associated with desquamation, extended to a large skin surface.

The plaques contain a more abundant lymphocyte infiltrate than the patch phase, arranged in a band at the level of the superficial dermis. The lymphocytes have hyperchromatic and pleomorphic nuclei with a "cerebriform" appearance. Epidermal tropism becomes even more evident and groups of lymphocytes are arranged to form characteristic “Darier-Pautrier micro-abscesses”.

The tumor phase is clinically manifested by the presence of nodule / tumor lesions often coexisting with patches and plaques (Figure 1.4).

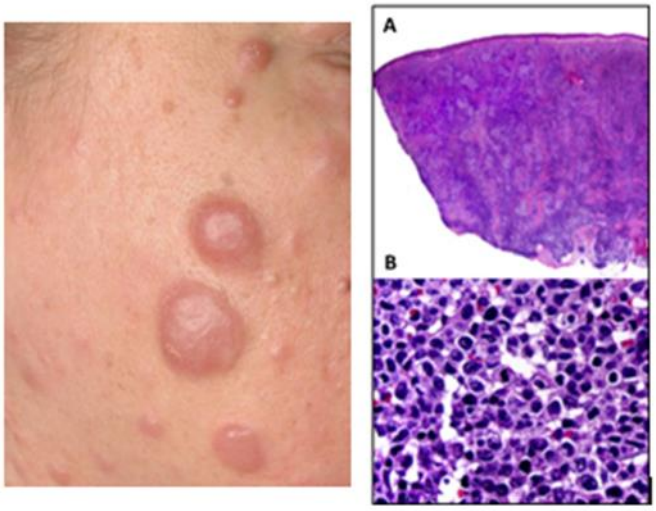

Figure 1.4: Clinical presentation and histopathology of a tumor-phase MF lesion. The tumor nodules show a diffuse lymphocyte infiltrate consisting of large and irregular lymphocytes (Mu˜noz-González H, et al., 2016).

Tumor lesions grow vertically and form brownish-red smooth surface nodules that can sometimes reach a few centimeters in size and undergo ulceration with the risk of infection (Girardi M, et al., 2004). They contain a lymphocytic infiltrate with a diffuse or nodular growth pattern. The neoplastic lymphocytes are localized throughout the thickness of the dermis and can extend to the subcutaneous level. The cells are large and pleomorphic, with a hyperchromatic nucleus and an evident nucleolus. (Figure 1.4 A and B).

The 2018 WHO / EORTC classification describes 3 clinical pathological variants of MF termed follicular mycosis fungoides, pagetoid reticulosis and granulomatous mycosis fungoides. These variants show differences in terms of lesion morphologies, distributions and clinical manifestations compared to ‘classic’ MF described above.

Diagnosis

Diagnosis of MF is accomplished through a combination of immunohistochemical and molecular investigations with clinical and histological findings. Immunophenotypically, neoplastic lymphocytes exhibit the typical markers of T Helper lymphocytes (CD4 +, CD2 +/-, CD3 +/-, CD5 +/-, CD7 +/- and CD26 +/-). Loss of CD7 followed by CD5 is common. In 20% of cases, the neoplastic cells have a CD8+ phenotype. In the advanced stage of the disease, neoplastic lymphocytes can begin to express the CD30 antigen, an event associated with a worse prognosis (Willemze R, et al, 2005).

Monoclonal rearrangement of the TCR receptor can be detected by PCR or Southern Blot. However, neither TCR rearrangement nor the loss of CD7 is specific for MF (Ferrara G, et al., 2008). High expression of PDCD1 (Programmed cell death 1) and TOX (thymocyte selection-associated high mobility group box protein) may be useful for diagnosis in the early stages of MF (Zhang Y, et al., 2012).

Staging and prognostic factors

The staging system adopted for MF is based on the TNMB system, where parameter T describes the extension / distribution of the primary skin involvement, parameter N reflects the extension of the pathology to the lymph node area, parameter M the presence of metastases and parameter B the involvement of peripheral blood (Olsen E, et al., 2007). Scores for involvement of the TNMB compartments can then be used to derive a clinical stage (I, II, III, IV) (Olsen E, et al, 2007). Stages range from IA and IB, which have low scores for skin involvement (T) and blood (B) involvement, to Stage IVA1, IVA2, and IVB, which are distinguished by high scores for B or visceral metastasis (M).

Proper staging and prognostic stratification require laboratory tests that include complete blood counts, liver function tests and measurement of blood levels of LDH. Imaging investigations, such as PET / CT, are useful for detecting visceral involvement. Most patients with MF are diagnosed with early-stage disease (stages I–IIA) (Maguire A, et al, 2020), which is considered localized. About one-quarter to one-third of patients with early-stage disease eventually progress to more advanced, late-stage disease. The median survival of MF patients decreases dramatically from Stage IA (35 years) to Stage IIB (4.7 years) to Stage 4B (1.4 years) (Olsen E, et al., 2007).

1.2.3 Treatment of MF

There are currently few effective therapies for the treatment of MF, which must take into consideration skin extension, staging, relapse, treatment refractoriness, patient age and any comorbidities (Jawed SI, et al., 2014). Treatments for the early stages (stage I and IIA) generally consist of a skin-directed therapy, and treatments for the late stages are generally systemic, and often combined.

"Skin-directed" therapies

Skin-directed treatment (SDT) is the first line of treatment for early-stage MF (IA to IIA), which is characterized by patches and/or plaques with little to no lymph node involvement and no visceral involvement (National Comprehensive in Oncology, 2021; Willemze.R, et al., 2018; Trautinger. F, et al., 2017). Corticosteroids, UV phototherapy, photochemotherapy, and radiotherapy allow for reasonable control of early disease (Hodak.E et al., 2015).

Narrow-band UVB (NB-UVB) phototherapy is recommended for patients with patch/thin plaque MF. Psoralen-UVA (PUVA), in which UVA is administered a few hours after an oral dose of psoralen, is given to patients with thick plaques (National Comprehensive in Oncology, 2021; Willemze.R, et al., 2018; Trautinger. F, et al., 2017; E.A. Olsen. E.A., et al., 2016). UV phototherapy (especially PUVA) is linked with skin aging and increased incidence of non-melanoma skin cancers. Careful follow-up for UV-related skin cancer is needed (Ohtsuka M, et al., 2020).

Another first-line treatment for early-stage MF is topical chemotherapy with Mechloretamine hydrochloryl-NH_2 or Carmustine-BCNU, which are alkylating drugs derived from nitrogenous mustards (Lessin SR, et al., 2013).

Radiotherapy (ranging from 1 to 35 Gy) can be used in the early and advanced stages if the disease is localized (Willemze R, et al., 2018; Chan DV, et al., 2012). TSEBI (total skin electron beam irradiation), in which the entire body surface is irradiated with electron beams to distribute the dose evenly and avoid damage to deep tissues, can be effective for treating stage T2-T3 MF (Trautinger F, et al., 2017).

For advanced-stage MF (≥ IIB) topical therapies are generally used in combination with systemic treatments (Willemze R, et al., 2018).

Systemic therapies

Systemic chemotherapy is administered to patients with relapsed or refractory disease or with advanced nodal or visceral disease at presentation.

Systemic chemotherapy agents include the retinoids acitretin and Bexarotene (Jawed SI, et al, 2014; Duvic M, et al., 2001). In poor responders, Bexarotene is combined with other anti-CTCL therapies, including PUVA, methotrexate, and alpha interferon (Whittaker S, et al., 2012). Interferon (IFN) therapy or combination therapy with IFN can be used for patients with advanced-stage disease (Olsen, E.A, et al., 2003).

Mono or multi-chemotherapy is considered for the advanced forms of MF that are resistant to previous treatments. The chemotherapeutic agents used alone are liposomal doxorubicin, gemcitabine, methotrexate, pralatrexate and chlorambucil (Trautinger F, et al., 2017; Dummer R, et al., 2012; Marchi E, et al., 2005).

Polychemotherapy regimens, used when there is nodal or visceral involvement (Willemze R, et al., 2005), include CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), CHOP-like, CVP (cyclophosphamide), vincristine, prednisone), MACOP-B (methotrexate, adriamycin, cyclophosphamide, vincristine, bleomycin and prednisone) (Trautinger F, et al., 2017). Multi-chemotherapy regimens increase the risk of opportunistic infections and further complications such as cardiotoxicity, nephrotoxicity, and myelosuppression. Although chemotherapy treatments lead to complete remission in some cases, the duration is very short (Wilcox RA, et al., 2016), and they do not significantly prolong survival (Hughes CF, et al., 2015).

Monoclonal antibody therapy represents another treatment alternative for patients with lymphoid malignancies. An example is Alemtuzumab, a recombinant humanized IgG1 monoclonal antibody targeting the CD52 glycoprotein expressed in normal and neoplastic T and B lymphocytes but not on haematopoietic progenitors. Alemtuzumab induces death of target cells mediated by the activation of the complement cascade, ADCC (antibody-dependent cell-mediated cytotoxicity) and the induction of apoptosis (Fraser G, et al., 2007). A problem related to the use of Alemtuzumab is the high risk of infectious complications (Wilcox RA, 2017). Brentuximab vedotin is an anti-CD30 monoclonal antibody conjugated to the antimitotic agent monomethyl auristatin E (Prince HM, et al., 2018). After binding to CD30 on the cell surface, the antibody is internalized, and the antimitotic agent is released inside the cell, inducing cell cycle arrest. Adverse effects related to Brentuximab vedotin include peripheral neuropathy, severe neutropenia, asthenia, nausea and alopecia (Trautinger F, et al., 2017).

Mogamulizumab, a humanized monoclonal antibody targeting the CCR4 chemokine receptor, shows promise for managing advanced MF (Duvic M, et al., 2015).

Histone deacetylase inhibitors (HDACi) represent another treatment option. The HDACi Romidepsin and Vorinostat have been approved by the FDA for patients with recurrence of CTCL or refractory to previous treatments (Whittaker SJ, et al., 2010; Prince HM, et al., 2009), but show modest effects (Whittaker SJ, et al., 2010; Piekarz RL, et al., 2009) and might provide greater benefit if used in combination with monoclonal antibodies and mono or polychemotherapeutic treatments (Alaibac M, et al., 2018). The autologous bone marrow transplant approach, already used for other haematological neoplasms, proved ineffective due to frequent relapses after a few months of treatment.

Currently available treatments for the advanced and refractory stages of MF are not curative and are used for palliative purposes (Wilcox RA, 2016). Therefore, the search for new therapeutic options is presently one of the most significant challenges for mycosis fungoides treatment.

1.3 Reactive oxygen species (ROS) and neoplasms

1.3.1 ROS and their effects at the cellular level

Reactive oxygen species (ROS) are a heterogeneous group of highly reactive molecules that derive from partial reduction reactions of molecular oxygen O_2 (Schieber M, et al., 2014).

ROS are physiologically produced by chemical reactions within the cells and play an essential role in signal transmission, functioning as second messengers (Turrens J. F., 2003). The major ROS producers are the enzymes belonging to the NADPH oxidase (NOXs) family. These enzymes were identified for the first time in leukocytes as responsible for the production of ROS in response to inflammatory stimuli (Holmstrom KM, 2014). At the mitochondrial level, the main source of ROS is the electron transport chain (ETC) located in the inner mitochondrial membrane, which produces superoxide anion ($O_2^{\cdot}$-) due to the reaction of leaked electrons with O_2 (Mailloux, R. J. et al.,2021; Brand, M. D. et al.,2020).

Superoxide anions can be converted to hydrogen peroxide (H_2O_2) either spontaneously or by a reaction catalysed by superoxide dismutase (SOD). In turn, H_2O_2 can be completely reduced to H_2O by thioredoxin (TRX) or glutathione (GSH) via redox reactions catalyzed by the enzymes thioredoxin peroxidase and glutathione peroxidase, respectively. Superoxide anions and H_2O_2 that escape these detoxifying mechanisms can generate peroxynitrite (ONOO-) and hydroxyl (OH-$^{\cdot}$) anions, respectively, which are very reactive species whose deleterious effects include lipid peroxidation, oxidation of DNA and proteins, and cell death by necrosis or apoptosis.

ROS are potent cell signaling messengers (Ray et al., 2012) that modulate signal transduction pathways involved in proliferation (MAP kinase, PI3 kinase, PTEN, protein-tyrosine-phosphatase), in the regulation of ROS homeostasis and of antioxidant genes (thioredoxin, Ref-1, Nrf2), oxidative stress within the mitochondria, apoptosis, aging (p66Shc), iron homeostasis (IRE-IRP), and DNA damage response (ATM) (Ray et al., 2012). ROS intervene in these pathways by modulating the redox state of cysteine residues present within kinases, phosphatases and other transduction factors, which alters their function (Groeger G, et al., 2009) (Veal E.A., et al.,2017).

The PI3K/AKT signaling pathway is a very important ROS-regulated pathway that can promote cell proliferation and survival. Under physiological conditions, the PI3K-AKT pathway is activated upon binding of growth factors, hormones, and cytokines to a specific tyrosine kinase receptor at the cell membrane. This leads to activation of PI3K, which converts PIP2 to PIP3. PIP3 in turn activates the kinase AKT whose diverse substrates include mTORC1. The activation of mTORC1 produces as a final effect an increase in protein synthesis and cell survival through the direct phosphorylation of effector proteins (Aoki M, et al., 2001).

Levels of PIP3 are normally limited by the phosphatase PTEN, which converts it back to PIP2 (Lee SR, et al., 2003). The oxidizing properties of H_2O_2 can inactivate PTEN through the formation of a disulfide bridge between cysteine residues 142 and 71 in the enzyme's catalytic site. Resulting activation of the PI3K/AKT/mTOR pathway, as well as others, allows cell survival and growth under conditions of ROS stress**.**

1.3.2 Cellular antioxidant mechanisms

Given ROS's multiple roles and potential adverse effects, their intracellular levels need to be finely controlled. Cells possess redundant detoxifying mechanisms based on enzymatic oxidation-reduction and chelation reactions.

Reduced glutathione (GSH) and thioredoxin (TRX) are essential for maintaining a low level of ROS. GSH is a tripeptide composed of glutamic acid, cysteine, and glycine. After glutathione peroxidase (GPX) has catalyzed the redox reaction of H_2O_2, transforming it into H_2O, the oxidized glutathione must be returned to its reduced form by another enzyme, glutathione reductase (GR). This enzyme uses NADPH as a source of reducing equivalents (Aoyama., et al., 2015). Similar to glutathione, thioredoxin also requires NADPH as a reducing agent (Weissbach., et al., 2001).

NADPH is mainly produced via the pentose phosphate pathway (PPP).

ROS indirectly control activation of the redox-sensing transcription factor Nrf2 (nuclear factor, erythroid 2 like 2, NFE2L2) (Sporn MB and Liby KT, 2012). Under normal conditions, this factor is sequestered and degraded at the proteasomal level by the Keap 1 protein (Kelch-like ECH-associated protein 1). As the levels of intracellular ROS increase, Keap 1 changes its conformation due to the oxidation of cysteine residues. This modification favors the release of Nrf2, which can reach the nucleus. At the nuclear level, Nrf2 associates with small proteins called MAFs to form heterodimers that bind to the ARE (antioxidant-response element) oxidative stress response elements, present in the regulatory regions of genes that contribute to the expression of proteins involved in antioxidant mechanisms (Ray et al., 2012). Among these are the genes coding for the catalytic and regulatory subunits of glutamyl-cysteine ligase, the rate-limiting enzyme for the biosynthesis of reduced glutathione. Nrf2 also plays a role in maintaining glutathione in a reduced form by controlling the expression of glutathione reductase 1, an enzyme involved in NADPH production. Other enzymes with roles in maintaining NADPH levels include ME1 (malic enzyme 1), isocitrate dehydrogenase 1 (IDH1), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase, thioredoxin and thioredoxin reductase 1 and enzymes involved in the de novo biosynthetic pathway of serine (Dinkova-Kostova A., Abramov A. Y., 2015) (DeNicola GM, et al., 2015).

1.3.3 ROS and neoplasms

Altered reactive oxygen species (ROS) homeostasis is considered one of the most important hallmarks of the tumor phenotype. Intracellular ROS are tightly regulated second messengers and constitute a homeostatic "rheostat" whose set-point controls several signal transduction pathways which influence cell proliferation, senescence, and death (Rusten et al. 2002). Normal cells must maintain an equilibrium between ROS and antioxidant mechanisms; tumor cells maintain much higher levels of ROS. These high levels of ROS are potentially toxic to the cancer cell and must be balanced by a concomitant increase in antioxidant response. To maintain ROS balance, cancer cells abnormally activate oxidative stress response pathways and metabolic pathways capable of producing antioxidant molecules (Figure 1.5).

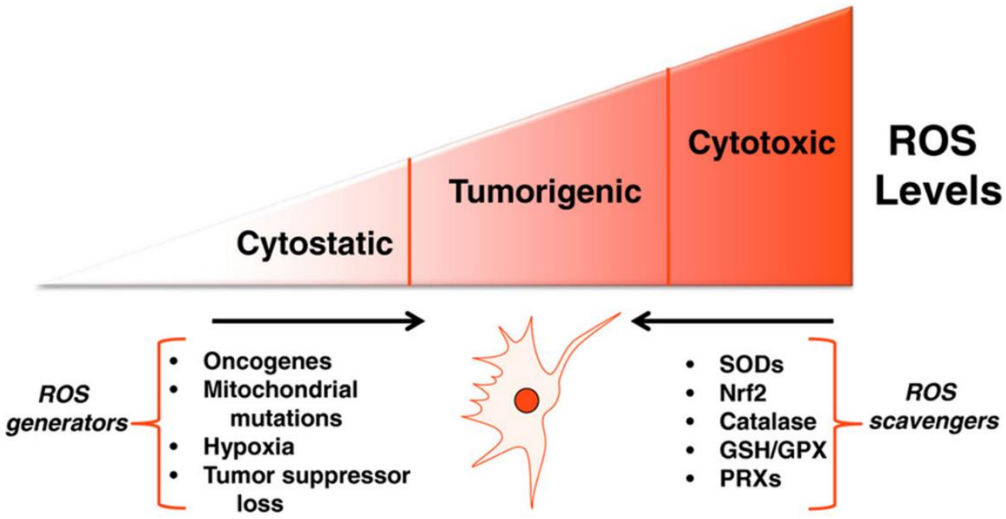

Figure 1.5: the balance between ROS production and scavenging allows cancer cells to keep ROS levels high but below toxic levels. Over-expression of oncogenes, inactivation of tumor suppressors, mitochondrial mutations and hypoxic conditions increase ROS levels which contribute to tumor transformation. Cancer cells also increase the activity of proteins and molecules with antioxidant function to allow the maintenance of ROS below toxic levels (Sullivan & Chandel, 2014). Balancing ROS generation and ROS scavenging allows cancer cells to remain in the tumorigenic range of ROS levels. Activation of mitochondrial ROS generation by oncogenes, mitochondrial mutations, hypoxia, or tumor suppressor loss increases ROS signaling to increase tumorigenicity. Tumor cells also express enhanced levels of antioxidant proteins that prevent increased ROS from reaching cytotoxic levels incompatible with growth.

As already reported, one of the most important is the pentose-phosphate pathway, which produces NADPH and ribose-5-phosphate as nucleotide precursors (Cairns et al., 2011). The folate cycle has recently emerged as an additional important source of NADPH in cancer cells (Fan J et al., 2014). The folate cycle operates at the cytoplasmic and mitochondrial levels and is a crucial metabolic pathway for cancer cells because it is coupled with the methionine cycle; it also produces the precursors necessary for synthesizing nucleotides (Yang, M., & Vousden, K. H, 2016). The amino acid serine takes part in this metabolic pathway and acts as a donor of carbon units to support the cycle.

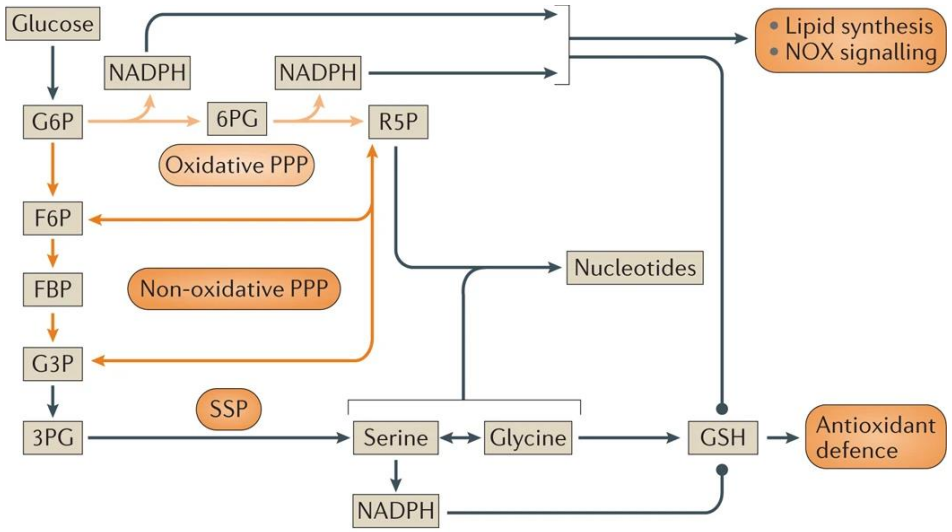

Figure 1.6: intersection of the metabolic pathways that allow the production of antioxidant defences and the production of nucleotides. The glycolytic intermediates of the oxidative phase and non-oxidative (Oxidative PPP- Non-Oxidative PPP) of the pentose phosphate pathway are used in order to produce ribose 5-phosphate (R5P) for the synthesis of nucleotides. Serine is also synthesized from a glycolytic intermediate (3PG). Serine can be transformed into glycine, and both can donate carbon units for nucleotide synthesis. The oxidative phase of the pentose phosphate pathway generates cytosolic NADPH in support of lipid synthesis, NADPH oxidase and the regeneration of glutathione (GSH) in a reduced form. Serine through the folate cycle can contribute to production of mitochondrial NADPH. Glycine, together with cysteine and glutamate, is a precursor for the synthesis of glutathione. In this way, the pentose phosphate pathway and the (de novo) serine biosynthesis pathway both contribute to the production of nucleotides and antioxidant agents to support cell proliferation and combat oxidative stress (Yang, M, et al., 2016).

Serine belongs to the group of non-essential amino acids and is synthesized starting from 3-phosphoglycerate, an intermediate of the glycolytic pathway. In physiological conditions, cells take up a large part of their serine from the environment. On the contrary, neoplastic cells increase the activation of the biochemical pathway that leads to the "de novo" synthesis of serine. In this way, tumor cells can implement the availability of nucleotide synthesis precursors and reduce power to counteract ROS-induced oxidative stress (Yang, M., & Vousden, K. H, 2016).

In this context, serine is involved in maintaining the redox balance since it acts as a precursor for synthesizing glycine and cysteine, which are glutathione precursors (Yang, M., & Vousden, K.H, 2016) (Figure 1.6). Serine is also involved in numerous other cellular anabolic processes as a component of many proteins and as a precursor of the synthesis of membrane lipids such as sphingolipids and phosphatidylserine (Kuge O, et al, 1998) (de Koning TJ, et al 2003) (Futerman AH & Riezman H, 2015).

In summary, both normal cells and tumor cells must balance ROS and antioxidant mechanisms. However, in tumor cells this equilibrium is reached at very high ROS levels and close to the toxicity

threshold. Therefore, the alteration of this balance represents a potential therapeutic target to affect neoplastic cells but not normal cells. The induction of tumor cell death through the use of compounds that, by increasing the quantity of ROS or by inhibiting the antioxidant mechanisms, can alter this balance, promising to be an attractive therapeutic alternative to selectively target the transformed cells (Figure 1.7).

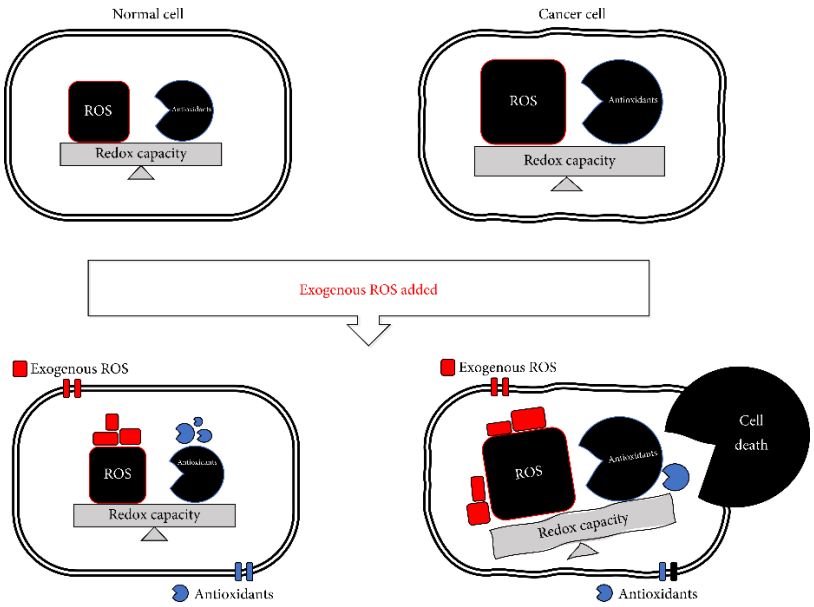

Figure 1.7: Differential ROS levels in normal and cancer cells: Normal cells have a lower basal ROS level than cancer cells. In normal cells, a moderate ROS level is essential to promote cell proliferation and survival whereas an excessive ROS level has detrimental effects such as tumor progression and angiogenesis. The redox balance in cancer cells is readily regulated by increasing antioxidant processes. Once the ROS level exceeds the redox capacity in cancer cells, severe oxidative stress occurs, resulting in cancer cell death by the activation of apoptosis, autophagic cell death, and necroptosis.

2. OBJECTIVES

Although the initial stages of mycosis fungoides have a moderate clinical course, 30% of patients relapse or prove to be refractory to currently used treatments and are destined to progress to advanced stages of the disease for which, in fact, there is not a curative treatment.

The studies described in this thesis evaluate the effects of some compounds capable of altering ROS homeostasis on mycosis fungoides cells cultured in vitro. For this purpose, we used CBR-5884 and Everolimus (RAD-001), which block the metabolic pathways that limit ROS levels, namely the serine pathway and the glucose 6-phospho dehydrogenase pathway, respectively.

The production of cytosolic and mitochondrial ROS and the effect on cell viability after treatment with the single compounds or with their association were analysed. The data obtained will help pave the way for the development of new therapeutic strategies based on the modulation of cellular ROS for the treatment of patients affected by mycosis fungoides who relapse or are refractory to the current therapies.

We also attempted to make MMAE cytotoxicity specific for MF cells by conjugating it to a monoclonal antibody (ab) recognizing Cutaneous Lymphocyte Antigen (CLA), a characteristics homing receptor expressed by MF cells, in order to evaluate the ability of the antibody-drug conjugate (ADC) to induce cell death.

3. MATERIALS AND METHODS

3.1 Cell culture

In order to test new possible therapeutic strategies for the treatment of MF *in vitro*, we used the cell line called MyLa CD4+ (Merck, Catalog No. 95051033), originally derived from the skin biopsy of a patient affected by plaque-phase MF, with negative serology for HTLV-1 (Kaltof K, et al.,1992).
Patient-derived immortalized cancer cell lines, although hard to establish, are easy to use, and provide primary platforms for evaluating molecular pathways associated with malignant transformation and identification of cancer cell intrinsic vulnerabilities for drug screening purposes. CTCL cells have been studied extensively to explore their genomic and transcriptomic similarities among cell lines (as a group), as well as between cell lines and patient samples (Van Doorn, R., et al., 2009; Laharanne, E., et al., 2010).
We routinely culture many T-lineage cell lines in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (FBS, Sial), 2 mM L-glutamine L glutamine, 100U/L penicillin and 20U/L streptomycin ('RPMI'). As the data sheet supplied with the MyLa CD4+ cells indicated that their culture medium requires supplementation with human serum and cytokines (IL-2 and IL-4), we initially cultured them with these additional components, and then gradually adapted them to growth in RPMI. The adapted cell line is herein referred to as 'MyLa'.
MyLa cells grow in suspension forming characteristic cellular aggregates, which indicate good viability.
The ideal seeding concentration is between 300,000 and 900,000 cells/ml and was determined by setting up cultures at different concentrations. Cell viability was evaluated by labelling cells with 0.4% Trypan Blue, a vital exclusion dye which, by entering cells that have lost the integrity of the cytoplasmic membrane, stains only dead cells, discriminating them from live cells. MyLa cells were grown in a humidified tissue culture incubator, at a temperature of 37°C, in a water-saturated, 5% CO_2 atmosphere.

3.2 Pharmacological treatments

Everolimus/RAD-001 (EVE, Selleckchem), dissolved in ethanol, and CBR-5884 (CBR, Merck), dissolved in DMSO (Hybrimax, Sigma-Aldrich), were utilized in this work. Monomethyl auristatin E (MMAE, Merck) was dissolved in DMSO. MMAE conjugated to anti-CLA antibody was kindly provided by Prof. M. Alaibac.

The cells were plated at 600,000 cells/ml in complete RPMI 1640 medium and treated with single compounds or in combination, as indicated in the figures. The solvents ethanol and DMSO were used as controls. The cells were treated for 24h and then analyzed as described below.

3.3 Flow cytometry

Flow cytometry is a technique that allows to quantify different parameters (morphology, expression of specific molecules detected by fluorescent antibodies/probes) in a suspension of cells. The flow cytometer consists of 4 main components: (1) a fluidic component, which transports the cells to the counting chamber; (2) a series of lasers, whose beams are crossed by cells; (3) photomultipliers, which detect the optical signal and convert it into an electrical signal; and (4) data-processing software. Flow cytometry permits analysis of individual cells in a sample in a very short time (seconds). We employed flow cytometry to analyze ROS as described in Section 3.4 and cell death as described in Section 3.6.

3.4 Calculation of combination index

The term synergy describes the condition in which the effect of a combination of drugs is greater than the sum of the effects of the single agents. The possible pharmacological cooperation between agents used in this work was evaluated using Compusyn software (ComboSyn Incorporated) to calculate the combination index (CI). Compusyn software uses an algorithm based on the Chou-Talalay method (Chou and Talalay, 1984) to calculate the CI. When the CI < 1 the effect of the compounds is synergistic, when the CI=1 the effect is additive, while when the CI>1 the effects of the compounds is antagonistic.

3.5 Analysis of cellular and mitochondrial ROS production

The ROS-sensitive fluorescent probes CellRox DeepRed and MitoSox Red (Thermo Fisher Scientific) were used to analyze cellular and mitochondrial ROS production, respectively. MyLa cells were plated in 12-well plates at $6x10^5$ cells/ml and treated with 10 μM Everolimus, 6.25μM CBR, or

the combination of the two; all treatments were performed in triplicate. After 24 hrs, aliquots of cells were placed in a 96-well plate and labeled with 2 μM MitoSox Red or with 5 μM CellRox DeepRed in a final volume of 180 μl; the probes were added directly to the culture medium. The cells were placed in a tissue culture incubator for 30 minutes and then analyzed by flow cytometer (BD Celesta™), equipped for sampling from 96-well plates.

3.6 Cell death assay

After 24h of treatment with the single compounds or with their combinations, aliquots of cells were labeled with 2 μg/ml propidium iodide (PI) and 2 μl annexin V conjugated to the Alexa 647 fluorophore (Thermo Fisher Scientific) in 300 μl RPMI medium for 10 min at room temperature, and then analyzed by flow cytometry.

PI is a chemical compound capable of penetrating exclusively into cells that have a damaged cell membrane, then binding stoichiometrically to the nuclear DNA. Annexin V, on the other hand, binds to phosphatidylserine, which is exposed on the outer surface of the cell membrane of apoptotic cells. Live cells are both PI- and Annexin V-negative, early apoptotic cells are Annexin V-positive and PI-negative, while both Annexin V and PI positivity indicate late apoptosis. Necrotic cells are positive for PI only. The specific cell death reported in the figures was calculated with the following formula:

$$SCD = [(\underline{CD_T - CD_{NT}})/(100 - CD_{NT})]x100$$

where CD_T indicates the percentage of dead cells in treated samples, and CD_{NT} indicates the percentage of dead cells in control samples.

3.7 Immunoblotting

SDS-polyacrylamide gel electrophoresis-immunoblotting is a standard technique that permits size-dependent separation of proteins in complex lysates and their identification/quantification using specific antibodies after transfer to a membrane support. After treatments described in the text, aliquots of cells were collected, washed with PBS, and then lysed in cell disruption buffer (Ambion) containing inhibitors of phosphatases and proteases (PhosphoSTOP and Complete, Roche). Proteins were separated by SDS/PAGE in 4–20% Tris-HEPES gradient gels (BioRad) and transferred onto nitrocellulose membranes (GE Healthcare). The membranes were saturated with 3% bovine serum albumin prepared in TBS (50 mM TRIS-Cl, pH 7.5, 150 mM NaCl)-0.01% Tween-20, and then incubated overnight with mouse anti-G6PD antibody (Santa Cruz Biotechnology, 1:1000) and rabbit

anti-GAPDH antibody (GeneTex International Corp., 1:10000). Membranes were then washed twice and incubated for 1h with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (Thermo Fisher Scientific, 1:5000). Chemiluminescent signals were detected using Lite Ablot Turbo (EuroClone) and a Cambridge UVITEC imaging system.

3.8 Statistical analyses and preparation of figures

Statistical significance was evaluated using the nonparametric Mann-Whitney rank sum test calculated with SigmaPlot version 13.0 software (Systat Software, Inc.). Statistical significance was considered at $p<0.05$. Graphs were generated using Sigmaplot version 13.0. Figures were prepared using Powerpoint.

4. RESULTS

4.1 Cell death induced by Everolimus.

Previous studies conducted in the laboratory of Prof. Ciminale have shown that by disturbing the homeostasis of ROS in T cell acute lymphoblastic leukemia (T-ALL) it is possible to obtain an effective induction of cell death, confirming the rheostat theory exposed by Rustin in 2002 (Silic-Benussi et al., 2018). Further studies demonstrated that Everolimus, an inhibitor of the mTOR pathway, increases cellular ROS and induces cell death in T-ALL cells (Silic-Benussi et al., 2022).

Based on the results obtained in the T-ALL system, we set out to verify whether inhibition of the mTOR pathway by Everolimus, could increase cellular ROS and constitute an effective strategy in the treatment of mycosis fungoides (MF), using the MF cell line MyLa as a model.

We first evaluated the cytotoxic activity of Everolimus in MyLa cells by treating them with different concentrations of Everolimus for 24 hours and then analyzing cell death by propidium iodide (PI) staining and flow cytometry. As shown in Figure 4.1, Everolimus induced a statistically significant increase in cell death in a dose-dependent manner.

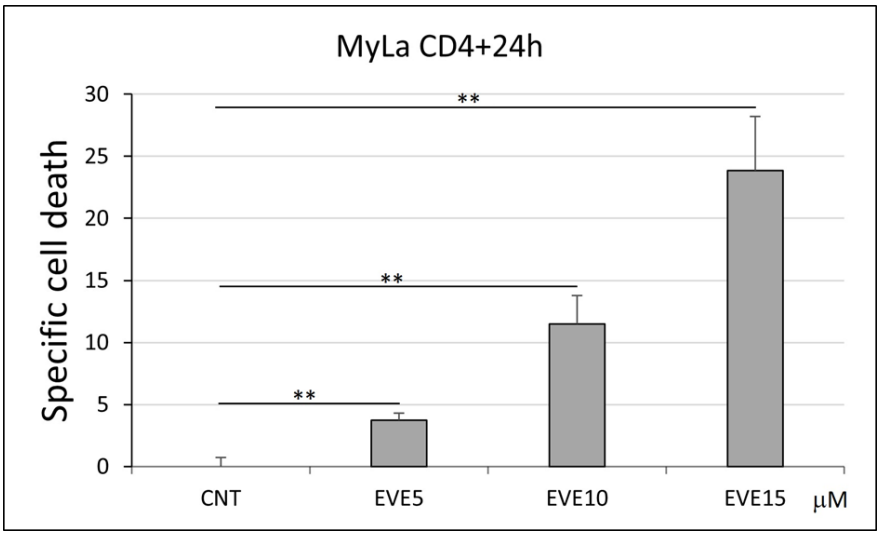

Figure 4.1: Cell death induced by Everolimus. The graph shows the death of MyLa cells, calculated as specific cell death, after 24h of treatment with increasing doses of Everolimus (EVE, 5- 10- or 15 μM). The mean values and standard error bars of three independent experiments performed in technical triplicates are represented. P-values were calculated with the nonparametric Mann-Whitney test.

4.2 Everolimus treatment reduces the levels of the PPP enzyme glucose-6-phosphate dehydrogenase.

In T-ALL cells, Everolimus was shown to reduce the levels of glucose-6-phosphate dehydrogenase (G6PD), the key enzyme of the PPP (Silic-Benussi et al., 2022). To determine if this occurs in MyLa cells, protein lysates of control- and Everolimus-treated MyLa cells were subjected to SDS-PAGE followed by immunoblotting to detect G6PD along with GAPDH, which served as a control for total protein quantity. Results showed that Everolimus reduced G6PD levels starting from the concentration of 10 μM.

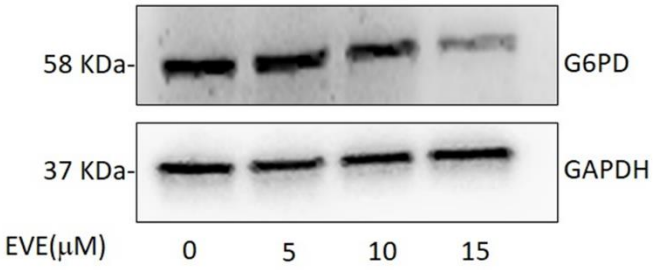

Figure 4.2: Everolimus reduces the levels of G6PD in MyLa cells. MyLa cells were treated with EVE (5 μM, 10 μM, 15 μM) for 24 hours and harvested for immunoblotting to detect G6PD expression levels. GAPDH was used as loading control.

4.3 Co-treatment with Everolimus and CBR-5884 induces death in MyLa cells.

As described in the Introduction, the Serine Synthesis Pathway plays an important role in maintaining the cellular redox balance (See Section 1.3.3). We therefore investigated the effects of inhibition of the Serine Synthesis Pathway in MyLa cells by treating them with CBR-5884 (CBR) alone or in combination with Everolimus.

Figure 4.3A shows the trend of cell death (calculated as specific cell death) induced by the different concentrations of the compounds. Co-treatment with Everolimus and CBR resulted in a statistically significant increase (**) in cell death compared to the single treatments, starting with the lowest doses.

Possible synergism between the 2 drugs was investigated by calculating the Combination Index (CI) with respect to the "fraction affected" (FA) (Chou and Talalay, 1984). The CI value less than 1 at low concentrations of Everolimus and CBR (Figure 4.3B) indicated the existence of a synergistic interaction. Based on these findings, we decided to use the 6.25 μM CBR and 10 μM Everolimus in subsequent experiments.

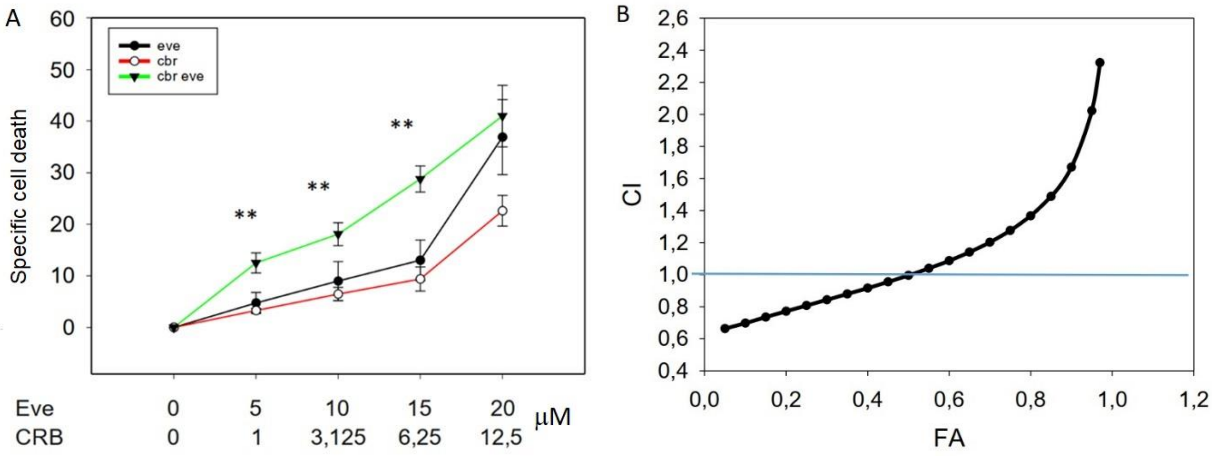

Figure 4.3: Cell death induced by Everolimus and CBR. The left-hand panel shows the death of MyLa cells, calculated as specific cell death, after 24h of treatment with increasing doses of CBR (red), EVE (black) and the combination CBR+EVE (green). The p-value was calculated using Mann Whitney's nonparametric test. The difference between the groups treated with the single agents and the groups treated with the combination was considered statistically significant with a p-value <0.01 (**). The right-hand panel shows the combination index of EVE and CBR in Myla cells was calculated using CompuSyn software. The FA (fraction affected) was calculated using specific cell death values measured as percentages of PI-positive cells.

4.4 Everolimus and CBR-5884 induce ROS and cell death.

To verify the correlation between the induction of cell death and changes in ROS, we analyzed the production of cellular and mitochondrial ROS after treatment of MyLa cells with CBR and Everolimus, using Cell-Rox and Mito-Sox fluorescent probes, respectively. Upon exposure to ROS, these probes emit a fluorescent signal that can be quantified by flow cytometry. In parallel, the effects on cell viability were measured by flow cytometric analysis after staining the cells with PI. The cells were treated for 24h with 10 µM Everolimus, 6.25 µM CBR and with both drugs and then labelled with the ROS probes and PI.

The graph in **Figure 4.4A** shows the fluorescence values of the Cell-Rox and Mito-Sox fluorescent probes for the cells treated with the drugs normalized against signals obtained for the untreated cells (CNT). In this analysis, cells treated with CBR showed increases in cellular ROS and mitochondrial ROS that were more pronounced that the increased obtained with Everolimus alone. However, the combination of Everolimus plus CBR did not result in a significant further increase in ROS levels (in other words, Everolimus did not increase the ROS-inducing effects of CBR).

The graph in **Figure 4.4B** shows the specific cell death after treatment with Everolimus, CBR and with the combination. The results confirmed that the co-administration of the two compounds can significantly increase the induction of cell death compared to the single compounds (p-value 0.001 between EVE and EVE+CBR; p-value of 0.034 between CBR and EVE+CBR).

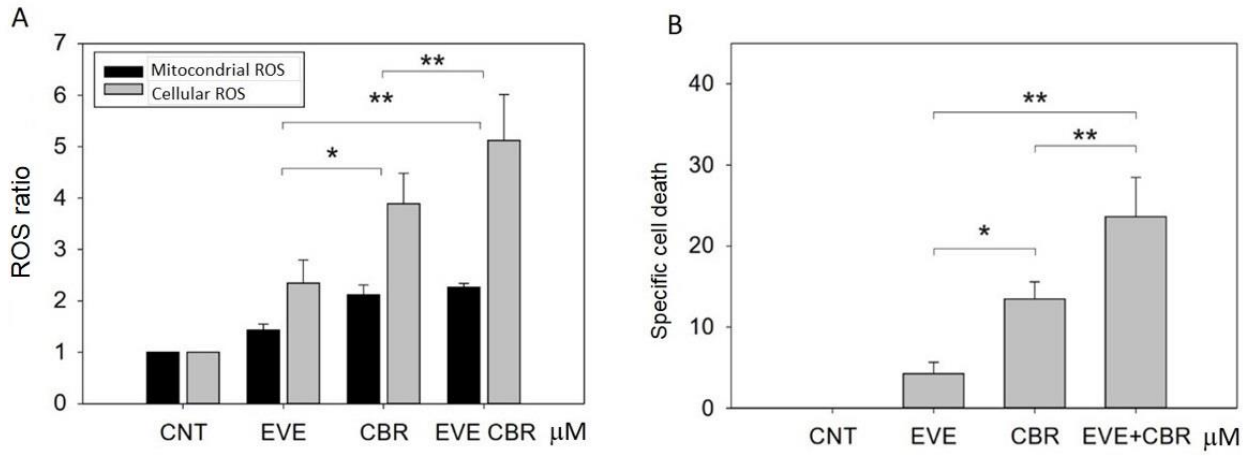

Figure 4.4: Treatment with CBR, EVE and EVE+ CBR increases cellular and mitochondrial ROS levels and induces cell death. Panel A shows the fluorescence values of the Cell-Rox (grey) and Mito-Sox (black) probes normalized with respect to controls (CNT), measured after 24h of treatment with EVE, CBR and with the combination of the two (EVE+CBR). Panel B depicts cell death, calculated as specific cell death, after 24h of treatment with EVE, CBR and with the combination of the two (EVE+CBR). The mean values and standard error bars of three independent experiments performed in technical triplicate are represented. P-values were calculated with the nonparametric Mann-Whitney test. Results with a p-value <0.05 (*), <0.01 (**) were considered as statistically significant.

4.5 Treatment with EVE+CBR induces apoptosis in MyLa cells.

In order to determine the prevalent death mechanism in MyLa cells treated with 10 μM Everolimus, 6.25 μM CBR and the combination of the two, cell death was measured by staining to detect Annexin V (a marker of apoptosis) and PI (a marker of necrosis or late apoptosis if the cells are also Annexin V-positive). In the resulting flow cytometry scatter plots shown in Figure 4.5, Annexin V-positive (early apoptotic) cells are represented by green dots in the lower right-hand quadrants, PI-positive (necrotic) cells shown as black dots in the upper left quadrant, and double-positive (late-apoptotic) cells are shown as red dots in the upper right quadrant. A comparison of percentages of cells presents in these populations showed that all the treatments induced apoptotic death measured as double positivity. EVE+CBR induced a substantially larger increase in the percentage of double-positive (late apoptotic) cells and a minor increase in PI-only positive (necrotic) cells compared to the single treatments.

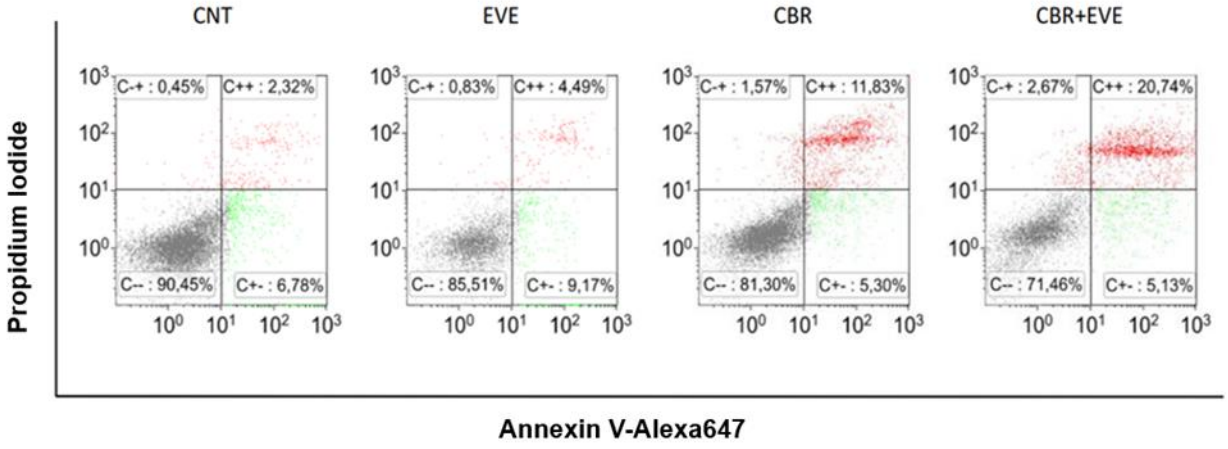

Figure 4.5: Cell death assay with Annexin V and PI. Shown are the cytograms obtained by labelling MyLa cells with Annexin V and PI after 24h of treatment with EVE (10μM), CBR (6.25 μM) or EVE+CBR. Results were analysed using Kaluza Analysis software (Beckman Coulter Life Sciences).

4.6 Cell death induced by MMAE and MMAE+ Anti-CLA-Ab treatment.

Most cytotoxic drugs used in cancer pharmacotherapy do not preferentially localize at the tumor site, causing undesired toxicities that restrict dose increases. This problem can be overcome by attaching the drug to a molecule that will selectively bind to and be taken up by the tumor cells. MMAE (monomethyl auristatin E) is an anti-mitotic agent which inhibits cell division by blocking the polymerization of tubulin. We attempted to make MMAE cytotoxicity specific for MF cells by conjugating it to a monoclonal antibody (ab) recognizing Cutaneous Lymphocyte Antigen (CLA), a characteristic homing receptor expressed by MF cells (See Figure 1.1). In order to evaluate the ability

of the antibody-drug conjugate (ADC) to induce cell death, MyLa cells were treated with MMAE, CLA-ab, or ADC (ab-MMAE) at the concentrations indicated in Figure 4.6. Results showed that MMAE was able to induce death in MyLa cells in a dose-dependent manner (Figure 4.6, left-hand panel). However, the ADC exhibited lower cytotoxic activity compared to MMAE or CLA-ab alone (Figure 4.6, right-hand panel).

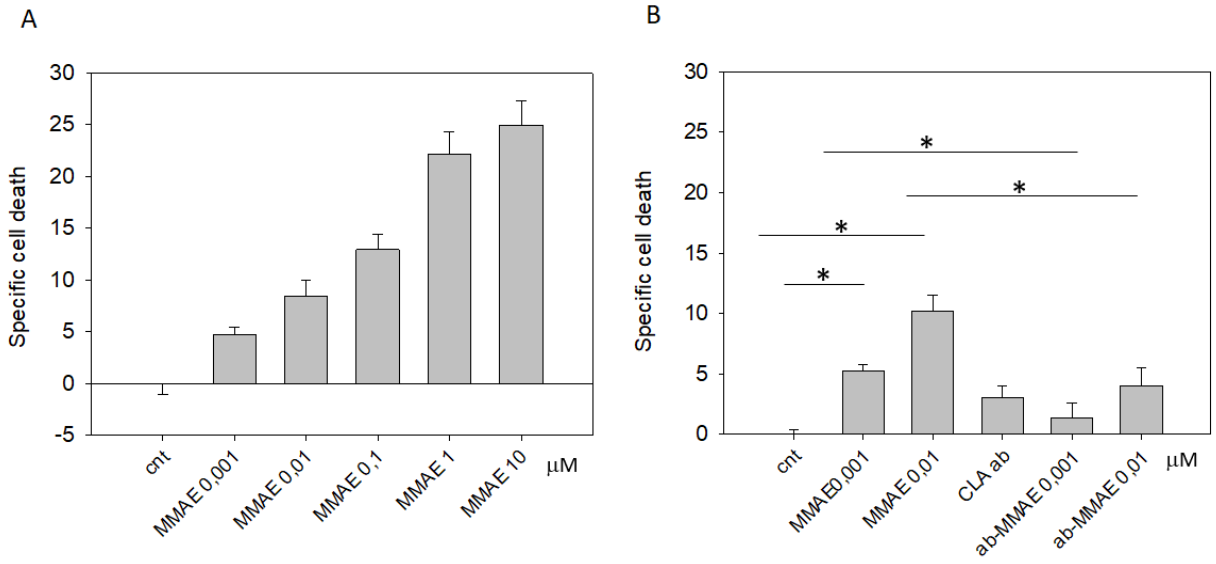

Figure 4.6: Treatment of MyLa cells with MMAE and ab-MMAE. The left-hand panel shows death of MyLa cells, calculated as specific cell death, after 24h of treatment with increasing doses of MMAE (indicated in µM). The left-hand panel shows death induced by MMAE and CLA-ab used as single agents, compared to the ADC (ab-MMAE). Statistically significant differences between the control cells and the cells treated with MMAE or ab-MMAE were calculated using the non-parametric Mann-Whitney test.

4.7 Detection of CLA and CLA-MMAE on/in MyLa cells.

The low cytotoxic activity observed for ab-MMAE prompted us to verify whether the MyLa cells were bound by the ADC and internalized it. To this end, we incubated MyLa cells with vehicle, CLA-ab, or ab-MMAE for 72 hours. Cells were then washed and, without fixing or permeabilizing them, incubated with FITC-conjugated secondary antibody and analyzed by flow cytometry. As shown in Figure 4.7, the percentages of FITC-positive cells were higher in both the CLA-ab treated sample (80.8%) and the ab-MMAE treated sample (62.7%) compared to the untreated control (1.83%). This result indicated that the ab-MMAE conjugate bound to the cell surface but perhaps was not efficiently internalized into the cells.

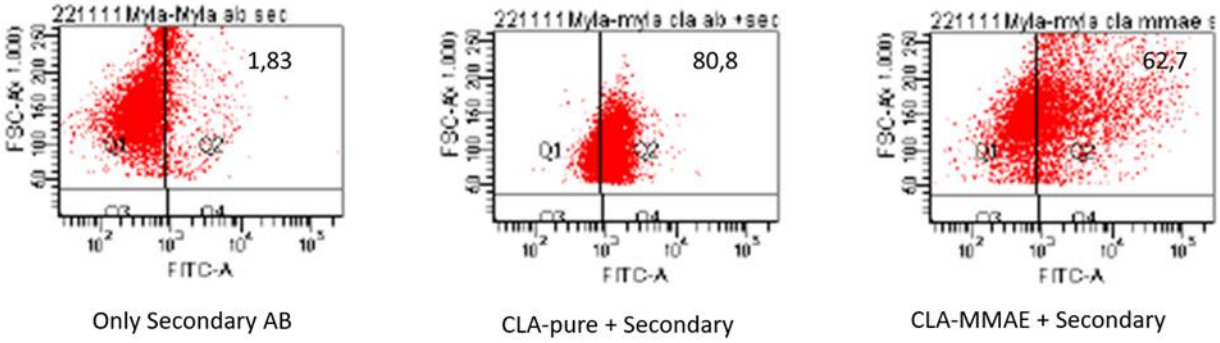

Figure 4.7: Detection of CLA and CLA-MMAE on MyLa cells. MyLa cells were cultured for 72h with CLA-ab and ab-MMAE and then labelled with the FITC-conjugated secondary antibody for 1h at RT. CLA-positive cells were detected by flow cytometry.

4.8 Co-treatment with Everolimus or Venetoclax does not increase killing by MMAE.

Because MMAE conjugation with CLA-ab failed to increase the anti-mitotic drug's specific cytotoxic effect, we tested other strategies to reduce the effective dose of MMAE. We hypothesized that the combination of MMAE with Everolimus or Venetoclax (a BCL2 inhibitor able to induce apoptosis in BCL2-overexpressing cells) could reduce the effective dose of MMAE.

To this end, we treated MyLa cells with increasing concentrations of MMAE (0.001-10 μM,) and 10μM Everolimus or 100 nM Venetoclax for 24 hours and measured cell death by PI staining and flow cytometry. As shown in Figure 4.8, the combination of MMAE plus EVE (Figure 4.8A) and MMAE plus Venetoclax (Figure 4.8B) did not increase specific cell death compared to MMAE used as a single agent.

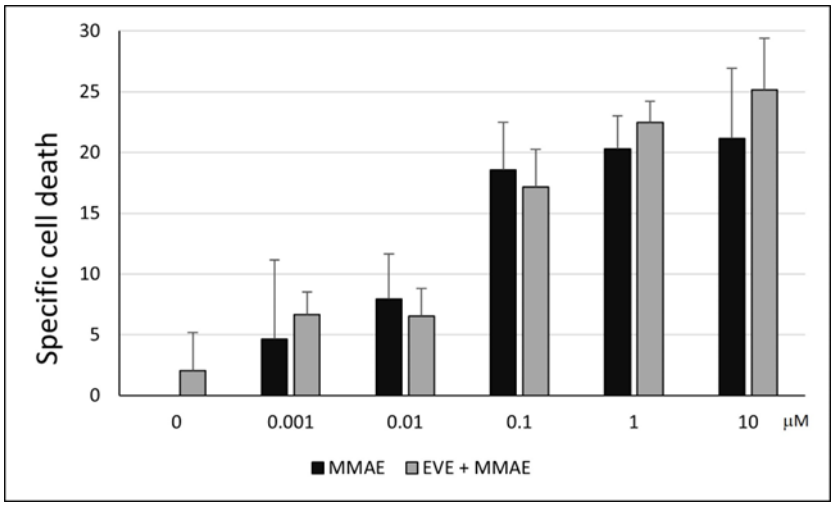

Figure 4.8 A: Treatment with MMAE+Everolimus. The graph shows the death of MyLa cells, calculated as specific cell death, after 24h of treatment with increasing doses of MMAE and the combination of MMAE+EVE (10µM). The combination of the two compounds did not increase death induced by the single treatment.

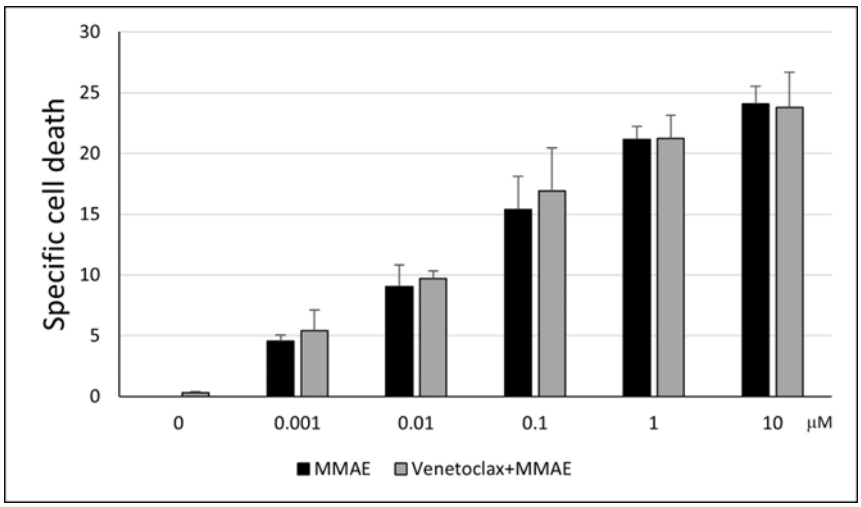

Figure 4.8 B: Treatment with MMAE+Venetoclax. The graph shows the cell death after 24h of treatment with increasing doses of MMAE and the combination of the MMAE+VEN (100 nM).

5. DISCUSSION

A variety of treatment strategies have been developed for MF patients. However, as pointed out in the Introduction, the outlook is dismal for patients who have MF beyond stage IIA. Advanced disease is essentially incurable, and the treatments currently used are primarily palliative. Thus, implementing new therapeutic strategies is indispensable for treating MF patients with no therapeutic alternatives.

The laboratory that hosted my thesis research is studying the manipulation of ROS homeostasis as a possible therapeutic strategy for the treatment of T-cell neoplasms. This strategy is based on the observation that cancer cells have more ROS than normal cells, and to maintain redox balance, they activate numerous metabolic pathways. Among these, one of the most important is the metabolic pathway of pentose-phosphates, which produces the NADPH necessary to restore the reserves of glutathione and thioredoxin. Experiments performed on the T-cell neoplasm T-cell acute lymphoblastic leukemia showed that the mTORC1 inhibitor Everolimus (Mittal SK, et al., 2009) raised the levels of ROS and reduces the levels of G6PD, the rate-limiting enzyme of the pentose phosphate pathway, and sensitized T-ALL cells to death stimuli (Silic-Benussi et al., 2022).

Everolimus is a drug already widely used in clinical practice as an immunosuppressant in transplant patients. Its use has also been approved for some advanced-stage tumors such as renal cell carcinoma, neuroendocrine tumors, and some forms of breast cancer (Porta C, et al., 2014).

Two clinical studies administered Everolimus at a daily dosage of 10mg to 16 patients with refractory T-cell lymphoma, 7 of whom had MF. The overall response rate to treatment was 44%, and the median progression-free survival was 4.1 months, indicating limited benefit (Witzig TE, et al., 2015).

To build on the laboratory's studies of ROS in T-cell neoplasms and to search for drug combinations that might improve the response of MF cells to Everolimus, I performed experiments with the MF cell line MyLa, which was originally established from an MF plaque of an elderly male patient with stage II disease (Kaltof K, et al.,1992). It must be pointed out that the use of a single cell line in these experiments represents a limitation of the study.

As shown in Figure 4.1, Everolimus induced a statistically significant increase in the death of MyLa cells in a dose-dependent manner. This effect was paralleled by a decrease in the levels of G6PD (Figure 4.2).

As described in the Introduction, the amino acid serine plays an important role in restoring antioxidant reserves in cancer cells. Serine is a precursor for synthesizing glycine and cysteine, glutathione precursors. In addition, it acts as a donor of carbon units to feed the folate cycle, whose importance as a source of NADPH for maintaining the redox balance in tumor cells has recently been emphasized.

I therefore tested the effects of CBR-5884, an inhibitor of de novo serine synthesis, on MyLa cells. Reults showed that CBR-5884 induced a consistent increase in ROS and death in MyLa cells (Figures 4.3 and 4.4). Furthermore, the combination of CBR-5884 with Everolimus further increased ROS accumulation (Figure 4.4) and synergistically increased death in MF cells (Figure 4.3). These observations, although preliminary, suggest that pharmacological inhibition of de novo serine synthesis might enhance the therapeutic effects of Everolimus in MF patients.

Most cytotoxic agents used for cancer therapy do not preferentially localize to the tumor site, causing unwanted toxicities (Krall N, et al., 2013). Therefore, antibodies represent an exciting strategy to target neoplastic cells selectively and can deliver molecules inside the cell, thus improving the therapeutic index of anticancer drugs. Monoclonal antibodies that recognize molecules expressed on the surface of lymphocytes have recently been introduced. Two of these, alentuzumab and mogalizumab target the CD4 and CD52 antigens, which are expressed by T lymphocytes and are, therefore, poorly selective for neoplastic lymphocytes. They therefore can cause a global reduction in circulating T lymphocytes, increasing the risk of severe immunosuppression.

Prof. M. Alaibac and colleagues recently tested the ability of an antibody against the CLA antigen conjugated to a photoactivable IRDye®700DX dye to selectively target MF cells. When exposed to red light, IRDye®700DX dye generated an oxygen singlet, a free radical capable of irreversibly damaging cancer cells. This approach yielded very promising results. Unfortunately, this line of research was abruptly interrupted as the IRDye®700DX molecule is no longer commercially available.

Brentuximab Vedotin (BV) consists of an anti-CD30 monoclonal antibody conjugated to monomethyl auristatin E (MMAE). Treatment with this preparation is very effective in patients with CD30 expression. CD30 is a surface antigen internalized once engaged by binding with its ligand. Thus, MMAE enters the cell, inhibiting tubulin polymerization, resulting in cell cycle arrest and apoptosis (Prince, H.M, et al., 2017; Van de Donk, N.W, et al., 2012). Gebleux and colleagues recently found that MMAE has good antitumor activity even when conjugated to antibodies that are not internalized. In this case, the proteolytic release of MMAE takes place in the extracellular matrix by specific proteases.

These results prompted us to test a CLA antibody-MMAE conjugate (ADC) provided by Prof. Alaibac on MyLA cells. Unfortunately, the ADC was not effective in inducing cell death (Figure 4.6). The failure of this approach may be due to several reasons, including i) the possible absence of specific proteases in MyLa cells that are needed to release MMAE from the antibody; or ii) treatment with an insufficient quantity of the ADC. These possibilities will be explored in future experiments

using additional MF cell lines and possibly with a new MF-targeting antibody conjugated with another photoactivable molecule.

6. CONCLUSIONI

MF is the most prevalent form of CTCL. Even though the early stages proceed slowly, about 30% of individuals develop advanced forms of the disease that are still incurable. Therefore, the development of new therapeutic strategies is fundamental.

With the present study we have contributed to demonstrate that the increase in ROS induced by the co-treatment with Everolimus and CBR-5884 is able to determine, compared to the treatment with the single compounds, a significant increase in death in cultured mycosis fungoides cells "in vitro".

We also tried a different therapeutic strategy using an antibody-drug conjugate (ADC), comprised of the drug MMAE and the monoclonal antibody (ab) targeting Cutaneous Lymphocyte Antigen (CLA),as a key homing receptor produced by MF cells. the results demonstrated that MMAE could induce death in MyLa cells in a dose-dependent manner, although the ADC had lesser cytotoxic effect than MMAE or CLA-ab alone.

The first data show that a therapeutic approach based on ROS regulation could be a promising therapeutic alternative for individuals who relapse or are refractory trently available treatments.

7. References

1.Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. 6th. Saunders Co p. 2000.

2.Abrams JT, Vonderheid EC, Kolbe S, Appelt DM, Arking EJ, Balin BJ. Sezary T-cell activating factor is a Chlamydia pneumoniae-associated protein. Clin Diagn Lab Immunol. 1999;6(6):895-905.

3.Alaibac M. Monoclonal antibodies against cutaneous T-cell lymphomas. Expert Opin Biol Ther. 2017;17(12):1503-10.

4.Aoki M, Blazek E, Vogt PK. A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(1):136-41.

5.Aoyama K, Nakaki T. Glutathione in Cellular Redox Homeostasis: Association with the Excitatory Amino Acid Carrier 1 (EAAC1). Molecules (Basel, Switzerland). 2015;20(5):8742-58.

6.Armerding D, Kupper TS. Functional cutaneous lymphocyte antigen can be induced in essentially all peripheral blood T lymphocytes. International archives of allergy and immunology. 1999;119(3):212-22.

7.Asadullah K, Docke WD, Haeussler A, Sterry W, Volk HD. Progression of mycosis fungoides is associated with increasing cutaneous expression of interleukin-10 mRNA. The Journal of investigative dermatology. 1996;107(6):833-7.

8.Bagherani N, Smoller BR. An overview of cutaneous T cell lymphomas. 2016; 5

9.Berger CL, Hanlon D, Kanada D, Dhodapkar M, Lombillo V, Wang N, et al. The growth of cutaneous T-cell lymphoma is stimulated by immature dendritic cells. Blood. 2002;99(8):2929-39.

10.Bradford PT, Devesa SS, Anderson WF, Toro JR. Cutaneous lymphoma incidence patterns in the United States: a population-based study of 3884 cases. Blood. 2009;113(21):5064-73.

11.Brand, M. D. Riding the tiger — physiological and pathological effects of superoxide and hydrogen peroxide generated in the mitochondrial matrix. Crit. Rev. Biochem. 2020; 55: 592–661

12.Burg G, Kempf W, Cozzio A, Dobbeling U, Feit J, Golling P, et al. Cutaneous malignant lymphomas: update 2006. J Dtsch Dermatol Ges. 2006; 4(11): 914-33.

13.Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nature reviews Cancer. 2011;11(2): 85-95.

14.Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. Blood. 2010;116(5): 767-71.

15.Chan DV, Aneja S, Honda K, Carlson S, Yao M, Katcher J, et al. Radiation therapy in the management of unilesional primary cutaneous T-cell lymphomas. The British journal of dermatology. 2012;166(5):1134-7.

16.Choi J, Goh G, Walradt T, Hong BS, Bunick CG, Chen K, et al. Genomic landscape of cutaneous T cell lymphoma. Nature genetics. 2015;47(9):1011- 9.

17.Chong BF, Wilson AJ, Gibson HM, Hafner MS, Luo Y, Hedgcock CJ, et al. Immune function abnormalities in peripheral blood mononuclear cell cytokine expression differentiates stages of cutaneous T-cell lymphoma/mycosis fungoides. Clin Cancer Res. 2008; 14: 646–53.

18.Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984; 22:27-55. doi: 10.1016/0065-2571(84)90007-4. PMID: 6382953

19.Criscione VD, Weinstock MA. Incidence of cutaneous T-cell lymphoma in the United States, 1973–2002. Arch Dermatol. 2007;143(7): 854-859.

20.Da Silva Almeida AC, Abate F, Khiabanian H, Martinez-Escala E, Guitart J, Tensen CP, et al. The mutational landscape of cutaneous T cell lymphoma and Sézary syndrome. Nat Genet. 2015; 47: 1465–70.

21.de Koning TJ, Snell K, Duran M, Berger R, Poll-The BT, Surtees R. L-serine in disease and development. The Biochemical journal. 2003;371(Pt 3):653-61.

22.DeNicola GM, Chen PH, Mullarky E, Sudderth JA, Hu Z, Wu D, et al. NRF2 regulates serine biosynthesis in non-small cell lung cancer. Nature genetics. 2015;47(12): 1475-81.

23.Dinkova-Kostova AT, Abramov AY. The emerging role of Nrf2 in mitochondrial function. Free radical biology & medicine. 2015;88(Pt B):179- 88.

24.Dobbeling U, Dummer R, Laine E, Potoczna N, Qin JZ, Burg G. Interleukin-15 is an autocrine/paracrine viability factor for cutaneous T-cell lymphoma cells. Blood. 1998;92(1):252-8.

25.Dobos G, Pohrt A, Ram-Wolff C, Lebbe C, Bouaziz JD, Battistella M, et al. Epidemiology of cutaneous T-Cell lymphomas: a systematic review and meta-analysis of 16,953 patients. 2020;12(10).

26.Dummer R, Goldinger SM, Cozzio A, French LE, Karpova MB. Cutaneous lymphomas: molecular pathways leading to new drugs. The Journal of investigative dermatology. 2012;132(3 Pt 1): 517-25.

27.Duvic M, Hymes K, Heald P, et al. Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II–III trial results. J Clin Oncol. 2001; 19: 2456–2471.

28.Duvic M, Martin AG, Kim Y, et al. Phase 2 and 3 clinical trials of oral Bexarotene (Targretin capsule s) for the treatment of refractory or persistent early-stage cutaneous T-cell lymphoma. Arch Dermatol. 2001; 137: 581–593.

29.Duvic M. Choosing a systemic treatment for advanced stage cutaneous T-cell lymphoma: mycosis fungoides and Sezary syndrome. Hematology Am Soc Hematol Educ Program. 2015; 529-44.

30.E.A. Olsen, E. Hodak, T. Anderson, J.B. Carter, M. Henderson, K. Cooper, H.W. Lim Guidelines for phototherapy of mycosis fungoides and sezary syndrome: a consensus statement of the United States cutaneous lymphoma consortium J Am Acad Dermatol. 2016; 74 :27-58

31.Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD. Quantitative flux analysis reveals folate-dependent NADPH production. Nature. 2014;510(7504):298-302.

32.Ferenczi K, Fuhlbrigge RC, Pinkus J, Pinkus GS, Kupper TS. Increased CCR4 expression in cutaneous T cell lymphoma. The Journal of investigative dermatology. 2002;119(6):1405-10.

33.Ferrara G, Di Blasi A, Zalaudek I, Argenziano G, Cerroni L. Regarding the algorithm for the diagnosis of early mycosis fungoides proposed by the International Society for Cutaneous Lymphomas: suggestions from routine histopathology practice. J Cutan Pathol. 2008;35(6):549-53.

34.Fraser G, Smith CA, Imrie K, Meyer R. Alemtuzumab in chronic lymphocytic leukemia. Curr Oncol. 2007;14(3):96-109

35.Fung MA, Murphy MJ, Hoss DM, Grant-Kels JM. Practical evaluation and management of cutaneous lymphoma. Journal of the American Academy of Dermatology. 2002;46(3):325-57; quiz, 58-60.

36.Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov. 2010; 9:775–89.

37.Gébleux R, Stringhini M, Casanova R, Soltermann A, Neri D. Non-internalizing antibody-drug conjugates display potent anti-cancer activity upon proteolytic release of monomethyl auristatin E in the subendothelial extracellular matrix. Int J Cancer. 2017 Apr 1;140(7):1670-1679. doi: 10.1002/ijc.30569. Epub 2016 Dec 30. PMID: 27943268

38.Girardi M. Cutaneous biology of gammadelta T cells. Adv Dermatol.2004;20:203-15.

39.Groeger G, Quiney C, Cotter TG. Hydrogen peroxide as a cell-survival signaling molecule. Antioxidants & redox signaling. 2009;11(11):2655-71.

40.Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nature reviews Molecular cell biology. 2014;15(6):411-21.

41.Hughes CF, Newland K, McCormack C, Lade S, Prince HM. Mycosisfungoides and Sezary syndrome: Current challenges in assessment,management and prognostic markers. Australas J Dermatol. 2016;57(3):182-91.

42.Iyer A, Hennessey D, O'Keefe S, Patterson J, Wang W, Wong G.K, Gniadecki R, Branched evolution and genomic intratumor heterogeneity in the pathogenesis of cutaneous T-cell lymphoma Blood Adv.2020; 4 (11): 2489-2500.

43.J. Kwon, S.R. Lee, K.S. Yang, Y. Ahn, Y.J. Kim, E.R. Stadtman, S.G. Rhee Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors Proc. Natl. Acad. Sci. U.S.A., 101 (47) (2004), pp. 16419-16424

44.Jawed SI, Myskowski PL, Horwitz S, Moskowitz A, Querfeld C. Primary cutaneous T-cell lymphoma (mycosis fungoides and Sezary syndrome): part II. Prognosis, management, and future directions. Journal of the American Academy of Dermatology. 2014;70(2): 40-42.

45.Kaltoft K, Bisballe S, Dyrberg T, Boel E, Rasmussen PB, Thestrup-Pedersen K. Establishment of two continuous T-cell strains from a single plaque of a patient with mycosis fungoides. In Vitro Cell Dev Biol. 1992 Mar;28A(3 Pt 1):161-7. doi: 10.1007/BF02631086. PMID: 1582990

46.Kim E.J., S. Hess, S.K. Richardson, S. Newton, L.C. Showe, B.M. Benoit, R. Ubriani, C.C. Vittorio, J.M. Junkins-Hopkins, M. Wysocka, A.H. Rook Immunopathogenesis and therapy of cutaneous T cell lymphoma J. Clin. Invest. 2005;115 (4): 798-812.

47.Kim EJ, Hess S, Richardson SK, Newton S, Showe LC, Benoit BM, et al. Immunopathogenesis and therapy of cutaneous T cell lymphoma. The Journal of clinical investigation. 2005;115(4):798-812.

48.Krall N, Scheuermann J, Neri D. Angew Small targeted cytotoxics: current state and promises from DNA-encoded chemical libraries. Chem Int Ed Engl. 2013 Jan 28;52(5):1384-402. doi: 10.1002/anie.201204631. Epub 2013 Jan 7. PMID: 23296451

49.Kuge O, Hasegawa K, Saito K, Nishijima M. Control of phosphatidylserine biosynthesis through phosphatidylserine-mediated inhibition of phosphatidylserine synthase I in Chinese hamster ovary cells. Proceedings of

the National Academy of Sciences of the United States of America. 1998;95(8):4199-203.

50.Laharanne E, Oumouhou N, Bonnet F, Carlotti M, Gentil C, Chevret E, Jouary T, Longy M, Vergier B, Beylot-Barry M, Merlio JP. Genome-wide analysis of cutaneous T-cell lymphomas identifies three clinically relevant classes. J Invest Dermatol. 2010 Jun;130(6):1707-18. doi: 10.1038/jid.2010.8. Epub 2010 Feb 4. PMID: 20130593

51.Laharanne, E.; Oumouhou, N.; Bonnet, F.; Carlotti, M.; Gentil, C.; Chevret, E.; Jouary, T.; Longy, M.; Vergier, B.; Beylot-Barry, M.; et al. Genome-wide analysis of cutaneous T-cell lymphomas identifies three Clinically relevant classes. J. Investig. Dermatol. 2010, 130, 1707–1718.

52.Lancet. 2017 Aug 5;390(10094):555-566. doi: 10.1016/S0140-6736(17)31266-7. Epub 2017 Jun 7. PMID: 28600132

53.Lessin SR, Duvic M, Guitart J, Pandya AG, Strober BE, Olsen EA, et al. Topical Chemotherapy in Cutaneous T-cell Lymphoma: Positive Results of a Randomized, Controlled, Multicenter Trial Testing the Efficacy and Safety of a Novel Mechlorethamine, 0.02%, Gel in Mycosis FungoidesTopical Chemotherapy in Cutaneous T-cell Lymphoma. JAMA dermatology. 2013;149(1):25-32.

54.Maguire A, Puelles J, Raboisson P et al. Early-stage mycosis fungoides: epidemiology and prognosis. Acta Derm Venereol 2020; 100: adv00013.

55.Mahalingam M, Reddy VB. Mycosis Fungoides, Then and Now... Have We Travelled? Advances in anatomic pathology. 2015;22(6):376-83.

56.Mailloux, R. J. An update on methods and approaches for interrogating mitochondrial reactive oxygen species production. Redox Biol. 45, 102044 (2021).

57.Marchi E, Alinari L, Tani M, Stefoni V, Pimpinelli N, Berti E, et al. Gemcitabine as frontline treatment for cutaneous T-cell lymphoma: phase II study of 32 patients. Cancer. 2005;104(11):2437-41.

58.Mittal SK, Sharma RK, Gupta A, Naik S. Increased interleukin-10 production without expansion of CD4+CD25+ T-regulatory cells in early stable renal transplant patients on calcineurin inhibitors. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology today. 1996;17(3):138-46.

59.Muñoz-González. H, A.M. Molina-Ruiz, L. Requena Clinicopathologic variants of mycosis fungoides Actas Dermosifiliogr. 2017;108(3): 192-208,

60.Narducci MG, Arcelli D, Picchio MC, Lazzeri C, Pagani E, Sampogna F, et al. MicroRNA profiling reveals that miR-21, miR486 and miR-214 are upregulated and involved in cell survival in Sézary syndrome. Cell Death Dis. 2011; 2: 151.

61.National Comprehensive Cancer network clinical Practice Guidelines in Oncology. Primary Cutaneous Lymphomas.2021

62.Nicolae-Cristea AR, Benner MF, Zoutman WH, van Eijk R, Jansen PM, Tensen CP, et al. Diagnostic and prognostic significance of CDKN2A/CDKN2B deletions in patients with transformed mycosis fungoides and primary cutaneous CD30-positive lymphoproliferative disease. The British journal of dermatology. 2015;172(3):784-8.

63.Ohtsuka M, Hamada T, Miyagaki T, Shimauchi T, Yonekura K, Kiyohara E, et al. Outlines of the Japanese guidelines for the management of primary cutaneous lymphomas 2020. J Dermatol. 2021;48: e49–71.

64.Olsen E, Vonderheid E, Pimpinelli N, Willemze R, Kim Y, Knobler R, et al. Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). Blood. 2007 ;110(6): 1713- 22.

65.Olsen EA. Interferon in the treatment of cutaneous T-cell lymphoma. Dermatol Ther. 2003;16(4):311-21.

66.Piekarz RL, Frye R, Turner M, Wright JJ, Allen SL, Kirschbaum MH, et al. Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2009;27(32):5410-7.

67.Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR Signaling in Cancer. Front Oncol. 2014 Apr 14; 4:64. doi: 10.3389/fonc.2014.00064. eCollection 2014. PMID: 24782981

68.Prince HM, Kim YH, Horwitz SM, Dummer R, Scarisbrick J, Quaglino P, Zinzani PL, Wolter P, Sanches JA, Ortiz-Romero PL, Akilov OE, Geskin L, Trotman J, Taylor K, Dalle S, Weichenthal M, Walewski J, Fisher D, Dréno B, Stadler R, Feldman T, Kuzel TM, Wang Y, Palanca-Wessels MC, Zagadailov E, Trepicchio WL, Zhang W, Lin HM, Liu Y, Huebner D, Little M, Whittaker S, Duvic M; ALCANZA study group. Brentuximab vedotin or physician's choice in CD30-positive cutaneous T-cell lymphoma (ALCANZA): an international, open-label, randomised, phase 3, multicentre trial.

69.Prince HM, Querfeld C. Integrating novel systemic therapies for the treatment of mycosis fungoides and Sezary syndrome. Best practice & research Clinical haematology. 2018;31(3):322-35.

70.Prince HM, Whittaker S, Hoppe RT. How I treat mycosis fungoides and Sézary syndrome. Blood. 2009; 114:4337–53.

71.Qin JZ, Zhang CL, Kamarashev J, Dummer R, Burg G, Dobbeling U. Interleukin-7 and interleukin-15 regulate the expression of the bcl-2 and cmyb genes in cutaneous T-cell lymphoma cells. Blood. 2001;98(9):2778-83.

72.R. Willemze, E. Hodak, P.L. Zinzani, L. Specht, M. Ladetto, ESMO Guidelines Committee Primary cutaneous lymphomas: ESMO clinical practice guidelines for diagnosis, treatment and follow-up Ann Oncol. 2018; 29: 30-40,

73.R.A. Wilcox, D.A. Wada, S.C. Ziesmer, S.F. Elsawa, N.I. Comfere, A.B. Dietz, A.J. Novak, T.E. Witzig, A.L. Feldman, M.R. Pittelkow, S.M. Ansell Monocytes promote tumor cell survival in T-cell lymphoproliferative disorders and are impaired in their ability to differentiate into mature dendritic cells Blood, 114 (14) (2009), pp. 2936-2944

74.Raess PW, Bagg A. The role of molecular pathology in the diagnosis of cutaneous lymphomas. Patholog Res Int. 2012:913523.

75.Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling. 2012;24(5):981-90.

76.S.G. Rhee, Y.S. Bae, S.R. Lee, J. Kwon Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation Sci. STKE: Signal Transduct. Knowl. Environ. 2000; 53.

77.S.R. Lee, K.S. Yang, J. Kwon, C. Lee, W. Jeong, S.G. Rhee Reversible inactivation of the tumor suppressor PTEN by H2O2 J. Biol. Chem., 277 (23) (2002), pp. 20336-20342

78.Salgado R, Toll A, Alameda F, Baro T, Martin-Ezquerra G, Sanmartin O, et al. CKS1B amplification is a frequent event in cutaneous squamous cell carcinoma with aggressive clinical behaviour. Genes Chromosomes Cancer. 2010;49(11):1054-61.

79.Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current biology: CB. 2014;24(10): R453-62.

80.Silic-Benussi M, Scattolin G, Cavallari I, Minuzzo S, Del Bianco P, Francescato S, et al. Selective killing of human T-ALL cells: an integrated approach targeting redox homeostasis and the OMA1/OPA1 axis. Cell death & disease. 2018;9(8):822.

81.Silic-Benussi M, Sharova E, Ciccarese F, Cavallari I, Raimondi V, Urso L, et al. mTOR inhibition downregulates glucose-6-phosphate dehydrogenase and induces ROS-dependent death in T-cell acute lymphoblastic leukemia cells.2022

82.Silic-Benussi M, Vittoria Raimondi, Ilaria Cavallari, Loredana Urso, Francesco Ciccarese, Gloria Scattolin, et al. mTORC inhibition increases reactive oxygen species and triggers death in T-lymphoblastic leukaemia (TALL) cells, 2019.

83.Sommer VH, Clemmensen OJ, Nielsen O, Wasik M, Lovato P, Brender C, et al. In vivo activation of STAT3 in cutaneous T-cell lymphoma. Evidence for an antiapoptotic function of STAT3. Leukemia. 2004; 18: 1288-95.

84.Sors A, Jean-Louis F, Pellet C, Laroche L, Dubertret L, Courtois G, et al. Down-regulating constitutive activation of the NF-kappaB canonical pathway overcomes the resistance of cutaneous T-cell lymphoma to apoptosis. Blood. 2006; 107:2354-63.

85.Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. Nature reviews Cancer. 2012;12(8):564-71.

86.Stolearenco.V, M.R.J. Namini, S.S. Hasselager, M. Gluud, T.B. Buus, A. Willerslev-Olsen, N. Odum, T. Krejsgaard Cellular interactions and inflammation in the pathogenesis of cutaneous T-Cell lymphoma Front. Cell Dev. Biol. 2020;8: 851.

87.Storz M, Zepter K, Kamarashev J, Dummer R, Burg G, Haffner AC. Coexpression of CD40 and CD40 ligand in cutaneous T-cell lymphoma (mycosis fungoides). Cancer Res. 2001;61(2):452-4.

88.Thode.C, A Woetmann, HH Wandall, MC Carlsson, K Qvortrup, CS Kauczok, M Wobser, A Printzlau, N Ødum, S Dabelsteen. Malignant T cells secrete galectins and induce epidermal hyperproliferation and disorganized stratification in a skin model of cutaneous T-cell lymphoma J. Invest. Dermatol. 2015; 135 (1): 238-246

89.Thorley-Lawson, D.A.2001. Epstein-Barr virus: exploiting the immune system. Nat. Rev. Immunol. 2001; 1:75-82.

90.Transplantation. 2009 Aug 15;88(3):435-41. doi: 10.1097/TP.0b013e3181af20fd. PMID: 19667950
Trautinger F, Eder J, Assaf C, Bagot M, Cozzio A, Dummer R, et al. European Organisation for Research and Treatment of Cancer consensus recommendations for the treatment of mycosis fungoides/Sezary syndrome - Eur J Cancer. 2017; 77:57-74.

91.Turrens JF. Mitochondrial formation of reactive oxygen species. The Journal of physiology. 2003;552(Pt 2):335-44.

92.van de Donk NW, Dhimolea E. Brentuximab vedotin. MAbs. 2012 Jul-Aug;4(4):458-65. doi: 10.4161/mabs.20230. Epub 2012 Jul 1. PMID: 22684302

93.Van de Donk, N.W.; Dhimolea, E. Brentuximab vedotin. MAbs 2012, 4, 458–465

94.Van Doorn R, van Kester MS, Dijkman R, Vermeer MH, Mulder AA, Szuhai K, et al. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. Blood. 2009;113(1):127-36.

95.van Doorn R, van Kester MS, Dijkman R, Vermeer MH, Mulder AA, Szuhai K, Knijnenburg J, Boer JM, Willemze R, Tensen CP. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. Blood. 2009 Jan 1;113(1):127-36. doi: 10.1182/blood-2008-04-153031. Epub 2008 Oct 1. PMID: 18832135

96.Vaque JP, Gomez-Lopez G, Monsalvez V, Varela I, Martinez N, Perez C, et al. PLCG1 mutations in cutaneous T-cell lymphomas. Blood. 2014;123(13):2034-43.

97.Veal EA, Underwood ZE, Tomalin LE, Morgan BA, Pillay CS. Hyperoxidation of Peroxiredoxins: Gain or Loss of Function? Antioxidants & redox signaling. 2018; 28(7):574-90.

98.Vivanco, C.L. Sawyer The phosphatidylinositol 3-Kinase AKT pathway in human cancer Nat. Rev. Cancer. 2002; 2 (7): 489-501.

99.Watanabe, R., Gehad, A., Yang, C., Scott, L. L., Teague, J. E., Schlapbach, C., Elco, C. P. et al., Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci. Transl. Med. 2015; 7: 279-39.

100.Weissbach H, Etienne F, Hoshi T, Heinemann SH, Lowther WT, Matthews B, et al. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Archives of biochemistry and biophysics. 2002;397(2):172-8.

101.Whittaker S, Oritz P, Dummer R, et al. Efficacy and safety of Bexarotene combined with psoralen-ultraviolet A (PUVA) compared with PUVA treatment alone in stage IB-IIA mycosis fungoides: results from the EORTC Cutaneous Lymphoma Task Force phase III randomized clinical trial (NCT00056056) Br J Dermatol. 2012; 163:678–687

102.Whittaker SJ, Demierre MF, Kim EJ, Rook AH, Lerner A, Duvic M, et al. Results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma.

Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2010;28(29):4485-91.

103.Wilcox RA. Cutaneous T-cell lymphoma: 2016 update on diagnosis, risk-stratification, and management. Am J Hematol.2016; 91:151–65.

104.Wilcox RA. Cutaneous T-cell lymphoma: 2017 update on diagnosis, risk stratification, and management. American journal of hematology. 2017;92(10):1085-102.

105.Wilcox, R. A., Cutaneous T-cell lymphoma: 2017 update on diagnosis, risk-stratification, and management. Am. J. Hematol. 2017; 92: 1085–1102.

106.Willemze R, Cerroni L, Kempf W et al. The 2018 update of the WHO-EORTC classification for primary cutaneous lymphomas. Blood. 2019; 133(16): 1703–14.

107.Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH, et al. WHOEORTC classification for cutaneous lymphomas. Blood. 2005;105(10): 3768-85.

108.Witzig TE, Reeder C, Han JJ, LaPlant B, Stenson M, Tun HW, Macon W, Ansell SM, Habermann TM, Inwards DJ, Micallef IN, Johnston PB, Porrata LF, Colgan JP, Markovic S, Nowakowski GS, Gupta M. The mTORC1 inhibitor everolimus has antitumor activity in vitro and produces tumor responses in patients with relapsed T-cell lymphoma. Blood. 2015 Jul 16;126(3):328-35. doi: 10.1182/blood-2015-02-629543. Epub 2015 Apr 28. PMID: 25921059

109.Yamanaka K, Clark R, Rich B, Dowgiert R, Hirahara K, Hurwitz D, et al. Skin-derived interleukin-7 contributes to the proliferation of lymphocytes in cutaneous T-cell lymphoma. Blood. 2006;107(6):2440-5.

110.Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. Nature reviews Cancer. 2016;16(10):650-62.

111.Zackheim HS, McCalmont TH. Mycosis fungoides: the great imitator. Journal of the American Academy of Dermatology. 2002;47(6):914-8.

112.Zhang Y, Wang Y, Yu R, Huang Y, Su M, Xiao C, et al. Molecular markers of early-stage mycosis fungoides. The Journal of investigative dermatology. 2012;132(6):1698-706.

113.Zhou, A.D. Attygalle, S.S. Chuang, T. Diss, H. Ye, H. Liu Angioimmunoblastic T-cell lymphoma: histological progression associates with EBV and HHV6B viral load Br J Haematol. 2007;138: 44-53