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Alkylphosphocholines and curcumin induce programmed cell death in cutaneous T-cell lymphoma cell lines

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ABSTRACT

While most patients with early-stage cutaneous T-cell lymphomas (CTCL) have a very good prognosis, the survival of patients with extensive tumour stage and visceral involvement remains extremely poor and necessitates the development of more effective treatment modalities. In this study, we evaluated the *in vitro* effects of two alkylphosphocholines (APCs, miltefosine and erufosine) and the polyphenolic compound curcumin on 5 human CTCL cell lines (Hut-78, HH, MJ, My-La CD4+ and My-La CD8+). All tested drugs showed considerable cytotoxic activity, as determined by the MTT dye reduction assay. The IC_{50} values of both APCs ranged from the low micromolar level (Hut-78 cells) to 60–80 μM (HH cells). The IC_{50} values of curcumin ranged from 12 to 24 μM. All tested drugs induced apoptosis, as ascertained by morphological changes, DNA fragmentation and activation of caspase cascades. Miltefosine and erufosine induced dephosphorylation of Akt in My-La CD8+ cells and phosphorylation of JNK in Hut-78 and My-La CD8+ cells. APCs increased the level of the autophagic marker LC3B in Hut-78 and MJ cells. Results from co-treatment with autophagy modulators suggested that the cytotoxicity of APCs in CTCL cells is mediated, at least in part, by induction of autophagy.

1. Introduction

Cutaneous T-cell lymphomas (CTCL) represent a heterogeneous group of rare non-Hodgkin lymphomas characterized by clonal expansion of neoplastic T-lymphocytes in the skin [1,2]. The annual incidence of CTCL in the United States has increased from 2.8 per million (1973–1977) to 9.6 per million (1998–2002) [3]. The two most common types of primary CTCL are mycosis fungoides (MF; 50–72% of all CTCL), which is generally indolent in behaviour, and Sézary syndrome (SS; 1–3% of all CTCL), an aggressive leukemic form of the disease [1]. Phenotypic analyses indicate that MF and SS are malignancies of CD4+ CD45RO+ skin-homing T-cells [4]. Recent data suggest that MF originates from skin-resident effector memory T-cells, whereas Sézary syndrome is a malignancy of central memory T-cells [5]. Mycosis fungoides classically presents with an indolent course and slow progression over years or sometimes decades [1]. The disease may evolve from patches to infiltrated plaques and eventually to tumours. Patients with Sézary syndrome present with erythroderma, circulating malignant T-cells (Sézary cells), and severe disabling pruritus with or without associated lymphadenopathy [1]. While the overall survival rate of patients with mycosis fungoides is 68% at 5 years and 17% at 30 years, patients with Sézary syndrome have an estimated 5-year survival of 24% [6,7]. The development and progression of CTCL is associated with pronounced immune dysregulation and a high risk of infections, which is the dominant cause of death in these patients [4]. Numerous immunological abnormalities are observed, including cytokine imbalances, T-cell exhaustion and direct inhibition by malignant T-cells, possibly mediated by CTLA4 [4]. Considerable evidence suggests that early CTCL has apoptotic defects (*e.g.* decreased and/or defective Fas expression, overexpression of Bcl-2) that render it more of a lymphoaccumulative than a lymphoproliferative disorder [1,8,9]. This is marked by

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a low apoptotic rate, low proliferative rate, relatively indolent clinical course, and no enhanced survival following conventional chemotherapy. Patients with early-stage disease generally have a very good prognosis and should be treated with skin-directed therapies; these include topical nitrogen mustard, corticosteroids, bexarotene, localized radiotherapy or psoralen plus ultraviolet A therapy [1,10]. If skin-directed therapies are ineffective or if the patient develops advanced stage disease, then systemic therapies are introduced, which may include α-interferon, bexarotene, photopheresis, denileukin diftitox, vorinostat, alemtuzumab, cytotoxic chemotherapy, or combination therapies [10]. However, the survival of patients with extensive tumour stage and visceral involvement remains extremely poor and the development of more effective modalities is an urgent necessity [4].

Alkylphosphocholines (APCs) are a class of antineoplastic compounds that exhibit significant cytotoxic and proapoptotic activity towards a vast number of tumour cell lines [11,12]. It is noteworthy that APCs are not myelotoxic and can even stimulate normal haematopoiesis [11,13–17] which implies that they could be used in combination treatment regimens in order to ameliorate the toxicity of conventional cytotoxic agents. APCs also spare normal B and T cells, as well as vascular endothelial cells [18]. APCs enhance the cytotoxicity of various conventional cytotoxic drugs [14–17,19] and sensitize tumour cells to radiation-induced cell death [20]. The complex mode of action of APCs involves disturbance of phospholipid metabolism and of membrane lipid raft-mediated signalling [12,18,21]. These result in cellular stress, deregulation of pro- and/or anti-apoptotic pathways, changes in protein kinase activity (*e.g.* Akt inhibition and JNK activation) and initiation of cell cycle arrest and apoptosis [16,17,19,22–27]. Miltefosine, the prototypic APC, has been evaluated as a topical therapy for CTCL in three small clinical trials and promising response rates have been observed [28–30]. However, no detailed mechanistic studies of miltefosine or other APCs have been performed in CTCL.

The polyphenolic compound curcumin is the major active component of the spice turmeric (*Curcuma longa*) that has been used for centuries in Indian traditional medicine [31]. Curcumin interacts with a wide variety of proteins modifying their expression and activity [32]. These include inflammatory cytokines and enzymes, transcription factors, and gene products linked to cell survival, proliferation, invasion, and angiogenesis [33]. Curcumin exerts antiproliferative and proapoptotic effects against various cancers *in vitro* and *in vivo* [34,35]. A recent *in vitro* study has demonstrated that curcumin induces apoptosis in CTCL cells [9].

The aim of the present study was to investigate and compare the effects of miltefosine, the novel APC erufosine and curcumin on CTCL cell lines.

2. Materials and methods

2.1. Drugs and chemicals

Miltefosine was obtained from ExperGen Drug Development GmbH (Vienna, Austria). Erufosine was synthesized and kindly provided by H. Eibl (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Both substances were dissolved in ethanol/PBS (1:1, v/v) to stock concentrations of 10 mM and stored at 4 °C. Curcumin, 3-methyladenine (3-MA), chloroquine diphosphate (CQ) and NH_4Cl were purchased from Sigma–Aldrich.

2.2. Cell lines and culture conditions

The human CTCL cell lines MJ and HH, originating from patients with MF and non-MF/SS aggressive CTCL, respectively, were obtained from the American Type Culture Collection (Rockville, MD, USA). The My-La CD4+ and My-La CD8+ cell lines, established from a single patient with MF, and the Hut-78 cell line, originating from a patient with SS, were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). MJ cells were grown in Iscove's modified Dulbecco's medium supplemented with 20% foetal bovine serum (FBS) and 2 mM L-glutamine (all from Lonza, Basel, Switzerland). The other cells were grown in RPMI-1640 (Lonza) medium enriched with 2 mM L-glutamine and 10% serum (FBS for Hut-78 and HH cells or human AB serum for both My-La variants). Culture medium for My-La CD4+ cells was additionally supplemented with recombinant human IL-2 and IL-4 (100 U/ml each; STEMCELL Technologies, Vancouver, Canada). My-La CD8+ cells required the addition of IL-2 only. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. MTT assay for cell survival and proliferation

Exponentially growing cells were seeded into 96-well microplates (100 μl/well at a density of 2×10^5 cells/ml) and exposed to various concentrations of the test compounds for 48 or 72 h, applied either alone or together with 3-MA (5 mM), CQ (20 μM) or NH_4Cl (10 mM). At least eight wells per treatment group were used (four for combination experiments with autophagy modulators). The cell survival fractions were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) dye reduction assay, performed as previously described [17]. The assays were conducted twice, with good reproducibility.

2.4. Apoptosis assays

Nuclear fragmentation was analyzed by DNA staining with Hoechst 33342. After treatment, cells were washed in PBS, spun on glass slides and stained with 5 μM Hoechst 33342 solution. The slides were kept in the dark at 4 °C until analysis with a fluorescence microscope.

Oligonucleosomal DNA fragmentation in apoptotic cells was determined using a Cell Death Detection ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. At least three wells were used for each experimental group.

The apoptosis assays were conducted twice, with good reproducibility.

2.5. Western blotting

Cell lysates were prepared and their protein content was measured as previously described [17]. Lysates were subjected to denaturing polyacrylamide gel electrophoresis on 4–20% Precise Protein Gels (Thermo Scientific, Rockford, IL, USA), adhering to the manufacturer's recommendations. The separated proteins were transferred to polyvinylidene difluoride membranes, which were thereafter processed as described previously [17]. The following antibodies from Santa Cruz Biotechnology (Heidelberg, Germany) were used: mouse monoclonal antibodies against caspase-9, caspase-3, poly (ADP)-ribose polymerase (PARP), actin, Bcl-X_L, JNK, p-JNK and LC3B (sc-17784, sc-56053, sc-8007, sc-8432, sc-8392, sc-7345, sc-6254 and sc-271625, respectively), rabbit polyclonal antibodies against NF-κB p65, Akt1/2/3 and p-Akt1/2/3 (Ser473) (sc-372, sc-8312 and sc-7985-R, respectively), a goat polyclonal antibody against caspase-8 (sc-6136) and a horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody (sc-2020). HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Thermo Scientific. Densitometric analysis of the blots was performed using the Quantity One 4.6.9 software (Bio-Rad, Hercules, CA, USA).

2.6. Statistics and evaluation of combination cytotoxic effects

Cell survival fractions were expressed as percentage of respective controls (not treated or treated only with any of the autophagy modulators – 3-MA, CQ or NH_4Cl). Values were presented as means with standard deviations (SD). Using these data points and the GraphPad Prism 6.01 program (GraphPad Software, San Diego, CA, USA), concentration–effect curves were fitted and the half-maximal cytotoxic concentrations (IC_{50}) were calculated, together with the respective 95% confidence intervals (CI). The Mann–Whitney statistical test was used to determine significant differences between experimental groups. If the combined treatment with APCs and autophagy modulators resulted in a survival fraction that deviated by more than 30% from the respective value for the single treatment, the combination cytotoxic effect was considered either synergistic or antagonistic, depending on the direction of deviation [36].

3. Results

3.1. Cytotoxic effects of APCs and curcumin

To analyze the effects of miltefosine, erufosine and curcumin on CTCL cell growth, cells were treated for 72 h with increasing concentrations of the compounds and the resulting changes in viability were measured by the MTT dye reduction assay. Concentration-dependent cytotoxicity was observed in all cell lines and for all treatments (Fig. 1). Miltefosine was most active against Hut-78 cells with an IC_{50} of 1.6 (95% CI: 1.1–2.3) μM (Fig. 1A). My-La CD8+ and MJ cells had intermediate sensitivity to miltefosine whereas My-La CD4+ and HH cells were more resistant. The IC_{50} value for the latter cell line was 82.9 (95% CI: 77.6–88.5) μM. Hut-78 cells were