Single-agent cytarabine is insufficient for the treatment of human mantle cell lymphoma in mouse xenograft model

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Mantle cell lymphoma is an aggressive type of B-cell non-Hodgkin lymphoma with adverse prognosis. It was demonstrated that alternation of CHOP and DHAP chemotherapy improved outcome of mantle cell lymphoma patients. However, which components of DHAP, cisplatin, cytarabine, or both, were responsible for the improved outcome remained unclear. To answer this question, antitumor efficacies of equally toxic doses of cytarabine, cisplatin, and three different combinations were compared *in vivo* using mouse xenograft models of mantle cell lymphoma. We demonstrated that cisplatin, alone or with cytarabine, is significantly superior to single-agent cytarabine in both eliminating lymphoma cells and suppressing their proliferation rate.

Key words: mantle cell lymphoma (MCL), cisplatin, high-dose cytarabin, DHAP

Mantle cell lymphoma (MCL) is an aggressive type of B-cell non-Hodgkin lymphoma characterized by frequent relapses and adverse prognosis. Therapy of MCL has been for decades based on anthracyclines and alkylating agents, namely on the CHOP (cyclophosphamide, doxorubicine, vincristine and prednisone) or CHOP-like regimen. In recent years the outcome of patients with MCL has been improved by dose-intensification of anthracyclines, addition of high-dose araC (HDAC) to the multi-agent chemotherapy [1], incorporation of anti-CD20 antibody rituximab (R) into induction and maintenance [2-3], and consolidation with high-dose therapy and autologous stem cell transplantation (ASCT) [4]. Recently, several studies showed that DHAP regimen (dexamethasone, high-dose araC and cisplatin) is highly effective as the frontline therapy of MCL patients [5-7]. Lefrere et al demonstrated that 4 cycles of DHAP were capable to convert insufficient responses (partial remissions or stable diseases) induced by 4 cycles of CHOP to complete remissions (CR) in as high as 84% of patients (22 of 26 patients) [5]. Recently, the randomized trial of The European Mantle Cell Network demonstrated that alternation of R-CHOP and R-DHAP increased significantly CR rates and time to treatment failure (TTF) compared to R-CHOP only [6]. Based on these results implementation of HDAC into induction regimen became the standard of care for all newly diagnosed younger MCL patients. The enthusiasm for HDAC in front-line therapy of MCL led even to attempts to avoid other chemotherapy agent in the front-line setting. However, the latest Nordic Lymphoma Group Mantle Cell Lymphoma 5 trial that evaluated "single"-agent HDAC (in combination with rituximab, but not other genotoxic agent standardly used in front-line therapy of MCL) was prematurely terminated because of insufficient efficacy [8]. Results from these clinical trials raised a crucial question, which of the two agents, Pt or araC (or both), actually contributed to the improved outcome of MCL patients. Despite that most clinicians (in our opinion)

Abbreviations: Mantle cell lymphoma (MCL), cisplatin (Pt), cytarabine (araC), high-dose araC (HDAC), CHOP (cyclophosphamide, doxorubicine, vincristine and prednisone), methotrexate (MTX), immunohistochemical (IHC), bone marrow (BM), twice daily (BID), overall survival (OS), DHAP (dexamethasone, high-dose araC and cisplatin), complete remission (CR), time to treatment failure (TTF), autologous stem cell transplantation (ASCT), rituximab (R), NOD-SCID-gamma (NSG), HyperCVAD (cyclophosphamide, vincristine, doxorubicin and prednison).

originally favored cytarabine (araC) over cisplatin (Pt), the recent Nordic Lymphoma Group trial 5 questioned this assumption. The main goal of this study was to identify, which of the two cytostatic components of the DHAP regimen, araC or Pt (or both), represents a key contributor of the improved clinical efficacy observed in newly diagnosed MCL patients treated with alternation of R-CHOP and R-DHAP compared to R-CHOP-only [6].

Materials and methods

MCL cell lines and culture conditions. Jeko-1 was obtained from the German Collection of Microorganisms and Cell Cultures, Hbl-2 was a kind gift of Prof. Martin Dreyling (University of Munich, Germany). Cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2.

Immunodeficient mice. The NOD.Cg-*Prkdc^scid Il2rg^{tm1Wjl}*/SzJ mice (referred to as NOD-SCID-gamma (NSG)) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). All animals were housed and maintained in a pathogen free environment in individually ventilated cages and provided with sterilized food and water. The experiment design was approved by the institutional animal care and use committee.

Experimental therapy of MCL xenografts. Jeko-1 and Hbl-2 cell line-based mouse models of MCL were established in the same way as previously described [9]. Briefly, 8 – 16 week old female NSG mice were inoculated with 1×10^6 Jeko-1 or Hbl-2 cells, and divided into 6 treatment cohorts (8-10 animals/cohort). Treatment was initiated in the middle of the estimated overall survival (OS) of untreated controls (which was day 18 and day 16 for Jeko-1 and Hbl-2-xenografted mice, respectively). The treatment was derived from the DHAP regimen. Pt and araC were administered on two consecutive days. Both agents were administered intraperitoneally (i.p.) as follows: 1. control group treated with PBS only; 2. single-agent cisplatin 240 µg; 3. single-agent cytarabine 10mg twice daily (BID) 12 hours apart; 4. three different combinations of both drugs designated as C1 (Pt 160 mcg + araC 2 mg BID), C2 (Pt 80 mcg + araC 3.5 mg BID), C3 (Pt 40 mcg + araC 5 mg BID). All treatment approaches were pre-tested to be equally toxic causing 10-20% early toxic deaths within 7 days of the drug

administration. When untreated controls developed signs of terminal disease, two mice from each cohort were subject to immunohistochemical (IHC) analysis of the selected murine organs. Remaining animals were euthanized by cervical dislocation when developed hint-leg paralysis or generalized inability to thrive. Differences in OS among treatment cohorts were calculated using Kaplan-Meier survival estimates set-up with the Graph-Pad Prism software.

Immunohistochemistry. Murine organs were removed, fixed in 4% formalin for 3 days, cut into tissue blocks, embedded in paraffin, and sectioned into transversal and longitudinal 6-7µm thick slices. Sections were stained with hematoxylin and eosin and Masson's blue trichrome. IHC staining for human CD20(L26) and Ki67/MIB1 (DakoCytomation) was performed. Antigen retrieval was done in citrate buffer solution (0.291% sodium citrate, 0.05% Tween 20, pH 6.0) using microwave for 3×5 minutes. Standard ABC (Avidin Biotin Complex) method was used for the detection with DAB (3,3'-Diaminobenzidine). Mayer's hematoxylin was used for counterstaining. Images were obtained using Olympus BX51 equipped with a DP25 digital camera. Data was analyzed by Image-Pro Plus 5.1 software within 20 samples from different organ areas (40 visual fields were observed). Experiments were carried out in duplicates (two mice for IHC analysis). The mean percent infiltration of murine tissues (bone marrow (BM) or spleen) with MCL cells (i.e. the percent of the area, which is occupied by CD20-positive or Ki67-positive cells) was calculated by the Image-Pro Plus 5.1 software and is shown (Figure 1B, Table 1).

Results

Mouse xenograft models of human aggressive MCLs were used to compare head-to-head anti-lymphoma efficacies of equally toxic doses of single-agent araC compared to single-agent Pt, and three different combinations of araC and Pt (C1-C3) [9]. Lymphoma-specific survival (defined as the time from lymphoma inoculation to lymphoma-caused demise of experimental animals) was significantly ($p<0.05$) prolonged in cohorts treated with single-agent Pt, and C1 combination compared to the other cohorts (Figure 1A, B). In addition, IHC analysis of the bone marrow (and the spleen in case of Jeko-1-bearing mice) demonstrated that mice from these treatment

Table 1. Results of immunohistochemical analysis showing percent infiltration (CD20) of murine bone marrow (BM) and spleen with MCL cells, and proliferation rate (Ki67) of MCL cells.

			Ctrl	Pt	araC	C1	C2	C3
Jeko-1	**% Infiltration (CD20) and proliferation (Ki67)**	**BM** CD20	86.7 ±2.8	10.3 ±1.8	44.5 ±3.2	0.5 ±0.4	30.9 ±1.6	45.7 ±7.8
		Ki67	84.9 ±3.2	1.4 ±0.2	2.5 ±2.1	1.6 ±1.6	3.1 ±1.9	6.8 ±5.1
		Spleen CD20	53.3 ±1.9	4.7 ±1.4	25.6 ±0.6	1.7 ±1.2	20.0 ±8.3	27.0 ±1.6
		Ki67	21.6 ±2.1	1.6 ±0.7	13.5 ±1.2	1.5 ±0.4	6.7 ±4.6	16.0 ±3.1
Hbl-2		**BM** CD20	95.2 ±4.8	19.2 ±7.7	29.9 ±5.4	21.8 ±5.2	37.6 ±6.1	41.0 ±8.5
		Ki67	89.1 ±5.0	0.5 ±0.0	14.2 ±6.4	3.7 ±1.6	21.5 ±4.1	28.5 ±5.3
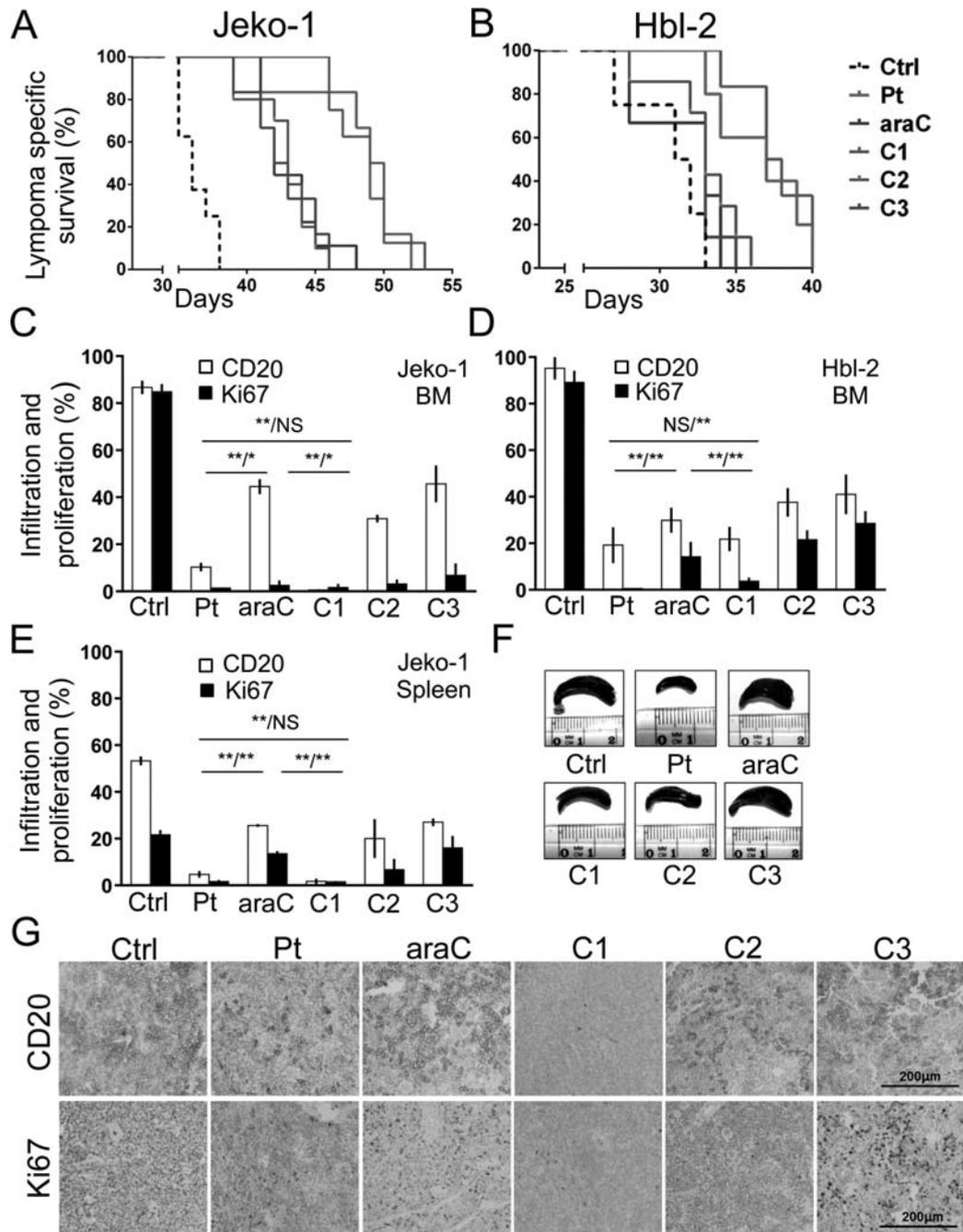
Figure 1. Anti-lymphoma efficacy of araC compared to Pt, and three different combinations of both drugs in mouse xenograft model of MCL
A-B Kaplan-Meier survival curves showing lymphoma specific survival of individual treatment cohorts of Jeko-1 (*A*) and Hbl-2 (*B*) xenografted mice. Single-agent cisplatin (Pt) and the C1 combination significantly (p<0.05) prolonged lymphoma specific survival in Jeko-1 and Hbl-2 xenografted mice compared both to untreated mice (Ctrl) and to the other cohorts. *C-E* Results of immunohistochemical analysis showing percent infiltration of murine bone marrow (BM) and spleen with MCL cells (anti-human CD20, white columns), and proliferation rate (Ki67, black columns) of the MCL cells. All 5 treatment approaches significantly (p<0.001) decreased the extent of infiltration (either by % of CD20- or Ki67-positive cells) of murine BM or spleen of Jeko-1 and Hbl-2 xenografted mice compared to untreated controls (Ctrl). Combination of Pt and araC (C1) significantly decreased the extent of infiltration (CD20-positive cells) of murine BM or spleen compared to single agent approaches in Jeko-1 xenografted mice. In case of Hbl-2 xenografted mice either the combination of Pt and AraC (C1) or single-agent Pt significantly decreased the extent of infiltration (CD20-positive cells) of murine BM compared to single-agent AraC. P values are shown for CD20/Ki67, NS= not significant (p≥0.05), * p<0.05 and ≥0.001, ** p<0.001.
F Murine spleens removed from the Jeko-1 xenografted mice, both from the untreated animals (Ctrl) and from the mice treated with indicated agent(s).
G Immunohistochemical analysis (anti-human CD20 and Ki67) of murine spleen of Jeko-1 xenografted mice (untreated controls (Ctrl) and the mice treated with indicated agent(s).

cohorts (Pt and C1) had both the lowest organ infiltration with human MCL cells (i.e. number of CD20-positive cells), and the most profound suppression of MCL cell proliferation rate (i.e. number of Ki67/MIB1-positive cells, see Figure 1C-E, G and Table 1). Accordingly, spleens isolated from mice treated with either Pt or C1 combination were smaller compared to those isolated from the other cohorts (Figure 1F).Our data indicate that single-agent Pt, or the combination of Pt and araC appear superior to equally toxic doses of single-agent araC in eliminating aggressive MCL cells.

Discussion

Large body of evidence suggests that araC-based strategies are superior to standard CHOP-like-based-only regimen. In addition to combinations of araC and Pt (DHAP), other strategies were tested in attempt to improve outcome of MCL patients. Romaguera et al demonstrated that alternating R-HyperCVAD (cyclophosphamide, vincristine, doxorubicin and prednison) and R-high-dose-methotrexate (MTX) / HDAC induced higher CR and prolonged OS compared to historical controls [2]. Another standardly used treatment strategy is the Nordic Regimen induction immunochemotherapy with rituximab and dose intensified CHOP (maxiCHOP) alternating with R-HDAC. R-maxiCHOP/R-HDAC regimen followed by high dose therapy with ASCT induced high CR rates and the 6-year overall and progression-free survival of 70% and 66%, respectively [3].

As there are currently no randomized trials that would directly compare these different approaches, it remains elusive, which of the agents or strategies are superior in improving outcome of MCL patients: whether hyper-fractionation of cyclophosphamide, dose-intensification of anthracyclines, addition of MTX, addition of Pt, addition of HDAC, or combination of more factors. To answer this question *in extenso* is far beyond the scope of this manuscript. Here, we focused on the simple question, which of the two agents in the DHAP regimen, Pt or araC (or both) are key contributors of improved efficacy of this regimen. Recently, the Nordic Lymphoma Group MCL trial 5 was prematurely terminated because of insufficient antilymphoma efficacy of front-line therapy based solely on high-dose araC in combination with rituximab [8]. To simulate the situation in patients with aggressive MCL disease, we used mouse xenograft models of aggressive metastatic human MCL, and subjected the MCL-bearing mice to equally toxic therapies consisting of Pt, araC and three different combinations of Pt+araC. Equal toxicity of individual treatments was absolute prerequisite that enabled us to compare their anti-lymphoma efficacy. We found that either single-agent Pt or C1 combination (containing the highest dose of Pt compared to C2 or C3 combination) belonged to the most potent treatment approaches. Single-agent araC and C3 combination (the combination with the highest dose of araC) were less effective suggesting araC monotherapy did not effectively suppress aggressive MCL xenografts. In conclusion

using these preclinical models we confirmed the results of the Nordic Lymphoma Group trial 5 concluding that single-agent araC is not appropriate treatment for biologically aggressive MCL in the clinical grounds. In translation the DHAP regimen appears superior to single-agent HDAC-based strategies. The results also indicate that Pt remains, despite its well-known toxic side-effects, a powerful cytostatic agent, at least in case of aggressive forms of MCL.

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