Inhibition of GST-pi Nuclear Transfer Increases Mantle Cell Lymphoma Sensitivity to Cisplatin, Cytarabine, Gemcitabine, Bortezomib and Doxorubicin

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Abstract. Purpose: Mantle cell lymphoma (MCL) is a chemoresistant lymphoma overexpressing the class pi glutathione-S-transferase (GST-pi). The nuclear localisation of GST-pi is induced by chemotherapy and is correlated to cell resistance. In this study, the effect of the Agaricus bisporus lectin (ABL), a GST-pi nuclear transfer inhibitor, on the chemosensitivity of MCL cells was investigated. Methods: The proliferation of three MCL cell lines was evaluated in the presence of doxorubicin (DOX), cisplatin (CDDP), cytarabine (Ara-C), gemcitabine (GEM) or bortezomib with or without ABL pre-treatment. Results: The cytotoxic activities of CDDP, Ara-C, GEM and bortezomib were increased in all cell lines. The DOX cytotoxic activity was enhanced in two of three cell lines. The inhibition of GST-pi nuclear transfer led to the potentialisation of all drug combinations. Conclusion: The inhibition of the nuclear transfer of GST-pi increases the MCL sensitivity to DOX, CDDP, Ara-C, GEM and bortezomib, alone or in combination.

Mantle cell lymphoma (MCL) is a rare but well-defined subtype of B-cell non-Hodgkin’s, distinguished notably by the presence of a specific chromosomal translocation, t(11;14)(q13;q32), leading to cyclin D1 overexpression (1-3). MCL is also characterised by a rapid acquisition of chemoresistance associated with a short duration of response and poor survival with a median survival around 3-4 years (4-8). Recently, gene expression profiling identified an MCL specific molecular signature with overexpression of genes involved in detoxification mechanisms such as MDR/ATP-binding cassette membrane proteins (ABCG2 and ABCC5) and the class pi glutathione-S-transferase (GSTP1) (9). GSTP1 overexpression in MCL was validated at the protein level (9, 10).

Glutathione-S-transferases (GST) comprise a family of widely distributed phase II detoxification enzymes that catalyze the conjugation of a broad variety of reactive electrophiles to the nucleophilic sulphur atom of the major intracellular thiol, the glutathione (GSH) (11). In addition, GSTs can bind GSH with varying affinities to a variety of aromatic compounds (12). Soluble GSTs are divided into distinct classes based on similarities in their primary structures and subtract specificities. Among the various GSTs, the class pi (GST-pi) has attracted recent attention. Elevated cellular levels of GST-pi are correlated with poor prognosis (13) and resistance to diverse common anticancer drugs (14, 15), and the introduction of GST-pi into various cells in culture has displayed protection against cytotoxic effects of doxorubicin (DOX) and cisplatin (CDDP) (16, 17). On the other hand, GST-pi inhibitors have been shown to restore sensitivity to alkylating agents in drug-resistant cells (18, 19) and GSTP1 antisense cDNA increased the sensitivity of cancer cells to various anticancer drugs, such as adriamycin, CDDP, melphalan and etoposide (VP-16) (20).
The presence of GST-pi in the nucleus of uterine cancer cells (21) and glioma cells (22) has been reported, and a negative correlation between the GST-pi nuclear localisation and patient survival was suggested. Treatment with doxorubicin (DOX), cisplatin (CDDP), irinotecan (CPT-11), VP-16 or 5-fluorouracil (5-FU) increased the amount of nuclear GST-pi, but not other GSTs in human cancer cell lines (23). Further studies clearly demonstrated the protective effect of nuclear GST-pi on DNA intercalation by DOX and DNA cross-linking by CDDP (17, 24) leading to the resistance of cells to DOX and CDDP. The mechanism involved in the nuclear transfer of GST-pi still remains unclear but some authors suggest that GST-pi is transferred to the nucleus through a protein transfer system both in the steady state and after exposure to anticancer drugs (24).

The edible mushroom lectin (Agaricus bisporus lectin, ABL) is known to be internalised efficiently into the cytoplasm of cultured cells, localised around the nucleus, and to inhibit the NSL-dependent nuclear protein import (25). A previous report presented evidence that ABL inhibits the nuclear transfer of GST-pi and increases the sensitivity of human colon cancer cells to DOX (24). Pretreatment of cells with ABL alone did not affect GST-pi in cytoplasm and had no effect on the DOX-dependent induction of GST-pi in cytoplasm. However, ABL decreased the amount of GST-pi in the nucleus, abolished the DOX-stimulated GST activity in the nucleus and enhanced the sensitivity to DOX.

In this study, the sensitivity of three human MCL cell lines to drugs usually used in the treatment of MCL in first line or at relapse [DOX, CDDP, cytarabine (AraC), gemcitabine (GEM) and bortezomib] with or without ABL were investigated, in order to examine whether the sensitivity of the cell lines to these drugs, tested alone or in combination, could be increased.

Materials and Methods

Compounds. Lyophilized powder of ABL was purchased from Sigma-Aldrich (Sigma-Aldrich Chimie, Lyon, France). Doxorubicin (DOX, Adriblastine®) and cytarabine (AraC, Aracytine®) were purchased from Pfizer (Pfizer, New York, USA), cisplatin (CDDP, Cisplatine®) from Merck (Merck, Lyon, France) and gemcitabine (GEM, Gemzar®) from Lilly (Lilly, Indianapolis, USA). Bortezomib (PS-341, Velcade®) was a kind gift of Pr. Charles Dumontet (INSERM U590, Lyon, France).

Cell culture. Three human MCL cell lines were cultured as follows: Granta-519 and REC-1 obtained from DSMZ collection (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS, streptomycin and penicillin, meanwhile UPN1, a kind gift of Pr. Vincent Ribrag, Institut Gustave Roussy, Villejuif, France, was cultured in α-MEM supplemented with 2 mM L-glutamine, 10% FBS, streptomycin and penicillin.

Cytotoxicity assay. Sensitivities of MCL cells to DOX, CDDP, AraC, GEM and bortezomib were determined using MTT assay with or without 10 h of pre-pretreatment with ABL at 40 μg/ml. Cells were seeded at a concentration of 2×10^4 cells/ml in a 96-well plate (20,000 cells/well) and exposed to different concentrations of DOX, CDDP, AraC, GEM or bortezomib or. After 48h, 20 μl of MTT (5 mg/ml) were added to each well. The absorbance of samples was measured at 550 nm. Cell sensitivities of all MCL cell lines were analysed three times under each condition. Inhibitory concentrations (IC_{50}) values were determined using semi-logarithmic curves and the influence of ABL pretreatment on IC_{50} was assessed using the Student’s t-test.

ABL and drug combinations. MTT assays were used to assess the ABL pre-treatment effects on cytotoxicity of drug combinations. Granta-519 and REC-1 were seeded in a 96-well plate as previously described, with or without 10 h pre-incubation with ABL at 40 μg/ml. CDDP, AraC and bortezomib were added in various combinations at IC_{50} concentrations previously defined. The absorbance of samples was measured at 550 nm and the percentages of viable cells were estimated compared to cells seeded in well without treatment. Theses MTT assays were carrying out three times and the influence of ABL pretreatment on drug combinations was assessed using the Student’s t-test.

Preparation of cytoplasmic and nuclear proteins. Granta-519 cells were cultured for 48h with DOX, CDDP, AraC, GEM or bortezomib with or without ABL pre-treatment. Cells were collected and washed twice in PBS before protein extraction. The cytoplasmic and nuclear proteins were prepared using a commercial kit (CellLytic NuCLEAR Kit, Sigma-Aldrich Chimie, Lyon, France). Briefly, cells pellets (1×10^6 cells) were treated with 100 μl of hypotonic buffer (100 mM Hepes, pH 7.9 with 15 mM MgCl₂ and 100 mM KCl, 1mM dithiothreitol, and protease inhibitor cocktail). After 15 minutes of in ice incubation, 0.6% of IPEGAL was added. After centrifugation of the sample (10,000 x g, 4˚C, 30 seconds), the supernatant was prepared as the cytoplasmic fraction. The debris were suspended in the extraction buffer ([20 mM HEPEs, pH7.9 with 4.5mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA and 25% (v/v) glycerol] and protease inhibitor cocktail) and vigorously agitated for 15 minutes. After centrifugation (20,000 x g, 4˚C, 5 minutes) the supernatant was prepared as the nuclear fraction.

Western blot assay. Immunological levels of GST-pi in the cells were estimated by western blotting. 15 μg of proteins from cytoplasmic and nuclear fractions were subjected to electrophoresis on SDS-polyacrylamide gel containing 12% acrylamide, transferred to nitrocellulose, and probe with the following antibodies: anti-human GST-pi (Abcam, Paris, France: 47709, 1/500), anti-human α-tubulin (Sigma-Aldrich Chimie, Lyon, France: T9026, 1/1,000) and anti-human pan-actin (NeoMarkers, Fremont, CA, USA: ACTN05, 1/10,000). Antigen-antibodies complexes were detected using peroxidase-coupled anti-mouse IgG with ECL reagents.

Results

ABL alone had no effect on MCL cell lines proliferation. It was first checked if ABL pre-treatment had any cytotoxic effect on MCL cells proliferation. When cells are cultured for 48 h with ABL at 40 μg/ml (the concentration used for further assay), their viability assessed by MTT was higher.
Figure 1. Effects of GST-pi nuclear transfer inhibition on the proliferation of MCL cell lines. Histograms represent the mean IC_{50} concentration of each drug both in absence and in presence of ABL pre-treatment: A/ IC_{50} of doxorubicin (DOX), B/ IC_{50} of cisplatin (CDDP), C/ IC_{50} of cytarabine (Ara-C), D/ IC_{50} of gemcitabine (GEM), E/ IC_{50} of bortezomib.
than 90%. It was concluded that ABL alone at 40 μg/ml had no impact on MCL cell lines proliferation. Drugs as single agent induced significant higher cytotoxicity when combined with ABL. To evaluate the effects of GST-pi nuclear transfer inhibition on MCL proliferation when chemotherapeutic agents are administered, the IC50 of DOX, CDDP, AraC, GEM and, bortezomib with or without ABL pre-treatment was determined in the three MCL cell lines. Treatment of all MCL cell lines with ABL followed by DOX resulted in a significant decrease of IC50 of DOX compared to DOX alone in two of three MCL cell lines (24.34 nM vs. 11.09 nM, p<0.05 for Granta-519; 1.67 μM vs. 1.29 μM, p<0.005 for REC-1; 60 nM vs. 40 nM, p=0.1 for UPN1) (Figure 1A). Regarding experiments with CDDP, ABL pre-treatment result in a significant decrease of IC50 in CDDP when associated with ABL compared to CDDP alone in all MCL cell lines (1.59 μM vs. 0.34 μM, p<0.0005 for Granta-519; 12.8 μM vs. 6.53 μM, p<0.005 for REC-1 and 3.22 μM vs. 1.89 μM, p<0.005 for UPN1) (Figure 1B). The sensitivities of all the MCL cell lines to AraC were significantly enhanced after the ABL pre-treatment with a AraC IC50 decrease compared to AraC alone (150 nM vs. 30 nM, p<0.005 for Granta-519; 990 nM vs. 180 nM, p<0.01 for REC-1; 33nM vs. 24nM, p<0.05 for UPN1) (Figure 1C). The cytotoxicity of GEM was significantly enhanced by the ABL pre-treatment in all MCL cell lines with a decrease of IC50 of GEM associated with ABL compared to GEM alone (4.53 nM vs. 2.03nM, p<0.01 for Granta-519; 12.14 μM vs. 0.13 μM, p<0.0005 for REC-1; 7.533 nM vs. 0.75nM, p<0.0005 for UPN1) (Figure 1D). Finally, treatment with ABL followed by bortezomib led to a significantly decrease of IC50 of bortezomib compared to bortezomib alone for all MCL cell lines (1.95 nM vs. 0.71 nM, p<0.005 for Granta-519; 7.40 nM vs. 3.28 nM, p<0.001 for REC-1; 3.52 nM vs. 2.24 nM, p<0.025 for UPN1) (Figure 1E). These results showed that used alone, each drug was associated with a significantly higher cytotoxicity when combined with ABL than without ABL, on all the 3 MCL cell lines, except DOX with which no significant difference was observed in one cell line (UPN1).
Significantly increased cytotoxicity of drug combinations after ABL pretreatment. REC-1 and Granta-519 cells were seeded in 96-well plates and treated with various combinations of AraC, CDDP and bortezomib at IC\textsubscript{50} with or without 40 μg/ml ABL pre-treatment (CDDP + AraC; CDDP + bortezomib; AraC + bortezomib; CDP + AraC + bortezomib; CDDP + AraC; CDPP + bortezomib; AraC + bortezomib; CDPP + bortezomib; AraC + bortezomib; CDDP + AraC + bortezomib). For each combination, the ABL pre-treatment of MCL cells led to a significant decrease of cell proliferation compared to combinations without ABL pre-treatment (Figure 2). For REC-1, the combination of AraC and CDDP resulted in 43.6% of cells being viable and ABL pre-treatment decreased the number of viable cells to 30.9% (p<0.05). The combination of AraC and bortezomib resulted in 50.3% of cells being viable and ABL pre-treatment decreased the number of viable cells to 27.7% (p<0.025). The combination of CDDP and bortezomib led to 36.8% of cells being viable and ABL pre-treatment decreased the number of viable cells to 18.9% (p<0.025). The combination of the three drugs resulted in 39.5% of cells being viable and ABL pre-treatment decreased the number of viable cells to 17.5% (p<0.005). For Granta-519, the combination of AraC and CDDP resulted in 32% of cells being viable and ABL pre-treatment decreased the number of viable cells to 22.6% (p<0.05). The combination of AraC and bortezomib resulted in 53% of cells being viable and ABL pre-treatment decreased the number of viable cells to 30.8% (p<0.01). The combination of CDDP and bortezomib led to 39% of cells being viable and ABL pre-treatment decreased the number of viable cells to 27.4% (p<0.05). The combination of the three drugs resulted in 32.9% of cells being viable and ABL pre-treatment decreased the number of viable cells to 24.7% (p<0.01). All these results indicate that AraC is the most sensitive drug to the nuclear transfer of GST-pi and that the best cytotoxicity is obtained by the combination of AraC, CDDP, bortezomib and ALB pre-treatment.

ABL effects on GST-pi localisation. ABL is known to be internalised in intact cells and to interfere with the transfer of nuclear proteins (25). Granta-519 cells pre-incubated with 40 μg/ml ABL for 10 h were treated with DOX, CDDP, AraC, GEM, or bortezomib for 6h. Western blot analysis showed that the pretreatment of the cells with ABL alone did not affect the GST-pi in cytoplasm or in the nucleus. Treatment with DOX, CDDP, AraC, GEM, or bortezomib alone led to an increase of GST-pi in the nucleus. Pre-treatment with ABL caused a lost in the amount of GST-pi in the nucleus (Figure 3). According to these results, the cytotoxicity of all drugs combined with ABL pre-treatment was in relation with the decrease level of GST-pi in the nucleus.

Discussion and Conclusion

MCL is a particularly chemoresistant subtype of small B-cell lymphoma whose treatment failure occurs rapidly (26). One of the potential factors of this short time to MCL relapse is the overexpression of several genes related to drug resistance, including GST\textsubscript{P1} (9, 10) that plays an important role in the phase II detoxification system. In this study, the influence of the inhibition of GST-pi nuclear transfer by ABL on the cytotoxicity of five chemotherapeutic reagents usually used in the treatment of MCL was investigated: four drugs whose targets are nuclear – DOX (27), CDDP (28), AraC and GEM (29)-, and one drug whose target is cytoplasmic and nuclear: bortezomib (30).

Among the 3 MCL cell lines used in this study, REC-1 was the most resistant MCL cell line. The GST-pi nuclear transfer inhibition by ABL enhanced the sensitivity of all MCL cell lines, including the more resistant cell line REC-1. It was also demonstrated that when treated with drug combinations at IC\textsubscript{50} concentrations, the sensitivity of MCL cell lines increased, whatever the drug combination. As it was found when cells were treated with single drug, the GST-pi nuclear transfer inhibition enhanced the cytotoxic activities of all drug combinations.

The main mechanism by which GST-pi acts in the cell protection from harmful chemicals is the conjugation to GSH. It has been demonstrated that cells treated with DOX or CDDP are able to form DOX-GSH or CDDP-GSH adducts leading to DOX and CDDP efficiency decrease (31). Although the GSH/GST-pi detoxification system is involved in the resistance to Ara-C, GEM and bortezomib, it has not been well established whether these drugs with aromatic structures can be conjugated directly to GSH. The cell treatments with cytotoxic drugs have been previously reported to increase the expression of GST-pi in both the cytoplasm and the nucleus, enhancing the cytoplasm and nuclear detoxification (23). In addition, the cell sensitivity to cytotoxic drugs has been described to be greater in nuclear GST-pi-negative than nuclear GST-pi-positive cells implying a role for nuclear GST-pi in the drug resistance (24). In this study, the inhibition of the GST-pi nuclear transfer resulted into an increased cytotoxicity of DOX, CDDP, AraC, GEM and bortezomib in all the MCL cell lines both in single administration and in combinations. These cell sensitivity increases are related to the decrease of the GST-pi nuclear level while no modification of the GST-pi cytoplasmic level was found. Although the GST-pi nuclear level decrease could contribute to reduce the nuclear detoxification, the pre-treatment with ABL had no effect on the cytoplasmic GST-pi activity, which could carry on the conjugation of drugs with GSH. The inhibition of the direct interaction between nuclear GST-pi and the drugs cannot explain entirely the enhancement of the MCL cell sensitivities.
Most agents used in chemotherapies act in specific ways, e.g., DOX by interfering with the topoisomerase II/DNA complex (27), CDDP by forming cross linkages inside or among the DNA chains (28), Ara-C and GEM by directly interfering with the de novo synthesis of nucleosides and nucleotides or inhibiting the DNA chain elongation (29), and bortezomib by inhibiting the proteasome (30). Moreover, all these agents also generate reactive oxygen species (ROS) causing cell oxidative stress that induce apoptosis (32-35). ROS attack electron-rich centers in DNA via aldehydes formed during lipid peroxidation process. Aldehydes possess high reactivity against DNA bases, especially guanine (36). The formation of DNA adducts leads to the generation of site-specific cleavages of double-stranded DNA (37, 38). Kamada et al. demonstrated that the nuclear GST-pi prevents the DNA damage and reduces the apoptosis induction by scavenging the formation of lipid-peroxide-modified DNA (39). The ABL pre-treatment, by inhibiting the nuclear transfer of GST-pi, increases the sensitivity of cells to oxidative stress (39). The enhancement of the MCL cell sensitivity to DOX, CDDP, AraC, GEM and bortezomib after the GST-pi nuclear transfer inhibition is the consequence of the less efficiency of the drug-induced-ROS detoxification in the nucleus.

Although it was demonstrated in this study that the inhibition of the GST-pi nuclear transfer by ABL is efficient at improving the cytotoxic effects of the five drugs that are most frequently used in MCL treatment, in first line or at relapse, these observations could not be replicated in an in vivo assay due to the large amount of ABL needed. Other GST-pi inhibitors are currently being investigated and the most promising is the canfosfamide (Telcyta®; Telik Inc, Palo Alto, CA, USA). Preclinical studies have suggested the activation of canfosfamide occurs when GST-pi splits canfosfamide into two active fragments: a glutathione analog fragment and an active cytototoxic fragment. The glutathione analog fragment remains bound to GST-pi, limiting the ability of GST-pi to inactivate other cancer drugs (40). In a phase I-IIa clinical trial for patients with advanced non-small cell lung cancer, the combination of canfosfamide with carboplatin and paclitaxel was well tolerated and active. In addition, patients receiving maintenance canfosfamide therapy had a prolonged median survival compared with those eligible for but not receiving maintenance therapy (41).

In conclusion, the GST-pi nuclear transfer inhibition in MCL cell lines led to a significantly higher enhancement of the cell sensitivity to several chemotherapeutic reagents, including anthracycline, alkylating agents, nucleoside analogues and proteasome inhibitor. Based on these results of the major impact of GST-pi inhibition in the enhancement of drug sensitivity in MCL, in vitro models, clinical trials are needed to investigate the effects of GST-pi inhibition with clinically available glutathione analogues in association with chemotherapeutic agents in patients with a MCL.

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References


