Cobalamin Potentiates Vinblastine Cytotoxicity Through Downregulation of *mdr-1* Gene Expression in HepG2 Cells

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Key Words

Cancer • MDR • Chemoresistance • Hepatocarcinoma cells • Cobalamin • Methylation

Abstract

Background: P-glycoprotein (Pgp), produced by multidrug resistance-1 gene (*mdr-1*), is a main mechanism developed by cancer cells to guard against anti-cancer drugs. Alterations of DNA methylation of the *mdr-1* gene promoter are known to be linked to *mdr-1* gene expression and are probably related to intracellular S-adenosyl-methionine. We here used HepG2 cells to determine the role of the methionine cycle (through the use of the Methionine-Synthase (MS) cofactor, cobalamin) on *mdr-1* gene expression. **Methods:** Semiquantitative RT-PCR of *mdr-1* gene, cellular retention of rhodamine-123, and vinblastine cytotoxicity were carried out on cells cultivated with and without cobalamin. Methylation status of the *mdr-1* gene promoter was determined by methylation-specific PCR. **Results:** Addition of cobalamin to the cells led to an increase in MS activity, to a significant decrease in *mdr-1* gene expression which is correlated to an increase in retention of the Pgp substrate Rhodamine 123. Furthermore, cobalamin

potentiated cell sensitivity to vinblastine to the same range as that of the Pgp blocker verapamil and prevented methotrexate-induced up-regulation of *mdr-1* gene expression. However, no modification in methylation of the *mdr-1* gene promoter was observed. **Conclusion:** Cobalamin downregulates *mdr-1* gene expression, as well as Pgp expression and function, and significantly increases cytotoxicity of vinblastine. The identification of this novel way of diminishing cellular resistance to the chemotherapeutic agent vinblastine holds promises of leading to better treatments for cancer patients.

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Introduction

Multidrug resistance (MDR) is one of the major mechanisms through which cancerous tumors become resistant to chemotherapy. Amongst the causes of multiple drug resistance, one of the best characterized is the overexpression of the *mdr-1* gene product, P-glycoprotein (Pgp). Pgp is a transmembrane protein of the ABC family that decreases cytotoxic activity of anticancer drugs, by acting as a multidrug efflux pump [1, 2]. Drugs trans-

ported by Pgp include alkaloids, vinblastine, colchicine, anthracyclines, actinomycin D and many others [3]. In order to revert MDR-mediated chemoresistance, numerous drugs blocking Pgp were tested in clinical trials. These include verapamil, cyclosporine, tamoxifen and progesterone. However, the results of these trials were not encouraging mainly because of deleterious side-effects [4]. Patients' clinical benefit has therefore not been clearly demonstrated in regards to the use of such modulators in circumventing the MDR phenomenon.

A reason behind such failure may be that the mechanism by which *mdr-1* is overexpressed in cancer cells is still little understood. *Mdr-1* gene expression is controlled by several mechanisms including action of transcription factors such as YB-1, c-Jun/c-fos, AP-1 and also by DNA methylation [5]. *Mdr-1* promoter methylation was shown to be also related to cancer invasiveness [6] and to cancer cell proliferation [7]. However, there are still no clear clues about the mechanisms triggering *mdr-1* gene overexpression in cancer.

Changes in DNA methylation patterns are often observed in cancer cells. Multiple observations linking DNA methylation to cancer are often explained by dysregulation of proto-oncogenes and/or genes involved in proliferation [8]. S-adenosyl-methionine dependent DNA methyltransferase activity was shown to be correlated not only to *mdr-1* expression and to chemoresistance [9] but also to cancer promotion and progression [10]. Through conversion to S-Adenosyl-Homocystein, S-Adenosyl-Methionine acts as a powerful methyl donor for CpG island methylation by DNA methyltransferases. Such a mechanism is consistent with the frequently reported methionine auxotrophy of cancer cells and with the tumorigenic effects of methyle-deficient diets. Methyle deficiency in tumor cells is also consistent with the commonly observed global hypomethylation of tumor cell DNA, despite normal or even high levels of DNA methyltransferase expression. The limiting factor of S-Adenosyl-Methionine synthesis is the biodisponibility of cellular methionine. This aminoacid originates from both external sources and from regeneration of homocystein through the methionine cycle in which the enzyme methionine synthase converts homocystein to methionine using methyltetrahydrofolate as a primary methyl donor, and cobalamin (vitamine B12) as a cofactor [11, 12].

Methionine synthase thus stands as an important key component of 1) the methionine metabolism, 2) the folate cycle and 3) S-Adenosyl-Methionine regeneration. The present investigation aims at testing the hypothesis that the methionine cycle and especially methionine synthase

may influence *mdr-1* gene expression through DNA methylation. We modified methionine synthase activity by manipulating cobalamin concentrations. The human liver cancer cell line HepG2 was chosen, as previous results indicated that its *mdr-1* gene expression is controlled by methylation [13]. Furthermore, HepG2 represents a good cellular model where the methionine cycle, the folate cycle and the three known DNA methyltransferases are fully active [14].

We found that cobalamin increases methionine synthase activity and down-regulates *mdr-1* gene expression in HepG2 cells, leading to an increased sensibility of these cells to vinblastine. However, no modification of the *mdr-1* promoter methylation status was detected.

Materials and Methods

Materials

Rhodamine 123 (Rh-123), 5'-aza-2'-deoxycytidine (5'-AZA), verapamil, trichostatin A (TSA), Cobalamin (Cbl), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and glybenclamide were obtained from Sigma (L'Isles d'Abeau Chesnes, France). Dulbecco's Modified Eagles Medium (DMEM), Penicillin G, Streptomycin and Foetal Calf Serum (FCS) were obtained from GIBCO (Invitrogen, Cergy Pontoise, France). All other chemicals were of cell culture grade.

Cell culture

The HepG2 cell line belongs to the laboratory. The cells were seeded and grown in basal DMEM supplemented with penicillin and streptomycin (100 U/ml each) and 10% of heat-inactivated FCS and were incubated in a humidified incubator (10% CO₂) at 37°C. Experiments were initiated when cells reached 90% of confluency. Cells were routinely screened for Mycoplasma contamination.

Measurements of Methionine Synthase activity

Cells were seeded at 6×10^3 cells/cm² onto 100 mm Petri dishes and cultured with or without 100 nM of cobalamin. Determination of methionine synthase activities was performed by a modification of the radioisotope assay described by Weissbach [15]. In brief, cell lysates (1 mg) were homogenized at 4°C in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.2) in presence of protease inhibitor cocktail (Sigma, L'Isles d'Abeau-Chesnes, France). After centrifugation (10,000 g, 4°C) for 3min to remove cell debris, the supernatant was used as crude extract. For measurement of apoenzyme activity, reaction mixture contained 0.25mM DL-homocystein, 29 mM DTT (used as reducing agent), 7mM β -mercaptoethanol, 0.25 mM S-Adenosyl methionine, 1 μ Ci (22 μ M) [methyl-¹⁴C]MeH₄F, crude extract and 50 μ M potassium phosphate buffer in a total volume of 800 μ l. The enzyme reaction was carried out under a N₂ atmosphere at 37°C for 1h in the dark and then stopped by heating at 95°C for 5min. The assay measures the amount of radioactive methio-

nine formed from 5-[¹⁴C]methyl-THF and Hcy. The mixture was passed through AG-1X8 (Cl-) columns (Bio-Rad, Marne-la-Coquette, France) and ¹⁴C radioactivity in the methionine containing fraction was measured. Holoenzyme were determined by the same procedure except that 25 μM of hydroxo-cobalamin was added to the reaction mixture. The formed radiolabeled methionine was measured in a Packard liquid scintillation counter. Enzyme activity was expressed as nmoles of product formed per hour and per mg of protein.

Cell treatment, RNA isolation and semi-quantitative RT-PCR for mdr-1

HepG2 cells were seeded onto 6-well plates (Becton Dickinson, Le Pont de Claix, France) and incubated with either Cobalamin (100 nM) or a mixture composed of 5'-AZA and TSA (1 μM / 165 nM) for 24 hours. This mixture was used as a positive control as it was shown to induce a significant increase in mdr-1 gene expression [5, 13]. When testing the effect of methotrexate (MTX), it was added to the cells at a 1 mM concentration with or without 100 nM Cbl and RNA was extracted over time (1 to 72 hours). Total RNA was extracted from the cultured cells according to the RNeasy Mini Kit extraction protocol, from Qiagen (Courtaboeuf, France). The extracted RNA was quantified by spectrophotometry and its integrity was checked by gel electrophoresis. 1 μg of total RNA was used for reverse transcription in a total volume of 20 μL using M-MLV reverse transcriptase (200U) and reagents from Invitrogen. Aliquots of 2 μL of cDNA were used for amplification. The sense and antisense primers were (for mdr-1), 5'-CCC ATC ATT GCA ATA GCA GG-3' and 5'-GTT CAA ACT TCT GCT CCT GA-3', and for gapdh, 5'-GGC CTC CAA GGA GTA AGA CC-3' and 5'-AGG GGT CTA CAT GGC AAC TG-3' respectively. The cycling conditions were 94°C for 120 seconds, followed by 37 (for mdr-1) or 25 (for gapdh) cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. Each primer set was amplified using an optimized number of PCR cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining under ultra-violet light. Identity of the observed band was thus confirmed by direct sequencing. For quantitation of cDNA, densitometric analysis of a digital image of the agarose gel was performed using the NIH 1.62 software.

Measurement of rhodamine 123 cellular retention

To demonstrate Pgp efflux pump activity the Neyfakh assay was used. This assay is based on reduced staining by fluorescent dyes of multidrug resistant cell lines due to an increased rate of dye efflux. If the mdr-1 encoded transporter is inhibited, cells will stain more intensively [16]. Cells cultivated into 24-well plates were incubated with Cobalamin (100 nM) or 5'-AZA/TSA (1 μM / 165 nM) for 24 hours. They were then incubated for 2 hours at 37°C in cell culture medium containing 2 μM rhodamine 123 (Rh-123). Glybenclamide (100 μM) was added as a standard inhibitor of ABC proteins in control experiments at the same time as Rh-123 and verapamil (1 μM) was used to specifically block Pgp activity. After incubation, the plates were rinsed with 0.1 M phosphate buffered saline (PBS)

and incubated in the respective media but without Rh-123 for 30 minutes as a washout period. The media were then discarded and intracellular Rh-123 was extracted for 1 hour at 4°C by adding 0.3 ml ethanol (95%). The rhodamine was quantified by measuring fluorescence (485 nm/538 nm) on a Perkin-Elmer LS-5 Luminescence Spectrometer.

Drug-sensitivity assay

The sensitivity of the HepG2 cells to vinblastine was determined using the MTT assay. Briefly, cells were seeded onto 96-well plates and grown until 90% confluency. Some of the plates were incubated with 100 nM Cobalamin for 24 hours. Plates were then incubated with increasing concentrations (from 1 nM to 30 μM) of vinblastine for 48h with or without 100 nM cobalamin, 10 μM verapamil or a combination of both. In other experiments cells were exposed to a constant concentration of vinblastine (100 nM) and increasing concentration of Cobalamin (1 nM to 30 μM). In other plates, cells pre-incubated for 72 hours with methotrexate were further incubated with increasing concentrations of vinblastine, with and without 100 nM Cobalamin. At the end of these experiments, cell medium was replaced with fresh drug-free medium containing 0.1 mg/ml of MTT and the cells were incubated for an additional 1 hour. Culture medium was removed and dye was extracted with DMSO. Absorbance of samples was read at 570 nm in a microplate reader. Negative control was conducted using only drug-free culture medium and positive control by using culture medium containing 1% of sodium azide leading to a 100% cell death. Experiments were performed at least three times and each concentration was tested in octaplates in each experiment. Viability was expressed as a percentage of untreated cells (mean ± SEM).

Methylation status analysis of the mdr-1 promoter

With regard to the effect of DNA methylation and histone deacetylation on gene expression, demethylation-induced gene reexpression was shown to be potentiated by TSA [17, 18]. This is probably due to the fact that DNA methyltransferases, which catalyze DNA methylation, are recruited by the HDACs present in the transcriptional inhibitory complex that bind to methylated DNA. We thus treated HepG2 cells for 24h with either 100 nM Cobalamin to be compared to cells incubated with the demethylation agent 5'-AZA (1 μM) in combination with TSA (165 nM). Genomic DNA from the cells was extracted using the QIAamp DNA Mini kit from Qiagen. 1 μg of DNA was subjected to sodium bisulfite modification using the CpGenome™ DNA Modification Kity (Intergen Inc). The modified DNA was used for a Methylation-Specific-PCR according to the Hideki and al. protocol [19], using either methylation-specific primers (MSP) or unmethylation-specific primers (USP) designed on the basis of the functional promoter sequence [20]. The sense and antisense primers were, for MSP, 5'-GGA ATT AGT ATT TAG TTA ATT CGG-3' and 5'-ACT CAA CCC ACG CCC CGA-3' and for USP, 5'-GGA ATT AGT ATT TAG TTA ATT TGG-3' and 5'-ACT CAA CCC ACA CCC CAA CA-3' respectively. The location of the forward and reverse primers, in which six CpG sites and two SP-1 sites are covered, and corresponding to amplified regions are shown in

Fig. 1. Activity of methionine synthase in HepG2 cells. Cells were incubated with or without 100 nM of Cobalamin. Methionine synthase activity was measured with (open bars) or without (dark bars) cobalamin in the reaction mixture showing presence of respectively total- and holo-enzyme. The data here present the mean values $\pm$ standard deviation of three independent experiments which were done in triplicates.

figure 7A. The forward and reverse primers are designed to cover the Sp-1 site at their 3'-prime end because both SP-1 sites are mandatory for the functional *mdr-1* promoter to be activated [21]. MSP products were analyzed on 2% agarose. 1 μ l of bisulfited-modified DNA was then amplified using a pair of universal primers in a total volume of 20 μ l of mixture. The sense (U-S) and antisense (U-AS) primers were, 5'-GGA AGT TAG AAT ATT TTT TTT GG-3' and 5'-ACC TCT ACT TCT TTA AAC TTA AA-3' respectively. The cycling conditions were 94°C for 120 seconds, followed by 37 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds. Negative control PCR amplification was performed by using both sets of primers and untreated DNA. Absence of PCR products confirms that unmodified DNA could not be amplified by modified sets of primers. Direct bisulfited DNA sequencing of the purified PCR products was also performed using each of the described universal primers according to the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Paris, France).

Statistical analysis

Results were expressed as the means $\pm$ SEM of at least triplicate measurements. To compare the effects of agents, values are presented as a percentage of effect in absence of treat-

Fig. 2. Mdr-1 expression evaluation by RT-PCR in HepG2. Total RNA (2 μ g) was amplified with the primers of *mdr-1* (upper) and *gapdh* (lower), which was used as an internal standard. The product of RT-PCR was detected by agarose gel electrophoresis. Quantitative analysis of expression levels of MDR-1 and GAPDH mRNA were estimated as described in Section 2 and the ratio of MDR-1 to GAPDH was calculated. Lane 1: Standard cell culture conditions, lane 2: cells incubated with cobalamin (100 nM), Lane 3: cells incubated with 5'-aza-2'-deoxycytidine (5'-AZA; 1 μ M) plus tricostatine A (TSA; 165nM). Data from one of twelve representative experiments.

ment. Statistical comparisons were based on the Student's t-test analysis using StatView software. Statistical significance: ** $p < 0.01$; *** $p < 0.001$.

Results

Cobalamin increases Methionine Synthase Activity in HepG2 cells

As a first insight we checked for methionine synthase (MS) activity upon addition of Cobalamin in the culture medium. As shown in Figure 1, addition of 100 nM of Cbl in the cell culture medium rose MS activity

from a low 0.02 ± 0.005 nmol methionine / hour per mg protein to a high 0.36 ± 0.025 nmol methionine/hour per mg protein. When Cbl is added to saturation during the enzymatic reaction, activities rose showing the amount of total holoenzyme present. These were 0.06 ± 0.01 nmol methionine/hour per mg protein and 0.59 ± 0.03 nmol methionine/hour per mg protein, for cells cultured in absence and in presence of 100 nM of Cbl, respectively.

Cobalamin decreases mdr-1 gene expression in HepG2 cells

We then used a semiquantitative RT-PCR to determine whether mdr-1 gene expression might be modulated by Cbl in the same conditions where MS activities were seen to increase. As indicated in Figure 2, a decrease in mdr-1 mRNA was found in presence of 100 nM Cobalamin as compared to standard cell-culture conditions. In contrast, and according to [13], a mixture of 5'-AZA (1 μ M) and TSA (165 nM) yielded an increase in mdr-1 expression. The calculated ratios of the MDR-1 mRNA to GAPDH mRNA in Cbl-treated cells and in 5'-AZA/TSA-treated cells were 0.32 ± 0.14 and 3.12 ± 0.25 , respectively.

Cobalamin decreases Pgp activity in HepG2 cells

The efflux of rhodamine 123 (Rh-123), which is a lipophilic cationic substrate of Pgp, correlates with Pgp function and is considered as a powerful index for cellular Pgp activity. We used Rh-123 to evaluate the effect of Cbl on the presence of functional Pgp (Figure 3). The efflux rate was estimated by measuring the amount of fluorescent dye remaining into the cells after washing and incubation in the presence of agents. The rate of Rh-123 retention was higher in cells incubated with Cbl (more than 2 fold). Addition of a 5'-AZA/TSA mixture led to slight but not significant decrease in Rh-123 retention. To determine how far Pgp is involved in intracellular drug accumulation, cells were incubated with glybenclamide (Gly), which is a general ABC protein blocker. Glybenclamide led to a significant increase in Rh-123 retention (about 2.5 fold). Verapamil, which is a specific Pgp functional inhibitor also led to a significant increase in Rh-123 retention (about 2 fold). This result supports the idea that the main part of increase in Rh-123 retention induced by Cbl is due to less amount of Pgp.

Cobalamin decreases resistance to vinblastine in HepG2 cells

Vinblastin is an antimitotic agent but is also a well-known substrate of Pgp and resistance of cancer cells to

Fig. 3. Cellular retention of Rhodamine 123 in HepG2 cells. Cells were incubated for 2 h with Rh-123 (2 μ M) and either 100 nM Cobalamin, a 5'-AZA/TSA mixture (1 μ M / 165 nM), 1 μ M verapamil (Vera) or 100 μ M Glybenclamide (Glib). After a 30min washout period in absence of dye and presence of agents, intracellular Rh-123 concentration was determined by fluorescence at 485-538 nm after extraction with ethanol. The data here present the mean values $\pm$ standard deviation of three independent experiments which were done in hexaplicates.

vinblastin is mediated by mdr-1 expression [22]. The effect of Cbl on the resistance of HepG2 cells to vinblastine was assessed by the determination of their chemosensitivity. HepG2 cells showed a resistance to vinblastine up to 300 nM. Higher concentrations led to a concentration-dependent toxicity with a calculated GI_{50} being about 5 μ M (Figure 4A). In presence of 100 nM of Cbl, a shift to the left was observed (with a calculated GI_{50} of 0.1 μ M). The observed effect was also dependent on the concentration of Cbl (Figure 4B), the Cbl concentration-dependent curve indicating a calculated GI_{50} for Cbl of 100 nM in the presence of a 100 nM dose of vinblastine. Without vinblastine, Cbl was shown as non-toxic, strongly indicative of a synergistic effect cobalamin on vinblastin-induced cytotoxicity.

We then compared the effect of Cbl to that of verapamil in regards to its relative potentialization of vinblastine-induced toxicity. Verapamil is a known Pgp inhibitor widely used to block Pgp function. It was consequently used in cancer combinational therapy [23-25]. As shown in figure 4C, verapamil shifted the vinblastine toxicity curve to the left, reaching a calculated GI_{50} of 1 μ M. Incubation with both verapamil and Cbl led to a calculated GI_{50} of 0.1 μ M which is same as the value obtained by Cbl alone. However, dose-response curve of vinblastine in presence of a mixture of verapamil and Cbl was linear whereas the dose-response curve of vinblas-

Fig. 4. Chemosensitivity of HepG2 cells was determined by MTT assay. (A) Vinblastine was added for 48h from 1 nM to 30 μ M to cells pre-incubated (light circles) or not (dark circles) for 24h with 100 nM of Cobalamin. (B) Increasing concentrations of Cobalamin were applied to HepG2 cells in absence (light squares) or in presence (dark squares) of a 100 nM dose of vinblastine. (C) Increasing concentrations of vinblastine were added for 48h in the presence of 1 μ M of verapamil (grey squares) or 1 μ M verapamil plus 100 nM Cbl (open circles) and compared to vinblastine alone (dark circles). (D) Increasing concentrations of verapamil were added for 48h in the presence of 100 nM of vinblastine (dark circles) or 100 nM vinblastine plus 100 nM Cbl (dark squares) and compared to verapamil alone (open circles). The data present the mean values $\pm$ standard deviation of three independent experiments which were done in octaplates. For some values, SD was less than size of points and was not represented. Results are expressed as a percentage of viable cells compared to untreated cells.

tine in presence of Cbl alone was biphasic. Figure 4D presents toxicity curves of increasing concentrations of verapamil in presence or not of either vinblastine or vinblastine plus Cbl (100 nM each). Verapamil alone elicited a toxic effect with a calculated GI_{50} of about 6 μ M. When 100 nM vinblastine was added to the cells, it shifted the verapamil dose-response curve to the left with a calculated GI_{50} of about 2 μ M. When Cbl and vinblastine (both at 100 nM) were added together to increasing doses of verapamil, the effect was additive. All three toxicity-curves converged to a maximal toxic effect of verapamil, achieved at 100 μ M.

Cobalamin prevents methotrexate-induced up-regulation of mdr-1 in HepG2 cells

Multidrug resistance is a phenomenon where mdr-1 plays a major role and which mainly consists in an increase of this gene's expression upon drug exposure. One

of the most common multidrug-resistance inducing drug is methotrexate [26]. As a consequence, methotrexate is currently only rarely used in cancer therapy. We thus determined whether Cbl could affect mdr-1 gene expression after long term methotrexate exposure. Incubation with 1 mM methotrexate for 72h led to an increase in mdr-1 gene expression (figure 5A). In contrast, no such increase was observed when cells were incubated with a mixture of methotrexate (1mM) and Cbl (100 nM). Furthermore, toxicity tests showed that HepG2 cells were totally insensitive to 1 mM methotrexate although HepG2 cells do not highly express mdr-1 (data not shown). Cells pre-treated with 1 mM methotrexate alone for 72h became more resistant to vinblastine exposure (Figure 5B). When cells were pre-treated for 72h with methotrexate in combination with Cbl, they presented a high vinblastine sensitivity as indicated by the significant shift to the left of the toxicity curve.

Cobalamin-dependent decrease in mdr-1 expression is not mediated by promoter methylation

Mdr-1 gene expression has been related to several transcription factors [5] and also to its promoter methylation status in other systems [19]. To examine the possible effect of Cbl on the methylation status of the promoter region of mdr-1 gene in HepG2 cells, we used two sets of primers to amplify part of mdr-1 gene promoter region (Figure 6A). Both the presence of PCR products from control HepG2 cells when MSP primers were used and the absence of PCR products when using USP primers indicates that the 5' promoter region of the mdr-1 gene is highly methylated in these cells (Figure 6B). A similar pattern was observed after incubating cells with 100 nM Cbl for 24 hours. This data suggests that Cbl induces no changes in the methylation status of the mdr-1 promoter. The presence of PCR products at 345 bp (a positive USP-PCR band) in 5'-AZA/TSA treated cells evidenced that mdr-1 promoter is partially unmethylated under these conditions. Direct bisulfite DNA sequencing confirmed the above results i.e. partial methylation of the 5'-AZA-treated samples and complete and similar methylation of both control and cobalamin-treated samples, at both the -110/-103 and -61/-51 sites.

Discussion

Many drugs used in cancer treatment are Pgp substrates. Understanding the physiological regulation of mdr-1 gene expression is important to design strategies aimed at improving efficiency of these drugs. Unfortunately, regulation of mdr-1 gene expression is still little understood. There is growing evidence that CpG methylation in the 5' regulatory region of mdr-1 gene is a dominant factor for silencing mdr-1 transcription [27]. Recent studies have shown that CpG methylation of mdr-1 promoter is a frequent event in prostate [28] and bladder [29] cancer and is related to cancer progression.

We herein evidenced for the first time that a vitamin, Cobalamin is able to downregulate mdr-1 gene expression suggesting a link between the methionine cycle and mdr-1 gene regulation. We also show that mdr-1 promoter is highly and similarly methylated in both control and Cbl-treated HepG2 cells. These data indicate that the downregulation of mdr-1 by Cbl is independent of the methylation status of mdr-1 promoter. In contrast, treatment with 5'-AZA/TSA resulted in a reduced methylation the promoter of mdr-1 gene, and in an increase in mdr-1 gene expression as already documented [13].

Fig. 6. Methylation status of the *mdr-1* gene promoter determined by methylation-specific PCR analysis. (A) The diagram of the promoter of human *mdr-1* gene and location of CpG sites. For determination of the methylation status of *mdr-1* promoter region, we used two primer pairs which amplify across the SpI sites located within the *mdr-1* promoter. (B) The methylation status of *mdr-1* promoter (92 bp) in HepG2 cells. Presence of PCR amplification products (MSP) indicates that -126 to -34 region was predominantly methylated in both control HepG2 cells and in HepG2 cells incubated with 100 nM Cobalamin. The presence of PCR products after USP amplification indicates the loss of methylation in 5'-AZA/TSA treated HepG2 cells.

However, it is noticeable that the chemical compound 5'-AZA leads to an artificial alteration of cell methylation reactions whereas Cbl may act in a more "physiological" way. Up to date, no physiological metabolic pathways were shown linked to *mdr-1* gene expression. *Mdr-1* gene expression is a hallmark of cancer cells and is very often increased after chemotherapy rendering it thus ineffective. Little is known about mechanisms that trigger regulation of *mdr-1* gene expression and especially mechanisms involved in its downregulation. Several transcription factors have been proved to be linked to *mdr-1* promoter's activation such as YB-1, NF-AT, c-Jun/c-Fos, [5]. However, no pharmacological trials can be set up from these data. *Mdr-1* gene expression, was also shown to be dependent on the cell methylation potential. Nonetheless, DNA methyltransferase inhibitors were proved to be poorly effective in improving chemotherapy. Several other clinical trials have been undertaken to inhibit Pgp (using Pgp blockers such as verapamil, cyclosporine, progesterone, alkaloids,...) with yet little results. This was because of interferences occurring between the used an-

ticancer drugs and these Pgp inhibitors or because of side effects of these inhibitors (biliary elimination or alteration of the cytochrome P450 system) [2]. New approaches to MDR therapy include downregulation of MDR proteins. However, the suggested formulae, i.e. antisense oligonucleotides, calcium inhibitors and SXR (steroids and xenobiotic receptor) antagonists are still debated [30].

The link between methionine synthase activity and *mdr-1* gene expression is unclear. The methionine cycle is a main cellular generator of SAM which is the methyl donor for most of the cell methylation reactions. Since we did not observe any modification of the *mdr-1* promoter methylation status, it is unlikely that the Cbl-induced decrease in *mdr-1* gene expression would involve modification in DNA methylation. Many regulatory systems exist that control *mdr-1* gene expression pre- or post-transcriptionally. For instance, protein kinase C activators which increase Pgp activity have been found to enhance *mdr-1* gene expression via both transcriptional and translational pathways [31]. Modulations in protein

stability, plasma membrane incorporation, mRNA stability and processing, gene transcription and gene amplification have each been reported for Pgp [32]. Amongst these, alterations in *mdr-1* expression that occur at the level of mRNA are perhaps the most frequently observed [33]. Although increased mRNA levels generally occur as a result of enhanced gene transcription rates, prolonged cellular exposure to several cytotoxic drugs have also been reported to induce *mdr-1* gene overexpression through both gene amplification [34] as well as through increased mRNA stability. However, none of these pathways have yet been described as being modulated by the methionine cycle. Recently, a possible regulatory Cbl / NFkB mechanism has been outlined by Wheatley et al. [35]. This mechanism could in turn control several cell functions such as TNFalpha and EGF production [36] and thus may be an important determinant of inflammation and of the coagulation cascade [35]. Since NFkB was also shown to regulate *mdr-1* mediated chemoresistance [37], it can be suggested that Cbl may downregulate *mdr-1* gene expression by preventing NFkB activation. Further investigations are now needed to understand the exact way by which Cbl may modulate *mdr-1* gene expression and especially an eventual role of NFkB in this phenomenon.

Important results of this study are the significant increase in drug sensitivity by cells incubated with cobalamin and the prevention of induction of *mdr-1* by methotrexate in presence of cobalamin. Cbl was shown to downregulate *mdr-1* gene expression leading to a de-

crease in both Pgp expression and its specific transport activity. As a consequence, it can be concluded that vinblastine sensitivity is a direct consequence of *mdr-1* expression in HepG2 cells. A 100 nM cobalamin concentration led to a dramatic increase in MS activity as already shown in cancer intestinal cells [38] and also in about a two log lower GI_{50} of vinblastine (Figures 4 and 5). However, the GI_{50} of cobalamin (100 nM) ranged above the physiological plasma concentration (0.1 to 1 nM). At these plasmatic Cbl concentrations, only a slight increase in sensitivity to vinblastine was observed. It is therefore tempting to suggest administering vinblastine to patients in combination with high doses of Cbl. Moreover, Cbl is not known to induce any toxic or side effects even at high doses. Of course, further investigations using animal models and clinical trials need to be performed first before issuing a possible use of Cbl in cancer chemotherapy. However, comparison between increased vinblastine cytotoxic in presence of Cbl, MTX or Pgp blockers such as verapamil indicates that Cbl is a possible innovative means of circumventing and/or preventing the MDR phenomenon which is worth further investigation.

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